The Photophysical and Photochemical Properties of Flavins (Isoalloxazines)

By P. F. Heelis
DEPARTMENT OF NATURAL SCIENCE, RESEARCH DIVISION,
NORTH E. WALES INSTITUTE, KELSTERTON COLLEGE,
CONNAH'S QUAY, DEESIDE, CLWYD CH5 4BR

1 Introduction

The term 'Flavin' refers to the yellow chromophoric and redox-active prosthetic group of a class of respiratory enzymes occurring widely in animals and plants, namely the flavoproteins. Flavins are based upon the nitrogen heterocycle 7,8-dimethylisoalloxazine, or to be more systematic, 7,8-dimethylbenzo[*g*]pteridine-2,4(3*H*,10*H*)-dione. The structures of the most important flavins are shown in Figure 1. Note that the IUPAC numbering system is employed throughout this article, although the older German (but still encountered) system is also given in (3).

Flavin photochemistry has been the subject of intense research over the past forty years.[1] Such efforts were prompted in part by the proposed involvement of flavin excited-states in several important photobiological and photochemical processes. Flavins are thought to be the most likely candidates for the photoreceptor pigment in the ubiquitous 'blue light' photoreceptive processes observed in a wide range of animals and plants. Such effects include the photoaccumulation (phototaxis) of unicellular algae and the photoresponse of fungal sporangiophores.[2] Flavin is also thought to be the light emitting chromophore in many bioluminescent bacteria. Further impetus to the study of flavin photochemistry came from their proposed role in the photochemical oxidation of dairy products[3] and the photodegradation of pesticides residues.

A prerequisite for a study of the excited-state properties of flavins is a knowledge of the chemistry of the ground state. Space restrictions, however, permit only a consideration of the redox and acid–base reactions of flavins (Figure 2). One electron reduction of a flavin in its normal oxidized form (Fl_{ox}) produces a free radical state or flavosemiquinone ($H\dot{F}l$, $\dot{F}l^{-}$, or $\overset{+}{H}_2\dot{F}l$). A further one electron reduction yields the fully reduced flavin, 1,5-dihydroflavin (H_2Fl_{red}) or its anion ($HFl^{-}{}_{red}$). Further details of relevant areas of flavin chemistry can be obtained

[1] G. R. Penzer and G. K. Radda, *Quart. Rev.*, 1976, **21**, 43, see also refs. 4 and 26.
[2] Various articles in 'Photoreception and Sensory Transduction in Aneural Organisms', ed. F. Lenci and G. Colombetti, Plenum Press, New York, 1980.
[3] A. Satter and J. M. deMan, *Crit. Rev. Food Sci. Nutrit.*, 1975, **7**, 13.

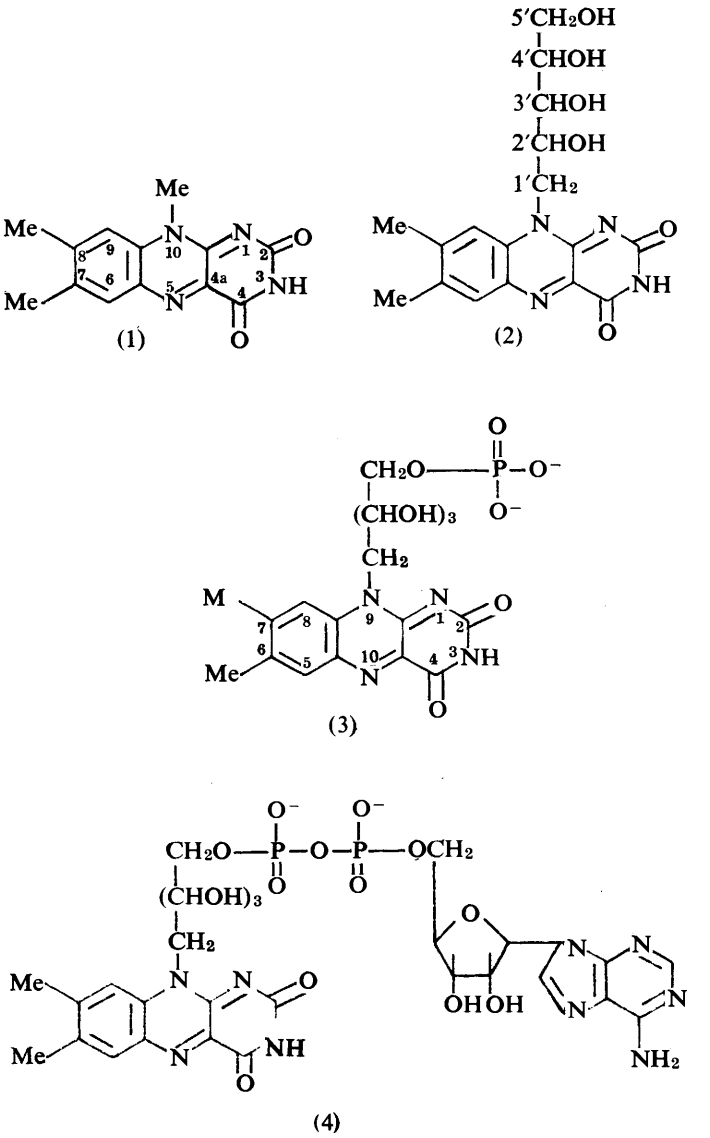


Figure 1 *Structural formulae of some common flavins; lumiflavin* (1), *riboflavin* (2), *flavin mononucleotide* (*FMN*) (3), *flavin adenine dinucleotide* (*FAD*) (4). *IUPAC numbering of the flavin nucleus on* (1) *and for the ribityl side-chain on* (2). *Alternative numbering on* (3)

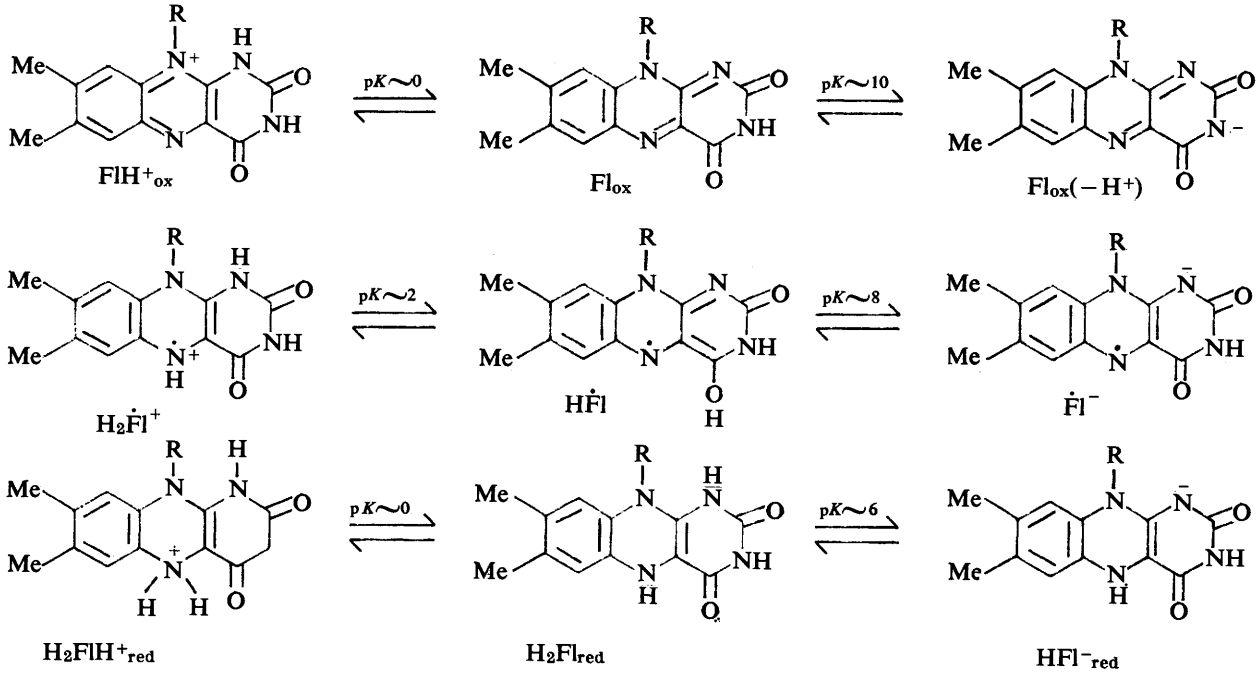


Figure 2 *Redox and acid-base equilibria of flavins*

from various reviews on their general chemistry,[4,5] free radical,[6] and enzymic properties.[7]

The absorption of electromagnetic radiation is a quantized phenomenon, and for radiation in the visible and ultraviolet regions sufficient energy is available to bring about changes in the electronic state of a molecule as well as changes in its vibrational and rotational energy levels. For the regions of the electromagnetic spectrum of relevance (200—800 nm), electronic excitation usually involves the promotion of one electron in either π bonding orbitals or a non-bonding orbital (when present) to the π^* antibonding orbitals (termed $\pi \rightarrow \pi^*$ or $n \rightarrow \pi^*$ transitions, respectively). Further, the unpaired electron spins in the bonding and antibonding orbitals may be antiparallel leading to a diamagnetic 'singlet state', or parallel, leading to a paramagnetic 'triplet state'. Much of what follows is an account of studies aimed at elucidating the energies, the properties, and the role of each excited state in the photochemical reactions of flavins. To aid in the discussion, the electronic and vibrational energy levels of a typical flavin are presented in Figure 3.

2 The Photophysical Properties of Flavins

A. Singlet States.—(i) *Visible and Ultraviolet Absorption and Fluorescence Emission Spectra.* The absorption spectrum of a flavin such as riboflavin in aqueous solution consists of four structureless peaks (Figure 4) centred at 446, 375, 265, and 220 nm. All four absorption maxima possess high molar extinction coefficients ($> 10^4 \text{M}^{-1}\text{cm}^{-1}$), indicative of $\pi \rightarrow \pi^*$ type transitions.

Quantum mechanical calculations have been reported employing most of the commonly used methods, including Huckel and extended Huckel,[8] CNDO,[9] MINDO, and SCF.[10] Most methods predict the lowest energy $\pi \rightarrow \pi^*$ transition to be close to 450 nm, in good agreement to the observed position (see Figure 3, transitions 1—3). The band exhibits virtually no change in position upon moving from water to less polar solvents, in agreement with this $\pi \rightarrow \pi^*$ assignment. Some fine structure is, however, now observed with the partial resolution of at least three vibronic (vibrational plus electronic) transitions,[11] as a result of a

[4] P. Hemmerich, *Fortschr. Chem. Org. Naturst.*, 1976, **33**, 451.
[5] K. Ohta, R. Wrigglesworth, and H. C. S. Wood, in 'Rodd's Chemistry of Carbon Compounds', ed. S. Coffey, Elsevier, Amsterdam, 1980, p. 237.
[6] H. Beinert in 'Biological applications of electron spin resonance', ed. H. M. Swartz, Wiley Interscience, New York, 1972, p. 351.
[7] Various articles in 'Flavins and Flavoproteins', ed. K. Yagi and T. Yamano, Japan Scientific Society Press, Tokyo, 1980.
[8] R. Norrestam, P. Kierkegaard, B. Stensland, and L. Torbjörnsson, *Chem. Comm.*, 1969, 1250.
[9] B. Grabe, *Acta Chem. Scand., Ser. A*, 1972, **26**, 4084.
[10] B. Grabe, *Acta Chem. Scand., Ser. A*, 1974, **28**, 363.
[11] J. K. Eweg, F. Müller, A. J. W. G. Visser. C. Veeger, D. Bebelaar, and J. D. W. Van Voorst, *Photochem. Photobiol.*, 1979, **30**, 463.

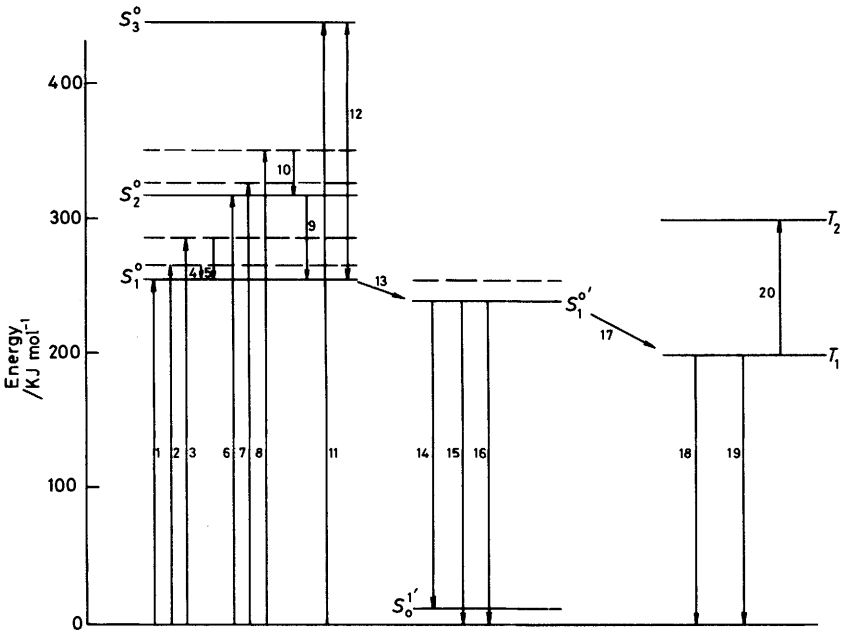


Figure 3 *Jablonski-type diagrams for flavins, giving the energies above the ground-state electronic (solid horizontal lines) and vibrational (dashed horizontal) levels. (a) Transitions:* 1, 2, 3, 6, 7, 8, 11, *and* 20, *absorption;* 14 *and* 15, *flourescence emission;* 18, *phosphorescence emission;* 16 *and* 19, *non-radiative deactivation;* 17, *intersystem crossing;* 4, 5, *and* 10, *thermal equilibration of vibrational levels;* 9 *and* 12, *non-radiative decay of upper excited-states to lowest-energy singlet-state. (b)* S = *singlet state,* T = *triplet state; subscript denotes electronic energy level and superscript denotes vibrational energy level. (c) Note* $S_1^{0\prime}$ = ^{1}Fl, T_1 = ^{3}Fl

reduction in solvent–solute interactions.[12,13] Recent studies at low temperature[11] (77 K) in 3-methylpentane have improved the resolution of these vibronic bands, allowing their assignment to the 0 → 0, 0 → 1, and 0 → 2 vibrational transitions superimposed upon the $S_0 \rightarrow S_1$ electronic transition (Figure 3; transitions 1—3, respectively). Supporting evidence for the above assignments has come from the temperature dependence of the absorption spectrum[14] and coherent anti-Stokes Raman spectroscopy (CARS).[15] As is commonly found for polyatomic molecules, because of Franck–Condon restrictions, the absorption maximum does not correspond to the 0 → 0 but to the 0 → 1 vibrational transition.

[12] K. Yagi, N. Ohishi, K. Nishimoto, J. D. Choi, and P. S. Song, *Biochemistry*, 1980, 19, 1553.
[13] J. K. Eweg, F. Müller, H. Van Dam, A. Terpstra, and A. Oskam, *J. Am. Chem. Soc.*, 1980, **102**, 51.
[14] F. Müller, S. G. Mayhew, and V. Massey, *Biochemistry*, 1973, **12**, 4654.
[15] P. K. Dutta and T. G. Spiro, *J. Chem. Phys.*, 1978, **69**, 3119.

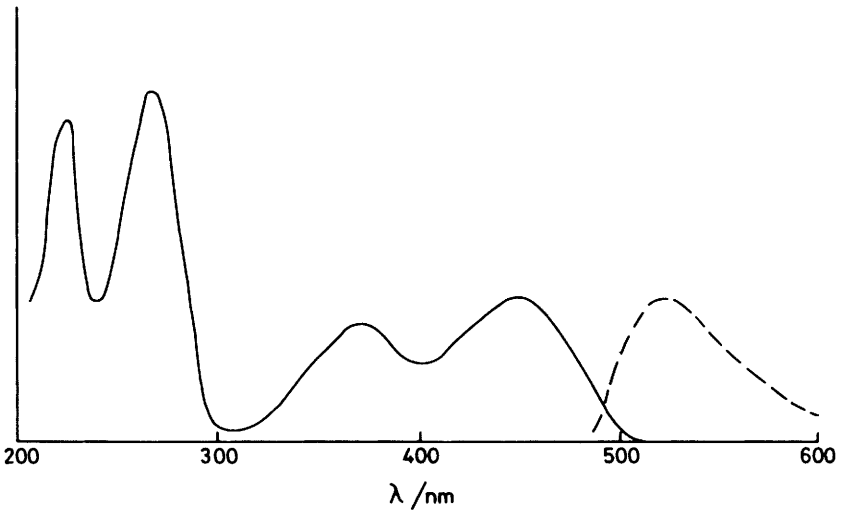


Figure 4 *The absorption* (——) *and flourescence emission spectra* (---) *of riboflavin in neutral aqueous solution*

The near u.v. absorption band at 375nm probably consists of a $\pi \rightarrow \pi^*$ transition, although MO calculations have not been too successful in predicting its position. Recent CNDO/S calculations[13] suggest that some mixing of an $n \rightarrow \pi^*$ transition involving the N-1 non-bonding electron pair with the $\pi \rightarrow \pi^*$ transition may occur. The increase in intensity of the 375nm band on increasing the solvent polarity may be due to an increase in the 'allowedness' of this transition. The red shift that is seen on moving to protic solvents may reflect a destabilization of the N-1 non-bonding electrons by hydrogen bonding.[12,13] Temperature difference spectra of the near u.v. band indicate the presence of three separate vibronic transitions (Figure 3, transitions 6—8), although they are not resolved in the absolute spectrum, even at 77 K.[11,14]

Little is known concerning the higher energy transitions at 265nm (Figure 3, transition 11) and at 220nm. Theoretical calculations predict that two transitions[16] (at 280 and 260nm) may contribute to the overall maximum at 265nm. Some support for this is provided by the reflectance spectra of crystalline 10-methylisoalloxazine, which reveals bands at 284nm and 257nm.[17] In addition, conventional absorption spectral studies coupled with linear dichroism of riboflavin in anisotropic sheets of polyvinyl alcohol suggest that another $\pi \rightarrow \pi^*$ transition occurs at 308nm.[18]

So far the presence of $n \rightarrow \pi^*$ type electronic transitions has not been discussed in detail. Several such transitions are possible due to the non-bonding electron

[16] M. Sun, T. A. Moore, and P. S. Song, *J. Am. Chem. Soc.*, 1972, **94**, 1730.
[17] M. W. Yu, C. J. Fritchie, A. F. Fucaloro, and B. G. Anex, *J. Am. Chem. Soc.*, 1976, **98**, 6496.
[18] R. Drabent, *Acta Phys. Pol.*, 1979, **A55**, 371.

pairs localized at N-1, N-5, N-3, N-10, C-2=O, and C-4=O. Although $n \rightarrow \pi^*$ transitions are of low probability due to symmetry and/or overlap restrictions, they are potentially intense in flavins due to the lack of molecular symmetry. However, the balance of evidence indicates that any $n \rightarrow \pi^*$ transitions are masked by the much more intense $\pi \rightarrow \pi^*$ transitions. In particular, theoretical calculations suggest that the lowest energy $n \rightarrow \pi^*$ transition primarily involves the N-5 lone pair and that its low intensity band is hidden under the second $\pi \rightarrow \pi^*$ absorption at 370 nm.[16,19] Earlier suggestions[20] that the circular dichroism spectra show evidence for an $n \rightarrow \pi^*$ transition in the 450 nm band have now been discounted.

The relative orientations of the transition moments of the 450 and 375 nm $\pi \rightarrow \pi^*$ transitions have been determined from fluorescence polarization data[21] (see p. 22), a value of *ca.* 30° being obtained. This value is similar to that of 38°, measured on macroscopically aligned flavins obtained by the dichroic bleaching method.[22] Of particular interest are recent attempts to define the orientation of the transition moments relative to the absolute molecular axis.[23] Linear dichroism of FMN, macroscopically aligned in lamellar liquid crystals, yielded the orientations shown in Figure 5. These results are also in reasonable agreement with

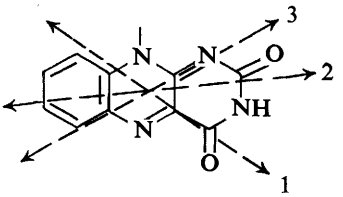


Figure 5 *Experimentally determined transition moments* (-----) *for absorption in the* 450 nm (1), 375 nm (2), *and* 265 nm (3) *absorption bands*

earlier studies of FMN in single crystals of the flavoenzyme flavodoxin.[24] A potential application of these data is the determination of the alignment of the flavin molecule *in vivo*, for example in photoreceptor organelles.

Flavins exhibit a bright yellow fluorescence (λ_{max} 520 nm, Figure 4) in aqueous solution. An essentially identical quantum yield of fluorescence (Φ_f) of 0.26 has been reported for riboflavin, lumiflavin, and FMN in dilute aqueous solution at pH 7.[25] Essentially the same value for Φ_f is obtained irrespective of the excitation wavelength (260—500 nm).[11] The wavelength independence of Φ_f indicates that

[19] M. Sun and P. S. Song, *Biochemistry*, 1973, **12**, 4663.
[20] D. W. Miles and D. W. Urry, *Biochemistry*, 1968, **7**, 279.
[21] G. Weber in 'Flavins and Flavoproteins', ed. E. C. Salter, Elsevier, Amsterdam, 1966, p. 15.
[22] A. Gordon-Walker, G. R. Penzer, and G. K. Radda, *Eur. J. Biochem.*, 1970, **13**, 313.
[23] L. B.-Å. Johansson, Å. Davidsson, G. Lindblom, and K. R. Naqvi, *Biochemistry*, 1979, **18**, 4249.
[24] W. A. Eaton, J. Hofrichter, M. W. McKinen, D. R. Andersen, and M. L. Ludwig, *Biochemistry*, 1975, **14**, 2146.
[25] G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, 1957, **53**, 646.

excitation to upper excited states ($S_{n>1}$) is followed by extremely rapid internal conversion to the S_1 level, without loss of energy due to emission from $S_{n>1}$ or intersystem crossing from $S_{n>1} \rightarrow T_{n\geqslant 1}$. This in turn suggests that spin–orbit coupling is not significant.[26]

In solvents less polar than water, the emission maxima is usually shifted to shorter wavelengths together with the partial resolution of some vibrational fine structure.[27] Recent low temperature studies[11] have assigned the emission maximum to the $S_1^0 \rightarrow S_0^1$ vibronic transition (Figure 3, process 14). In frozen solution, quite a large blue shift in the emission maxima occurs.[11] This is attributed to a change in the most intense vibronic transition from the $S_1^0 \rightarrow S_0^1$ to the $S_1^0 \rightarrow S_0^0$ transition (Figure 3, transition 14 is replaced by 15).

The quantum yield of flavin fluorescence is dependent on the solvent polarity, *e.g.* $\Phi_f = 0.26$ in aqueous solution, but rises to 0.47 in acetonitrile.[27] In attempting to interpret such effects, it is important to recognize that Φ_f is dependent on the relative magnitudes of several competing processes *i.e.* non-radiative internal conversion, fluorescence, and intersystem crossing [Figure 3, processes 16, 14 (or 15), and 17, respectively]. Probably only the latter two processes are important for flavins as Φ_f and $\Phi_{\text{intersystem crossing}}$ = unity [See section B (i)]. It has been suggested that the degree of hydrogen bonding between the flavin and the solvent may be an important factor influencing the fluorescence efficiency.[12] Hydrogen bonding may also influence the degree of vibronic spin–orbit coupling in the S_1^0 state, hence affecting the rate constant of intersystem crossing, and thereby Φ_f. Interestingly, in dry carbon tetrachloride solutions, the vibrational resolution of the long wavelength absorption band disappears upon the addition of hydrogen bonding solutes, *e.g.* phenol.[12] These changes are accompanied by a decrease in Φ_f. Molecular orbital calculations have predicted that, upon the basis of electron density, the flavin N-1 position is the most likely site for hydrogen bonding.[12]

Fluorescence polarization spectra of flavins have been determined by several investigators in viscous or rigid media (to prevent rotational depolarization). The emission polarization for 450 nm excitation, approaches the maximum value theoretically attainable, suggesting that the emission and absorption transition moments are very nearly parallel.[23] The excitation spectra of flavins show a constant degree of polarization over the 450 nm and 375 nm absorption bands, confirming that only one electronic transition contributes significantly to each band.[16] In addition this suggests that individual vibrational transitions within each absorption band do not result in marked torsional motions. Similar considerations apply to the polarized emission spectra, which again exhibit a constant degree of polarization over the whole emission band.[16]

The radiative lifetime of the lowest excited singlet state has been estimated as 12 ns (1.2×10^{-8} s), using the Birks–Dyson formula.[11] Direct measurements using time resolved techniques have yielded values around 5 ns for the fluorescent

[26] P. S. Song in 'Flavins and Flavoproteins', ed. H. Kamin, University Park Press, Baltimore, 1971, p. 37.
[27] A. J. W. G. Visser and F. Müller, *Helv. Chim. Acta*, 1979, **62**, 593.

lifetimes of riboflavin, lumiflavin, and FMN. FAD possesses a somewhat shorter lifetime (2.3 ns).[28]

It should be noted that the energy of the lowest vibrational level of the S_1^0 state formed upon light absorption (Figure 3) is not usually exactly equal to that of the singlet state responsible for fluorescence emission, $S_1^{0'}$ (Figure 3). This energy difference is termed the Stokes loss and is due to re-orientation of the solvent electric dipole to the essentially 'new' solute, *i.e.* the excited singlet state (S_1^0) to produce the 'relaxed' species ($S_1^{0'}$). Recent measurements of the magnitude of the Stokes loss have yielded a value of 10 kJ mol^{-1} in 3-methylpentane,[11] although higher values are expected in more polar solvents.

The shape of the fluorescence emission spectrum and its intensity are both pH dependent. At high pH (> 9) a pronounced decrease in fluorescence intensity, but little change in spectral distribution is observed.[29] This is compatible with deprotonation of the flavin in both the ground and excited singlet state, with both states possessing similar pK_a values (*i.e.* ≈ 10). Similarly, at low pH (< 3.5) a decrease in the fluorescence emission intensity is observed from which an apparent pK_a, for ^{1}Fl of $\sim$ 2, can be derived[29] (compare pK_a, Fl_{ox} = 0). In contrast, the weak emission spectrum attributed to the cationic singlet state (^{1}Fl—H$^+$) shows a blue shift relative to the spectrum of the neutral form (^{1}Fl).[27] Application[30] of the Förster–Weller thermodynamic cycle to these results predicts the pK_a of ^{1}Fl to be lower than that of Fl_{ox}. Fluorescence lifetime studies[30] have resolved this discrepancy by showing that protolytic equilibria in the excited singlet state are not completely established (Equation 1) owing to the extremely short lifetime of ^{1}Fl–H$^+$.

$$^1Fl + H_3O^+ \rightarrow {}^1Fl\text{–}H^+ + H_2O \qquad (1)$$

Hence results obtained from the pH *vs* intensity plots do not represent a conventional 'pK_a' type situation. The fluorescence of the cation of riboflavin (^{1}Fl–H$^+$) can in fact only be observed at low temperatures (77 K). However, some cationic flavins are fluorescent even at room temperature,[27] particularly those possessing a covalent bridge between the N-1 and N-10 positions. This again suggests that the rigidity of the chromophore plays an important role in determining the rate of radiationless transitions. It is of particular interest that such model cations are much more fluorescent in non-polar solvents than in aqueous solution, emphasizing the importance of interactions between the charged species and the solvent dipole.

The absorption spectra of most flavoenzymes in aqueous solution can be divided into two distinct classes. In the first (type A), the flavin portion of the spectrum is essentially identical to that of protein-free flavins. In the second (type B) the absorption spectrum exhibits a variable degree of vibrational resolution in the 450 nm band together with a blue shift of the 375 nm band, similar in fact to free flavins in non-polar solvents. Hence it has been suggested

[28] Ph. Wahl, J. C. Auchet, A. W. J. G. Visser, and F. Müller, *FEBS Lett.*, 1974, **44**, 23.
[29] F. Kavanagh and R. H. Goodwin, *Arch. Biochem.*, 1948, **20**, 315.
[30] N. Lasser and J. Feitelson, *J. Phys. Chem.*, 1975, **79**, 1344.

that 'type A' spectra represent the flavin chromophore freely exposed to the bulk aqueous phase.[31] In contrast, a 'type B' spectrum may indicate that the flavin chromophore is wholly or partly 'buried' in the hydrophobic portion of the protein, *i.e.* in close contact with the aromatic amino-acids. The above interpretation has, however, recently been challenged by Visser and co-workers.[11] These authors suggest that the orientational constraints or rigidity imposed by the binding of the flavin to the protein is sufficient to account for the vibrational resolution in the absorption spectrum of 'type B' flavoenzymes.

For some flavoenzymes, the absorption spectrum may undergo a transition from one type to the other (usually A → B) upon the addition of small molecules such as the enzyme's substrate or an inhibitor. These changes could be variously attributed to alterations in the polarity of the flavin's environment or to the rigidity of the flavin chromophore. Whichever view is correct the observed changes almost certainly reflect changes in the tertiary structure of the protein chain.

The flavin fluorescence is often markedly quenched (or totally absent when the flavin is enzyme-bound). By analogy with considerations discussed for the absorption spectra, both polar and non-polar environments for the flavin moiety are indicated by the shape of the emission spectrum. Again, alternative explanations involve increased vibronic resolution resulting from the rigidity imposed by protein binding. This last interpretation may explain the pronounced blue shift observed in the fluorescence emission of the flavoenzyme transhydrogenase upon addition of $NADP^+$. Changes in the rigidity of the flavin chromophore upon the binding of $NADP^+$ could result in changes in the relative intensities of the vibronic transitions within the emission band without any change in their energy. Indeed, such a situation would parallel that described earlier for frozen solutions of free flavin in 3-methylpentane.[11] Two flavoenzymes that have received particular attention are lipoamide dehydrogenase (LAD) and D-amino-acid oxidase. (D-AAO). The flavin fluorescence of LAD is highly polarized even in aqueous solution, indicating that the rotational motion of the flavin is highly restricted and is therefore limited to the slow 'tumbling' of the protein itself.[32] Time-resolved fluorescence studies indicate that the two FAD molecules present in LAD are in different environments. The enzyme D-AAO exists in a monomeric form, possessing one FAD molecule, or in a dimeric form. The fluorescence lifetimes of FAD in the monomer and dimer are 130 and 40ps, respectively, indicating a substantial increase in FAD–protein interactions upon dimerization of the enzyme.[33] These lifetimes are only about 5% of those obtained for free FAD, and this is attributed to dynamic quenching by the protein.

(ii) *Fluorescence Quenching*. Flavin fluorescence can be reduced (quenched) in the presence of a wide range of compounds or ions.[34] Studies of fluorescence quench-

[31] K. V. Rajogopalan, F. O. Brady, and M. Kanda, *Vitam. Horm. (N.Y.)*, 1970, **28**, 303.
[32] A. J. W. G. Visser, H. J. Grande. F. Müller and C. Veeger, *Eur. J. Biochem.*, 1974, **45**, 99.
[33] N. Nakashima, K. Yoshihara, F. Tanaka, and K. Yagi. *J. Biol. Chem.*, 1980. **255**, 5261
[34] M. A. Slifkin in 'Charge transfer interactions of Biomolecules', Academic Press, New York. 1971, p. 132; D. B. McCormick, H. C. Li, and R. E. MacKenzie. *Spectrochim. Acta. Part A*, 1967, **23**, 2353.

ing have received much impetus from the observation that enzyme-bound flavin fluorescence is usually markedly lower (or even absent) than free-flavin fluorescence. An understanding of the mechanism of fluorescence quenching in flavoenzymes might therefore be expected to yield information regarding the environment of the flavin chromophore.

Two distinct mechanisms of fluorescence quenching have been recognized. In the first (termed the static mechanism), association may occur between the ground-state flavin and the quencher molecule resulting in the formation of a non-fluorescent (or less fluorescent) complex. In the second (termed the dynamic mechanism), excitation of the flavin may occur normally, but a collision with a quencher molecule may take place during the lifetime of ^1Fl resulting in the degradation of the electronic energy to heat.

The static mechanism appears to be dominant in fluorescence quenching by most organic molecules including aromatic hydrocarbons,[34] and the amino-acids tryptophan, tyrosine, histidine, and methionine.[35] The nature of the binding forces in flavin–amino-acid complexes is still a matter of investigation. Hydrophobic stacking of the isoalloxazine ring with the aromatic moieties of the amino-acids may be the dominant force in aqueous solution.[35] In addition, specific interactions *via* hydrogen bonding between the tyrosine hydroxy-group and the flavin may occur. A series of compounds have been prepared by McCormick and co-workers[36] in which the amino-acids tryptophan, tyrosine, histidine, or phenylalanine are attached *via* a peptide linkage to either the N-3 or N-10 position of the flavin chromophore. N.m.r. studies suggest that such peptides are associated intramolecularly in water in such a manner that the aromatic portions of the flavin and amino-acid are in a planar orientation. In addition, some dynamic quenching of the flavin fluorescence is suggested to occur as the fluorescence lifetime of the flavin–peptide is lower than expected.[37]

Flavin adenine dinucleotide (FAD) is the most common form of flavin to be found in enzymes. The absorption spectrum of FAD in aqueous solution differs only slightly from that of FMN, although the extinction coefficient at 260nm is significantly less than the sum of FMN and ADP. The ORD spectrum of FAD is substantially different from FMN, exhibiting a pronounced Cotton effect in the 260nm region.[38] The fluorescence emission intensity of FAD is only 20% as intense as that of FMN or RF in neutral aqueous solution,[39] the lower fluorescence efficiency of FAD being due to intramolecular complexing between the adenine and flavin moieties.[39] Time-resolved fluorescence studies indicate that the complexed or closed structure does not unfold within the excited singlet-state lifetime.[28] N.m.r. spectroscopy of FAD suggests that the adenine group lies over the benzenoid subnucleus of the flavin.[40]

[35] R. E. MacKenzie, W. Föry, and D. B. McCormick, *Biochemistry*, 1969, **8**, 1839.
[36] M. C. Falk, P. G. Johnson, and D. B. McCormick, *Biochemistry*, 1976, **15**, 639.
[37] A. J. W. G. Visser, T. M. Li, H. G. Drickamer, and G. Weber, *Biochemistry*. 1977, **16**, 4883.
[38] I. M. Gascoigne and G. K. Radda, *Chem. Comm.*, 1965, 534.
[39] D. B. McCormick in 'Molecular associations in Biology', ed. B. Pullman, Academic Press. New York, 1968. p. 377.
[40] R. H. Sarma. P. Dannies, and N. O. Kaplan, *Biochemistry*, 1968, **7**, 4359.

Comparatively little is known concerning the detailed mechanism of fluorescence quenching in flavin complexes, including FAD and flavoenzymes. Flash photolysis studies of flavin peptides,[41] FAD,[42] and some flavoenzymes[43] show that the yield of the excited triplet-state is reduced in parallel with the fluorescence intensity. Hence it follows that enhanced intersystem crossing (Figure 3, process 17) is not a principal mechanism of fluorescence quenching. Further, electronic energy transfer from the lowest excited singlet of the flavin to the quencher molecules singlet state is energetically unfeasible. Hence fluorescence quenching most likely proceeds by the enhanced non-radiative interconversion of $S_1 \rightarrow S_0$ (Figure 3, transition 16). This could be accomplished by electron transfer from the quencher (Q) to the flavin (Equation 2) as recently suggested.[44]

$$(^1\mathrm{Fl} \cdots\cdots \mathrm{Q}) \rightarrow \dot{\mathrm{Fl}}^- + \dot{\mathrm{Q}}^+ \qquad (2)$$

However no such free radicals are observed upon flash photolysis of flavin peptides,[41] FAD, or flavoenzymes.[43] Alternatively, partial charge transfer may occur, but spin inversion within the intermediate radical pair ($\mathrm{Fl}^{\gamma-} \cdots\cdots \mathrm{Q}^{\gamma+}$) may lead to deactivation rather than the formation of free radicals (Equation 3).

$$^1\mathrm{Fl} \cdots\cdots \mathrm{Q} \rightarrow (\mathrm{Fl}^{\gamma-} \cdots\cdots \mathrm{Q}^{\gamma+}) \rightarrow \mathrm{Fl} + \mathrm{Q} + \text{heat} \qquad (3)$$

Dynamic quenching is established as the quenching mechanism in the case of ionic quenchers such as iodide ions and transition-metal cations. The efficiency of iodide quenching of fluorescence seems to depend upon the flavin involved,[45] *e.g.* quenching cross-sections of 0.91, 0.68, and 0.56 are obtained for lumiflavin, riboflavin, and FMN, respectively. The lower value obtained for riboflavin compared to lumiflavin possibly reflects steric hindrance by the ribityl side-chain. The still lower value found for FMN is probably due to electrostatic repulsion between the iodide ion and the negatively charged phosphate groups of FMN. Halide ions are expected to quench fluorescence by heavy-atom spin–orbit perturbation leading to enhanced intersystem crossing (Figure 3, transition 17). However, no increase in the yield of the triplet state (^{3}Fl) is observed[46] upon fluorescence quenching by Br^- hence an alternative 'charge-transfer' type mechanism has been proposed (Equation 3).

Dynamic fluorescence quenching by transition metal cations may occur by at least three different mechanisms.[47] Electronic energy transfer may be involved in the case of Fe^{2+} or Cr^{3+} (*e.g.* Equation 4).

$$^1\mathrm{Fl} + \mathrm{Fe}^{2+}(^5T_2) \rightarrow \mathrm{Fl}_{ox} + \mathrm{Fe}^{2+}(5E) \qquad (4)$$

[41] D. B. McCormick, M. C. Falk, F. Rizzuto, and G. Tollin, *Photochem. Photobiol.*, 1975, **22**, 175.
[42] G. Colombetti, F. Lenci, J. F. McKellar, and G. O. Phillips, *Photochem. Photobiol.*, 1975, **21**, 303.
[43] A. J. W. G. Visser, F. Müller, and J. D. W. Van Voorst, *Biochem.. Biophys. Res. Commun.*. 1977, **77**, 1135.
[44] N. Nakashima, K. Yoshimura, F. Tanaka, and K. Yagi, *J. Biol. Chem.*, 1980, **255**, 5261.
[45] S. Bergstrom, *Chem. Scr.*, 1976, **9**, 193.
[46] N. Lasser and J. Feitelson, *Photochem. Photobiol.*, 1975, **21**, 249.
[47] A. W. Varnes, P. B. Dodson, and E. L. Wehry, *J. Am. Chem. Soc.*. 1972. **94**, 946.

Secondly, ions such as V^{3+} may quench by spin–orbit perturbation. Finally, quenching by electron transfer is also possible with reducing ions such as Fe^{2+}.

(iii) *Delayed Fluorescence.* Under special conditions two distinct types of delayed (or long-lived) fluorescence emission are observed from flavins. In the first, termed E-type fluorescence, emission spectrally identical to the ordinary fluorescence of flavins is observed, but with a much longer lifetime ($\approx$ 100 ms).[22] It arises *via* thermal activation of the lowest excited triplet state back to the singlet state followed by fluorescence emission from the latter. It is hence only observed in environments where the triplet state is sufficiently long-lived for this to occur, *e.g.* in rigid media. The magnitude of the energy difference between the singlet and triplet states is critical, as the probability of thermal activation is proportional to $e^{-E/RT}$, where 'E' = the transition energy (Figure 3, process 17 reversed). From the temperature dependence of delayed fluorescence, a value for 'E' of $\sim$22 KJ mol^{-1} was derived.[22]

The second type of delayed fluorescence, termed P-type, has been reported only recently. It arises by fluorescence emission from an excited dimer (or higher aggregate) formed in solvents of very low polarity.[11]

B. The Triplet State.—Flavins exhibit an orange-red (λ_{max} 610 nm) phosphorescence at low temperatures (< 150 K).[16] The quantum yield of phosphorescence (Φ_p) is generally quite low (*e.g.* Φ_p = 0.0012 in ethanol at 77 K).[48] This contrasts with flash photolysis data, which show that intersystem crossing is very efficient (Φ = 0.7), at least at room temperature in fluid solution.[49] Hence it would appear that the lowest excited triplet state (^{3}Fl) decays predominantly by non-radiative internal conversion (Figure 3, transition 19). The lifetime of phosphorescence at 77 K lies in the range 0.1 to 0.2 seconds, the exact value depending on the flavin and the solvent matrix.[50] This relatively long lifetime together with the polarized phosphorescence data are consistent with ^{3}Fl being predominantly $\pi\pi^*$ in character.[16] In fluid solution at room temperature, the lifetime of ^{3}Fl is much shorter[49] (10—100 μs), probably due to dynamic quenching by the ground-state flavin. Electron proton resonance (e.p.r.) spectra of ^{3}Fl have been determined at 77 K.[6,51] The zero field splitting energies of the three sub-levels (corresponding to M_s = −1, 0, +1) are amongst the lowest determined for phosphorescent bi- or tri-cyclic molecules, indicating extensive delocalization of the two unpaired electrons. Qualitatively, the unpaired electron spin density distribution resembles that of the flavosemiquinone ($H\dot{F}l$).

The phosphorescence of the flavin cation (in an acidic ethanol glass at 77 K) shows a blue shift relative to that of the neutral form.[51] Hence, application of the Forster–Weller cycle predicts that ^{3}Fl is less basic than the ground state (*i.e.* pK_a, $^3Fl < 0$). In contrast, flash photolysis of flavins in solution at room temperature clearly shows that ^{3}Fl is more basic than Fl_{ox}, with a pK_a of 4—5 being deter-

[48] A. Bowd, P. Byrom, J. B. Hudson, and J. H. Turnbull, *Photochem. Photobiol.*, 1968, **8**, 1.
[49] M. S. Grodowski, B. Veyret, and K. Weiss, *Photochem. Photobiol.*, 1977, **26**, 341.
[50] P. S. Song and W. E. Kurtin, *Photochem. Photobiol.*, 1969, **10**, 211.
[51] J. M. Lhoste, A. Haug, and P. Hemmerich, *Biochemistry*, 1975, **5**, 3290.

mined for the former.[52,53] Some support for the latter pK_a value and a possible explanation for the contrasting phosphorescence data have come from calculations of the π-electron densities on the pyridine-like nitrogens.[54] Assuming that such electron densities can be taken as an index of relative basicities, the results predict that while the N-1 position is more basic than N-5 in Fl_{ox}, the reverse should be true for $^3Fl_{ox}$. Hence the phosphorescence emission observed at 77 K would be from ^3Fl protonated as in the ground state, *i.e.* at N-1 (the proton would be fixed at this position at 77 K). In contrast, in fluid solution ^3Fl is predicted to be protonated at N-5 and hence the pK_a value determined by flash photolysis refers to this latter triplet-state.

Evidence from phosphorescence data for a dimeric triplet-state ($^3Fl_2$) has been reported by Song and co-workers.[55] This species emits phosphorescence of a lower energy than ^3Fl, but possesses a shorter lifetime.

3 The Photochemical Reactions of Flavins

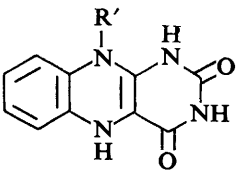
A. Classification and Mechanisms.—Flavin photochemistry can be conveniently divided into three categories; photoreduction, photodealkylation, and photoaddition. The following description of each reaction is by necessity somewhat idealized and hence the original publications should be consulted for further details. In particular, it should be realized that, depending upon the structure of the flavin and the reaction conditions, some or all of the photoreactions which follow may occur simultaneously.

(i) *Photoreduction.* Both intermolecular and intramolecular photoreductions are known. The intermolecular reaction occurs in the presence of a wide range of substrates (RH) including amino-acids, α-hydroxycarboxylic acids, thiols, aldehydes, and unsaturated hydrocarbons.[56a] Intermolecular photoreductions (Equation 5) yield either free 1,5-dihydroflavins (H_2Fl_{red}) or its alkyl derivatives [adducts, $R—Fl_{red}H$, (5)—(8)].

$$^*Fl + RH \rightarrow \underset{(\text{or } RCO_2^-)}{H_2Fl_{red}} \quad \text{or} \quad R—Fl_{red}H \qquad (5)$$

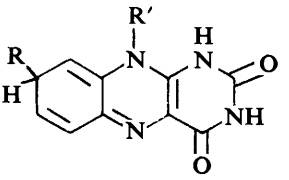
documented example of the influence of substrate structure is the photoreduction of flavins by cycloalkenes.[56b] With cyclopentadiene as substrate, quantitive yields of the C-4a adduct are found. In this case formation of the unsubstituted flavin Whether substituted ($RFl_{red}H$) or unsubstituted (H_2Fl_{red}) reduced flavins are formed appears to depend primarily upon the type of substrate (Table 1). A well

[52] S. Schreiner, U. Steiner, and H. E. A. Kramer, *Photochem. Photobiol.*, 1975, **21**, 81.
[53] S. Schreiner and H. E. A. Kramer in 'Flavins and Flavoproteins', ed. T. P. Singer. Elsevier. Amsterdam, 1976, p. 793.
[54] P. S. Song, *Photochem. Photobiol.*, 1968, **1**, 311.
[55] P. S. Song, T. A. Moore, and W. E. Kurtin, *Z. Naturforsch., Teil B.* 1972, **27**, 1011.
[56] (*a*) W. R. Knappe and P. Hemmerich, *Z. Naturforsch. Teil B*, 1972, **27**, 1032; W. R. Knappe and P. Hemmerich, *Liebigs Ann. Chem.*, 1976, 2037. (*b*) M. Brustlein, W. R. Knappe. and P. Hemmerich, *Angew. Chem., Internat. Ed. Engl.*, 1971, **10**, 804.



H_2Fl_{red}

(5)

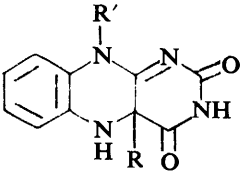


C^{8a}-$RFl_{red}H$

(6)

N^5-$RFl_{red}H$

(7)



C^{4a}-$RFl_{red}H$

(8)

(Equation 11*b*) is energetically unfavourable since the leaving group $R^+(C_5H_5^+)$ is anti-aromatic. In contrast, cyclohexa-1,4-diene yields H_2Fl_{red} since in this case process (11*b*) forms the energetically stable residue $C_6H_6(+H^+)$.

The reaction conditions may also play an important role, *e.g.*, when phenylacetic acid is the substrate. Depending upon the temperature, solvent, and/or pH, C-4a, N-5, or C-8a adducts are formed.[56a]

In all cases flavin reduction is observed spectrally under anaerobic conditions.[56] In the presence of oxygen, H_2Fl_{red} is re-oxidized[57] according to the following overall equation.

$$H_2Fl_{red} + O_2 \rightarrow H_2O_2 + Fl_{ox} \qquad (6)$$

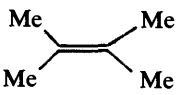
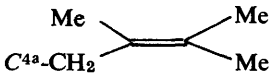
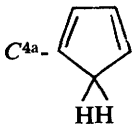
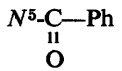
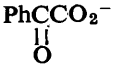
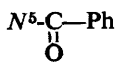
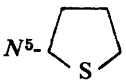
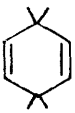
Detailed studies of process (6) show that the reaction is actually quite complex, with the transient formation of the flavosemiquinone anion and the superoxide anion ($\dot{O}_2^-$) being detected. The corresponding re-oxidation reaction of the adducts ($R—Fl_{red}H$) depends upon the position of attachment of the alkyl residue.[58a]

Two opposing mechanisms have been put forward for the intermolecular

[57] V. Massey, G. Palmer, and D. Ballow in 'Oxidases and related redox systems', ed. T. E. King, H. S. Mason, and M. Morrison, University Park Press, Baltimore, 1973, p. 25.

[58] (*a*) G. Blankenhorn and P. Hemmerich, *Tetrahedron*, 1979, **35**, 1129. (*b*) D. Clerin and T. C. Bruice, *J. Am. Chem. Soc.*, 1974, **96**, 5571.

Table 1 *Intermolecular photoreduction of flavins–reduced flavins formed with selected substrates*

Substrate	*Reduced flavin formed*
	
	
	
	
	
$Me_3CSCH_2CO_2^-$	C^{4a}-CH_2SCMe_3 + CO_2
$PhCH_2CO_2^-$	$C^{8\alpha}$-CH_2Ph + CO_2
$CH_2{=}CH{-}CH_2{-}CO_2^-$	C^{4a}-$CH_2{-}CH{=}CH_2$ + CO_2
$PhCH(OH)CO_2^-$	H_2Fl_{red}
	H_2Fl_{red}

photoreductions of flavins. In the first, photoreduction proceeds *via* an initial one-electron equivalent [either a hydrogen atom (Equation 7*a*), or an electron

(Equation 7*b*)] transfer from the substrate to the flavin yielding a flavosemiquinone radical.

$$*Fl + RH \rightarrow \begin{cases} \dot{H}\dot{F}l + \dot{R} & (7a) \\ \dot{F}l^{\cdot -} + \dot{R}H^{+} & (7b) \end{cases}$$

For carboxylate anions as substrates, we can rewrite Equation 7*b*:

$$*Fl + RCOO^{-} \rightarrow \dot{F}l^{\cdot -} + RCOO\cdot \qquad (8)$$

Equation 8 may then be followed by decarboxylation (Equation 9).

$$R{-}\overset{\dot{O}}{\underset{|}{C}}{=}O \rightarrow \dot{R} + CO_2 \qquad (9)$$

The final products could then be formed by disproportionation of the flavosemiquinone radical (Equations 10*a*, *b*) or by radical addition (Equation 10*c*).

$$\dot{H}\dot{F}l + \dot{H}\dot{F}l \rightarrow H_2Fl_{red} + Fl_{ox} \qquad (10a)$$

$$\dot{R} \rightarrow \text{Products} \qquad (10b)$$

$$\dot{H}\dot{F}l + \dot{R} \rightarrow RFl_{red}H \qquad (10c)$$

Diametrically opposed to the above view is the suggestion that photoreduction proceeds *via* a single two-electron transfer process[56a] [either a group transfer (Equation 11*a*) or a hydride transfer (Equation 11*b*)].

$$*Fl + RH \rightarrow RFl_{red}H \qquad (11a)$$

$$*Fl + RH \rightarrow HFl^{-}{}_{red} + R^{+} \qquad (11b)$$

The formation of the unsubstituted reduced flavin (H_2Fl_{red}) could also occur by protolysis (Equation 12*a*) of the first-formed substituted derivative (R—$Fl_{red}H$).[4]

$$RFl_{red}H + H_2O \rightarrow H_2Fl_{red} + ROH \qquad (12a)$$

It is difficult to ascertain the importance of process (12*a*) where H_2Fl_{red} is formed in protic solvents. In at least one case, for indole-3-acetic acid as substrate, process (12*a*) is slow enough to be studied.[58b]

Both the above mechanisms envisage that the reactive species involved (previously denoted *Fl) is the flavin triplet-state (^3Fl) in its free (*i.e.* uncomplexed) form. Good evidence for triplet-state involvement is the fact that intermolecular photoreductions are invariably inhibited by concentrations of excited-state quenchers (*e.g.* iodide ions) far too low to affect the much shorter-lived excited singlet-state.[59] Specific evidence in favour of a one-electron pathway (Equation 7) came from conventional flash-photolysis studies,[60,61] which showed that the triplet state was quenched by the addition of phenol or EDTA as substrates, and that the yield of HFl was increased. Furthermore, the flavosemiquinone radical (HFl) decayed by second-order kinetics, as expected for Equations

[59] B. Holmström, *Ark. Kemi*, 1964, **22**, 329.
[60] S. P. Vaish and G. Tollin, *Bioenergetics*, 1970, **1**, 181.
[61] S. P. Vaish and G. Tollin, *Bioenergetics*, 1971, **2**, 61.

10*a* or 10*b*. More detailed evidence for the involvement of ^{3}Fl comes from laser flash-photolysis experiments with amino-acids and phenylacetic acids as substrates.[62,63] These studies demonstrated that the decay of ^{3}Fl following laser excitation was concomitant with the build up of HFl absorption. Clearly the mere observation of HFl does not prove its involvement as the sole, or even major, intermediate in photoreduction. However, quantitative evaluation of flash photolysis spectra have shown that HFl is the only intermediate present immediately ($< 1\mu$s) after the reaction of ^{3}Fl with indoles or aromatic carboxylic acids.[62,63] Hemmerich and co-workers[79] have suggested that an initial two-electron primary process (Equations 11*a* or 11*b*) could be followed by comproportionation of H_2Fl_{red} (Equation 12*b*) to produce HFl. However, the rapid formation of the latter precludes such a process, as reaction 12*b* is far too slow.

$$H_2Fl_{red} + Fl_{ox} \rightarrow 2HFl \qquad (12b)$$

Further evidence for one-electron primary process has been obtained by use of the 'spin trap' 2-methylnitrosopropane[64] (NtB) which effectively prevents the formation of $RFl_{red}H$ in the presence of aromatic carboxylic acids as substrates. Further, the characteristic e.s.r. spectrum of the NtB adduct of the substrate radical (R) was observed, providing convincing evidence for the involvement of process 7. Finally, in some cases the products expected from the reactions of R have been observed, *e.g.*, dipyridyl is detected in the photoreduction of flavins by pyridine.[65]

The only direct evidence for the involvement of two-electron transfer has been obtained for borohydride as the substrate. Flash-photolysis studies[66] show clearly that $HFl^-{}_{red}$ is formed directly from ^{3}Fl by hydride transfer (Equation 11*b*).

In conclusion, therefore, firm evidence for one-electron primary processes has been obtained for substrates such as indoles, phenols, and carboxylic acids, whereas two-electron transfer is only proven for the obligatory hydride donor, borohydride. It remains to be seen which mechanism applies to substrates such as unsaturated hydrocarbons.

Several compounds (*e.g.* phenols and indoles) which have been shown by flash photolysis to be good electron donors nonetheless do not yield significant amounts of reduced flavins under conditions of continuous irradiation: this was thought to be due to the radical back reaction[61] (Equation 13).

$$HFl + R \rightarrow Fl_{ox} + RH \qquad (13)$$

However, a recent analysis of the light-intensity dependence of the yield of

[62] P. F. Heelis. B. J. Parson, G. O. Phillips, and J. F. McKellar, *Photochem. Photobiol.*, 1978. **28**, 169.
[63] P. F. Heelis and G. O. Phillips, *Photobiochem. Photobiophys.*, 1979, **1**, 63.
[64] M. Novak. A. Miller. T. C. Bruice, and G. Tollin, *J. Am. Chem. Soc.*, 1980, **102**, 1465.
[65] A. de Kok and C. F. A. H. Peters. *Z. Naturforsch., Teil B*, 1972, **27**, 1021.
[66] P. Hemmerich. W. R. Knappe. H. E. A. Kramer. and R. Traber. *Eur. J. Biochem.*, 1980. **104**, 511.

H_2Fl_{red} and the kinetics of decay of $\dot{H}\dot{F}l$, showed that an additional process (Equation 14) is needed to account for the low efficiency of photoreduction.[67]

$$\dot{H}\dot{F}l + RH \rightarrow Fl_{ox} + \dot{R}H_2 \qquad (14a)$$

$$\dot{R}H_2 + \dot{R} \rightarrow 2RH \qquad (14b)$$

Evidence for a net two-electron oxidation of substrates such as methionine by two separate one-electron transfers has been obtained by flash photolysis.[67] In this case, reaction 15 competes efficiently with the disproportionation of $\dot{H}\dot{F}l$ (Equation 10*a*).

$$\dot{H}\dot{F}l + \dot{R} \rightarrow HFl^{-}_{red} + \dot{R}^{+} \qquad (15)$$

Finally, some alternative primary processes involving dimeric triplet-states or a triplet–ground-state reaction are worthy of consideration. Spectroscopic and thermochemical studies[14,68*a*] suggest that flavins may be weakly associated in most solvents. Flash photolysis studies[68*b*] showed the formation of ion radicals, possibly *via* dissociation (Equation 16) of a dimeric triplet-state[55] ($^3Fl_2$) formed by excitation of such an aggregate (dimer ?).

$$^3Fl_2 \rightarrow \dot{F}l^{+} + \dot{F}l^{-} \qquad (16)$$

The electron-deficient cation radical (Fl^{+}) may then react further with added substrates (Equation 17).

$$\dot{F}l^{+} + RH \rightarrow Fl_{ox} + \dot{R} + H^{+} \qquad (17)$$

Note that the overall stoicheiometry of reactions 16—17 is identical to that of the more conventional scheme proposed earlier (Equations 7 and 10) and might therefore be difficult to distinguish in practice.

A further alternative has been proposed following flash photolysis studies of 3-methyl-lumiflavin. In this scheme,[66] the reaction of the flavin triplet-state with the ground state generates the oxidized and reduced radicals (Equation 18), which may then react further, as in Equation 17.

$$^3Fl + Fl_{ox} \rightarrow \dot{F}l^{+} + \dot{F}l^{-} \qquad (18)$$

Such a reaction represents an example of the so called D–D reaction, well known from dye photochemistry.

Further studies are clearly needed in order to distinguish between the various pathways proposed to account for intermolecular photoreductions.

The intramolecular photoreduction reaction involves dehydrogenation of the hydroxyalkyl side-chain at the N-10 position to yield a variety of ketonic or aldehydic functions in the side chain with or without partial loss of some of the

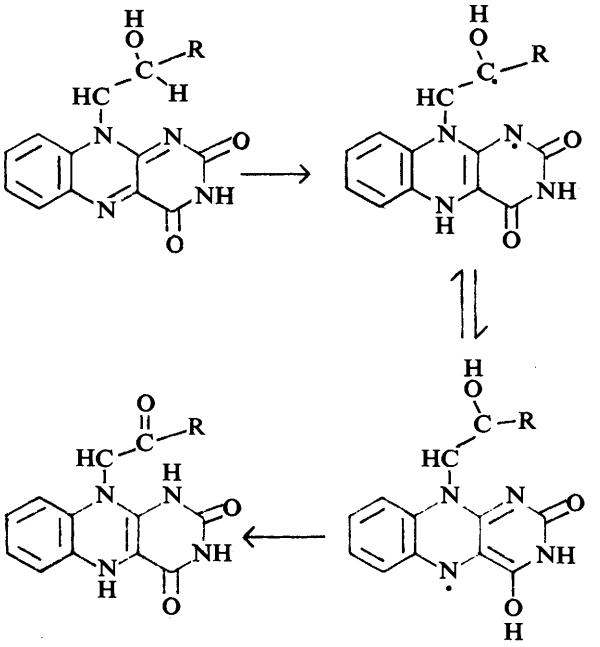
[67] P. F. Heelis, B. J. Parsons, G. O. Phillips, and J. F. McKellar, *Photochem. Photobiol.*, 1979, **30**, 343.

[68*a*] M. J. Medina de Gonzalez and N. Langermann. *Arch. Biochem. Biophys.*, 1977, **180**, 75.

[68*b*] S. G. Ballard, D. C. Mauzerall, and G. Tollin, *J. Phys. Chem.*, 1976, **80**, 341.

side-chain carbon-atoms.[69] Riboflavin has been extensively studied but presents a complex product-distribution, including 2′ and 4′ keto-derivatives together with formylmethyl flavin (Flavin—$\overset{1'}{C}H_2\overset{2'}{C}HO$), this last compound being formed *via* combined photoreduction and dealkylation.[69] Studies[70] of a variety of mono-hydroxyalkyl flavins show reactivities in the order 2′ > 3′ > 5′ > 6′ > 4′. This structural dependence probably reflects the importance of sterics factors, as dehydrogenation may proceed by attack of the back-bent side-chain upon the azomethine subgroup at C-4a—N-5 of the flavin nucleus, rather than peri-attack at N-1.[4] The pH dependence of intramolecular photoreduction of riboflavin or simpler derivatives suggests that the cationic triplet ($^3FlH^+$) may react differently from the neutral form (^3Fl).[69]

The mechanism of intramolecular photoreduction has been more difficult to ascertain than that of the intermolecular reaction. Again, both two- and one-electron equivalent transfers have been proposed as the primary photochemical process.[4,71] A kinetic isotope effect of 2.7 ($K_{H/^2H}$) has been observed[72,73] for replacement of a α-hydrogen in the side chain (—CH_2—C^2H_2OH), but no such effect has been seen for replacement of hydroxyl hydrogen.[73] This is consistent



Scheme 1

[69] W. L. Cairns and D. E. Metzler, *J. Am. Chem. Soc.*, 1971, **93**, 2773.
[70] C. S. Yang and D. B. McCormick, *J. Am. Chem. Soc.*, 1965, **87**, 5763.
[71] B. Holmström, *Ark. Kemi*, 1964, **22**, 329.
[72] W. M. Moore and C. Baylor, jun., *J. Am. Chem. Soc.*, 1969, **91**, 7170.
[73] W. M. Moore and R. C. Ireton, *Photochem. Photobiol.*, 1977, **25**, 347.

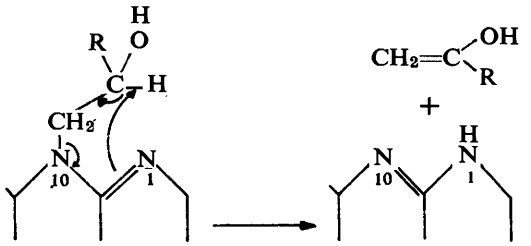
with a primary process involving hydrogen atom abstraction from the α-CH bond, leading to the formation of a biradical intermediate[73] (Scheme 1). Subsequent disproportionation of the biradical could then lead to the formation of a ketone. Alternative mechanisms that have been proposed include the intermediacy of an N-1—N-10 bridged compound and/or electron exchange between the biradical (Scheme 1) and Fl_{ox}. Evidence for hydrogen-atom transfer as the primary process is not, however, conclusive. Flash photolysis studies[61] of riboflavin in the absence of added substrates have detected an intermediate whose spectral characteristics are similar to that of HFl, *i.e.* as expected for the biradical intermediate. However, the intermediate detected may result from processes 16 or 18 rather than from intramolecular hydrogen transfer, particularly as lumiflavin, which possesses no hydroxylic side-chain, yields almost identical amounts of the radical-like intermediate.[61]

Quenching studies[69] indicate that intramolecular photoreduction may proceed by reaction both of the singlet and of the triplet states. Reactions from the singlet manifold, despite its short lifetime, are not unlikely for an intramolecular reaction since a substrate (side chain) is in close proximity. Calculations suggest that for efficient intramolecular hydrogen-transfer to occur the side chain should be co-planar with the main flavin ring-system.[74] The rate of intramolecular photoreduction is particularly sensitive to the solvent polarity. It has been suggested that this is due to changes in the conformation of the side chain in different solvents,[73] resulting from reorganization of the solvent sheath around the hydroxy-group(s).

(ii) *Photodealkylation.* Photodealkylation is strictly an intramolecular reaction, yielding an alloxazine and an alkene. It is a particularly important reaction as most, if not all, flavins ultimately undergo dealkylation as the final intramolecular reaction. Dealkylation involves synchronous breakage of the N-10—C-1′ and C-2′—H bonds (Scheme 2) in a *cis*-periplanar conformation with direct proton transfer.[4] An analogous reaction occurs for model flavins substituted at N-10 with some alkyl (not methyl or ethyl) or cycloalkyl groups. Photolysis in acetonitrile produces the alloxazine 'lumichrome' plus the corresponding alkene (or cycloalkene).[75] Although not a dealkylation, the decarboxylation of synthetic flavins possessing N-10 alkanoic acid substituents is formally similar. For example, photolysis of the N^{10}-(1′-methylethanoic acid) flavin yields lumichrome and acrylic acid.[76]

Comparatively little is known about the mechanism of photodealkylation. As for intramolecular photoreduction, flash photolysis studies have not provided any convincing information concerning the intermediates involved. Two alternative processes have, however, been proposed. The first involves homolytic fission of the N-10—C-1′ bond in the biradical intermediate previously mentioned (Scheme 1).[73] The second mechanism proceeds by a synchronous process[26] which does not

[74] P. S. Song and W. M. Kurtin, *Mol. Photochem.*, 1969, **1**, 1.
[75] M. Gladys and W. R. Knappe, *Chem. Ber.*, 1974. **107**, 3658.
[76] W. R. Knappe, *Chem. Ber.*. 1975. **108**, 2422.



Scheme 2

involve radical intermediates (Scheme 2). The excited singlet-state is thought to be the major intermediate involved in photodealkylation of riboflavin, since lumichrome formation is not inhibited by triplet-state quenchers.[69] Quenching studies indicate that intramolecular photodecarboxylation and dealkylation of model compounds can each proceed by both triplet- and singlet-state reactions.[75,76] Photodecarboxylation of flavin-10-acetic acid proceeds by initial attack on ^{3}Fl of a second molecule in the ground state, to produce a biflavin intermediate,[76] which undergoes protolysis ultimately to yield lumichrome. In contrast, photodecarboxylation of flavin-10-propanoic acid at low pH proceeds *via* a triplet mediated intramolecular reaction leading to a N-1—N-10 bridged structure. In more alkaline solution complete dealkylation occurs *via* both the singlet and triplet states.

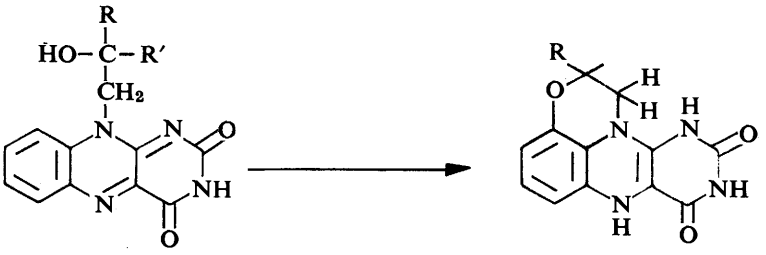
(iii) *Photoaddition*. This reaction follows Equation 19 and yields hydroxy- or alkoxy-dihydroflavins as first intermediates.

$$^*\text{Fl} + \text{ROH} \rightarrow \text{RO—Fl}_{\text{red}}\text{H} \qquad (19)$$

Intermolecular photoaddition has been reported where ROH is the solvent[77] (R = H or alkyl) and the solvent residue ('RO') adds to the C-6 or C-9 positions of the benzenoid subnucleus. An analogous reaction occurs with cyanides or with ammonia.[78] Intramolecular photoaddition[79,80] occurs if a free hydroxy-group is present at the C-2′ position yielding the intermediate shown in Scheme 3. It is of some interest that the presence of divalent ions (*e.g.* HPO_4^{2-}) is required for the intramolecular reaction to occur.[79] Although the reason for this is unknown at present, re-orientation of the C-2′ hydroxy-group to facilitate reaction is presumably involved.

Photoaddition appears to proceed by nucleophilic attack (of CN^- or NH_3) upon the flavin triplet-state, as shown by recent flash photolysis studies. This can be considered to be analogous to the well known photohydration reactions shown by other nitrogen heterocyclic compounds (*e.g.* uracil and phenazine[81]). Photoaddition by water or intramolecular addition of a hydroxy-group appears to

[77] G. Schöllhammer and P. Hemmerich, *Eur. J. Biochem.*, 1974, **44**, 561.
[78] R. Traber, H. E. A. Kramer, W. R. Knappe, and P. Hemmerich, *Photochem. Photobiol.*, 1981, **33**, 807.
[79] M. S. Jorns, G. Schöllhammer, and P. Hemmerich, *Eur. J. Biochem.*, 1975, **57**, 35.
[80] M. S. Jorns and P. Hemmerich, *Z. Naturforsch., Teil B*, 1972, **27**, 1040.
[81] 'Photochemistry of Heterocyclic Compounds', ed. O. Buchardt. Wiley Interscience. New York, 1976.



Scheme 3

proceed by reaction of ^{1}Fl and is hence the predominant photoreaction only if the triplet state is suppressed (*e.g.* by oxygen quenching).[77,79]

(iv) *Miscellaneous Photoreactions.* Space does not permit the consideration of many other interesting studies of flavin photochemistry. However, the reader may wish to consult original studies dealing with photo-electron ejection,[82] electronic energy-transfer,[83] photochemistry of flavins anisotropically aligned in polymers[84] and lipid vesicles,[85] cyclization of flavins possessing a phenyl substituent at N-10,[86] the use of ^{13}C n.m.r. in monitoring flavin photoreactions,[87] photohydrolysis and ring-cleavage reactions,[88] photodissociation of water by flavins absorbed to solid supports,[89] and finally the photodehalogenation of model flavins.[90]

(v) *Photochemistry of Flavoproteins.* Flavoprotein photochemistry is of particular relevance to photoreception in biology as the flavin photoreceptor-pigment would almost certainly be enzyme-bound rather than a free flavin. To date, though, the identification of the precise flavin or flavoenzyme responsible for photoreception has not been achieved. Action spectra for the photoresponse of the unicellular alga *Euglena gracilis* resemble those of flavins in a non-polar environment, suggesting a lipophilic site or lipoprotein.[2]

The photochemistry of pure preparations of a number of flavoenzymes has been studied by several groups. In general flavoenzymes are found to be photochemically inert, in agreement with the almost complete quenching of the fluorescence and triplet yields of the flavin upon enzyme binding (see p. 24). Photoinactivation of some flavoenzymes (*e.g.* glucose oxidase) occurs upon irradiation with visible light in the presence of oxygen.[91a] Such effects, however, may be due to the photo-oxidation of the protein moiety by free flavins (invariably

[82] N. Getoff, S. Solar, and D. B. McCormick, *Science*, 1978, **201**, 616.
[83] J. Posthuma and W. Berends, *Biochem. Biophys. Acta*, 1966, **112**, 442.
[84] R. Drabent and B. Siodmiak, *Acta Phys. Pol.*, 1975, **A47**, 837.
[85] W. Schmidt and P. Hemmerich, *J. Membrane Biol.*, 1981, **60**, 129.
[86] W. R. Knappe in 'Flavins and Flavoproteins', ed. T. P. Singer, Elsevier, Amsterdam, 1976, p. 788.
[87] M. D. D. MacMurchie and R. J. Cushley, *Can. J. Chem.*, 1978. **56**, 1045.
[88] F. Yoneda, Y. Sakuma, and K. Shinozuka, *J. Chem. Soc., Chem. Commun.*, 1977, **6**, 175.
[89] G. N. Lyalin, *Biofizika*, 1975, **20**, 535.
[90] V. Massey, M. Husain, and P. Hemmerich, *J. Biol. Chem.*, 1980, **225**, 1393.
[91] (*a*) G. H. Schmid, *Phytochemistry*, 1971, **10**, 2041. (*b*) D. B. McCormick in 'Flavins and Flavoproteins', ed. H. Kamin, University Park Press, Baltimore. 1971. p. 154.

present to a small extent). Intermolecular photoreduction of many flavoenzymes under anaerobic conditions has been observed with substrates such as nicotine or EDTA.[91b] In many cases, however, photoreduction of trace amounts of free flavins is the primary process, followed by electron transfer from free HFl to the enzyme-bound flavin. A similar catalysed photoreduction has been described recently upon addition of trace amounts of deazoflavins.[92a] As noted earlier, some flavoenzymes (*e.g.* D-amino-acid oxidase) do exhibit fluorescence and triplet formation. In these cases true or direct photoreduction of the flavin moiety may occur. In addition, D-amino-acid oxidase exhibits a unique reversible photo-release of FAD, apparently without damage to the binding site.[92b]

B. Further Considerations of Triplet-state Reactivity.—A major factor leading to the predominance of triplet-state reactions for flavins (and many other organic compounds) is the long lifetime of the triplet state compared to the singlet state. However, triplet states possessing $\pi\pi^*$ symmetry are generally expected to be inactive in photo-redox reactions as is indeed found for a large range of nitrogen heterocycles.[81] Flavins appear to be an exception, but this may be explained by a mixing of the lowest $^3\pi\pi^*$ state with upper $^3n\pi^*$ states *via* vibronic and spin–orbit coupling, producing a hybrid triplet-state.[26]

Recent studies using laser flash-photolysis[63] have attempted to distinguish between true one-electron uptake by ^{3}Fl (Equation 7*b*) and hydrogen atom abstraction (Equation 7*a*). Some substrates appeared to react preferentially by electron donation and others by hydrogen atom donation. It is of interest that the former substrates are generally more reactive towards $^3FlH^+$ than ^{3}Fl, whereas for the latter substrates the reverse applies. Indeed, this behaviour is in agreement with thermodynamic considerations,[93] which predict that the free-energy change upon electron uptake by $^3FlH^+$ is more negative than for ^{3}Fl. Further calculations have shown that the reverse is true for hydrogen atom abstraction.[94]

Recent laser flash-photolysis studies[95] determined the influence of the solvent dielectric constant upon electron abstraction by ^{3}Fl from both Fl_{ox} and 2,6-dimethylphenol. No solvent effects were observed, other than those due to solvent viscosity, which suggests the intermediacy of a dipolar species in electron transfer (Equation 20).

$$^3Fl + RH \rightarrow (\overset{\gamma^-}{F} \cdots\cdots \overset{\gamma^+}{RH}) \rightarrow \overset{\cdot}{Fl}^- + RH^+ \qquad (20)$$

C. Flavin-sensitized Photo-oxidations.—In the presence of oxygen, flavins are well known to sensitize or catalyse the oxidation of a wide range of substrates

[92] (*a*) V. Massey and P. Hemmerich, *Biochemistry*, 1978, **17**, 9. (*b*) G. Colombetti, F. Lenci, J. F. McKellar, and G. O. Phillips, *Photochem. Photobiol.*, 1975, **21**, 303.
[93] E. Vogelmann, S. Schreiner, W. Rauscher, and H. E. A. Kramer, *Z. Phys. Chem. (Frankfurt am Main)*, 1976, **101**, 321.
[94] P. F. Heelis, B. J. Parsons, and G. O. Phillips, *Biochim. Biophys. Acta*, 1979, **587**, 455.
[95] I. Ahmad and G. Tollin, *Biochem.*, 1982, in the press.

e.g. amino-acids,[96] proteins,[97] DNA and nucleotides,[98] and lipids.[99] In the case of micro-organisms as 'substrates', photodamage to cellular components may even result in cell death.[100]

The mechanisms of photo-oxidations in general have been extensively reviewed.[101] As might be expected from the previous discussion of photo-reductions, flavins are frequently found to photo-oxidize by electron abstraction from the substrate (a so called radical or type I mechanism). In this case the mechanism differs from those given previously for photoreduction in that reactions of both flavosemiquinone and substrate radicals with oxygen are now possible. Either of these two reactions may also prevent back reactions of $\dot{H}Fl$ and $\dot{R}$ (Equation 13) and hence result in efficient photo-oxidation of substrates such as phenols and indoles. In addition, re-oxidation of the reduced flavin (Equation 6) serves to re-cycle the flavin sensitizer.

Flavins are also able to sensitize photo-oxidations by non-radical (type II mechanism) processess, which involve energy transfer from ^3Fl to oxygen in its ground state ($^3O_2$, Equation 21).

$$^3Fl + {}^3O_2 \rightarrow Fl_{ox} + {}^1O_2 \qquad (20)$$

The excited singlet-state of oxygen so formed ($^1O_2$) is well known as a reactive intermediate and can in turn result in the photo-oxidation of a wide range of compounds.

Since both oxygen and the substrate are competing for the same excited state (^3Fl), the relative contribution of the two different mechanisms depends upon the rates of reaction of oxygen and the substrate with ^3Fl and the subsequent reaction of $^1O_2$ with the substrate. Hence, whichever mechanism predominates depends upon the type of substrate and reaction conditions (pH, solvent, concentrations *etc.*). This is well illustrated by the photo-oxidation of the amino-acid methionine, which is thought to proceed by a type I process in aqueous air-saturated solution to yield methional. In contrast, in non-aqueous solution (where the oxygen concentration is much higher) methionine is photo-oxidized by a type II process, to yield methionine sulphoxide.[62]

Photo-oxidations may also proceed by direct triplet–triplet energy-transfer from ^3Fl to the substrate, providing that the triplet-state energy of the substrate lies below that of ^3Fl ($\approx 200\,KJ\,mol^{-1}$). Such a mechanism may operate in the flavin sensitized photo-oxidation (or photoisomerism) of retinol,[102] bilirubin,[103] and stilbenes.[104]

[96] G. R. Penzer, *Biochem. J.*, 1970, **116**, 733.
[97] J. M. Rolden, F. Calero, and P. J. Aparicio, *Z. Pflanzenphysiol.*, 1978, **90**, 467.
[98] W. T. Speck, C. C. Chen, and H. S. Rosenkranz, *Pedriatr. Res.*, 1975, **9**, 150.
[99] H. W. S. Chan, *J. Am. Oil Chem. Soc.*, 1977, **54**, 100.
[100] O. M. Pereira, J. R. Smith, and L. Packer, *Photochem. Photobiol.*, 1976, **24**, 237.
[101] C. S. Foote in 'Free Radicals in Biology', vol. 2, ed. W. A. Pryor, Academic Press, New York, 1976, p. 85.
[102] S. Futterman and M. H. Rollins, *J. Biol. Chem.*, 1973, **248**, 7773.
[103] D. V. Sanvordeker and H. B. Kostenbouder, *J. Pharm. Sci.*, 1974, **63**, 404.
[104] A. Gordon-Walker and G. K. Radda, *Biochem. J.*, 1970, **120**, 673.