The Chemistry and Biological Function of Isoalloxazines (Flavines)

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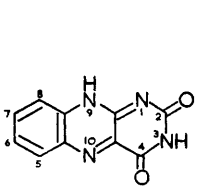
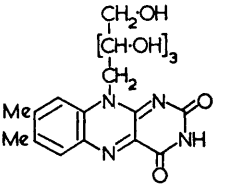
1 Introduction

The significance of derivatives of isoalloxazine (1) in biological systems was recognised in the early 1930's. Warburg and Christian discovered an enzyme containing a yellow chromophore[1] (now known as the 'old yellow enzyme') and this was closely followed by the isolation of a yellow fluorescent vitamin (riboflavine) by Kuhn, György, and Wagner-Jauregg.[2] The early chemical work on riboflavine has been summarised by Wagner-Jauregg[3] and some other excellent reviews are available on a number of different aspects of flavine coenzymes.[4,5]

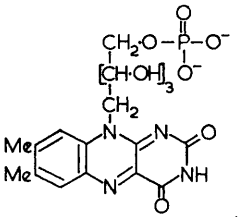
It is now common to refer to 6,7-dimethyl-9-alkylisoalloxazines as flavines and we shall follow this practice. Three biochemically important flavines are known: riboflavine (2), flavine mononucleotide (3), and flavine-adenine dinucleotide (4). Some others have been reported.[6]



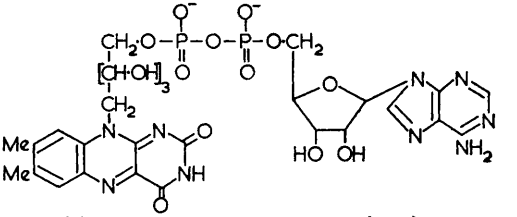
(1) Isoalloxazine



(2) Riboflavine



(3) Flavine mononucleotide (FMN)



(4) Flavine-adenine dinucleotide (FAD)

[1] O. Warburg and W. Christian, *Naturwiss*, 1932, **20**, 688.
[2] R. Kuhn, P. György, and T. Wagner-Jauregg, *Ber.*, 1933, **66**, 317, 576.
[3] T. Wagner-Jauregg in 'The Vitamins', ed. W. H. Sebrell and R. S. Harris, Academic Press, New York, 1954, vol. III, p. 299.
[4] H. Beinert in 'The Enzymes', ed. P. D. Boyer, H. Lardy, and K. Myrbäck, Academic Press, New York, 1960, vol. II, p. 339.
[5] P. Hemmerich, C. Veeger, and H. C. S. Wood, *Angew. Chem.*, 1965, **77**, 1.
[6] Ref. 4, p. 397.

The purpose of the present Review is to discuss aspects of the chemistry of flavines which are related, or at least potentially related, to their biological function. We hope it will become clear that this involves the application of ideas from different disciplines of chemistry.

The Biological Functions of Flavines.—Riboflavine is a well-known vitamin,[3] and its action is closely associated with the function of flavine nucleotides in biochemical oxidations. FMN and more often FAD function as coenzymes in the oxidation of amino-acids, amines, carboxylic acids, reduced nicotinamide nucleotides, thiol groups, etc. Flavines appear to play a part in bacterial dehydrogenation, and nitrate assimilation in fungi and plants. They are also implicated in many other systems like that of thyroid iodination.[7] Flavines are involved in several photobiological processes. For instance, FMN participates in photosynthetic phosphorylation in isolated chloroplast,[8] and it enhances bacterial bioluminescence.[9] Crystalline riboflavine is present in the eyes of some fish and mammals.[10] It might also be a photoreceptor in phototropism of plants.[11]

The chemical problems raised by these natural functions are of three general types: the properties related to interaction with proteins, catalytic function, and photobiology.

2 Non-covalent Interactions of Flavines

A. Spectra.—*Visible and ultraviolet.*—The electronic spectrum of riboflavine in water consists of four bands centred around 220, 265, 375, and 447 mμ (Figure 1). The spectrum of FMN is identical while that of FAD differs in several respects (see Section 2*b*). The precise positions of the absorption maxima and the extinction coefficients depend on the environment of the flavine chromophore, and a knowledge of these effects is essential for the understanding of the state of flavines in biochemical systems.

Solvents affect the position of the 375 mμ band, generally shifting it to shorter wavelengths with decreasing solvent polarity. For instance, for 3,6,7,9-tetramethylisoalloxazine (3-methyl-lumiflavine) the absorption maxima in different solvents are: 444, 369 in water; 448, 362 in formamide; 448, 344 in NN-dimethylformamide, and 450, 347 mμ in chloroform.[12] Good correlations between transition energies and an empirically derived solvent parameter (*Z*-value) have been observed in mixed aqueous solvents.[13] We find that a similar correlation holds for different non-aqueous solvents. It is difficult to decide how important

[7] Ref. 4, p. 412.
[8] F. R. Whatley, M. B. Allen, and D. I. Arnon, *Biochim. Biophys. Acta*, 1959, **32**, 32.
[9] W. D. McElroy and H. H. Seliger in 'Advances in Enzymology', ed. F. F. Nord, Interscience, New York, 1963, vol. XXV, p. 119.
[10] A. Pirie, *Nature*, 1960, **186**, 352.
[11] K. V. Thimann and G. M. Curry, 'Comparative Biochemistry', ed. M. Florkin and H. S. Mason, Academic Press, New York, 1960, vol. 1, p. 281.
[12] H. A. Harbury, K. F. LaNoue, P. A. Loach, and R. M. Amick, *Proc. Nat. Acad. Sci.*, 1959, **45**, 1708.
[13] J. Koziol and E. Knobloch, *Biochim. Biophys. Acta*, 1965, **102**, 289.

the hydrogen-bonding contribution is to the spectral shifts because solvent polarity and ability to form hydrogen bonds often run parallel.

The position of the visible band is hardly affected by solvent but the band splits into several inflections in a non-polar environment.

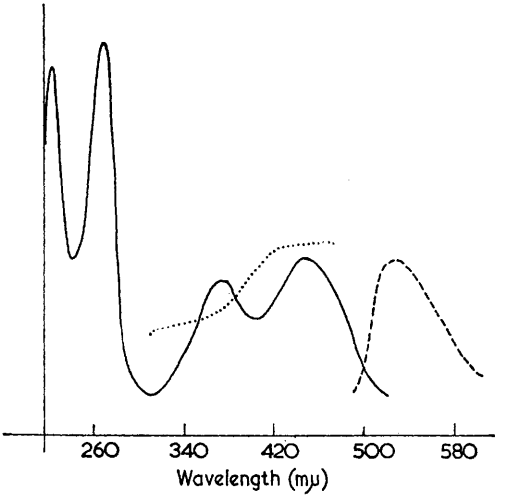


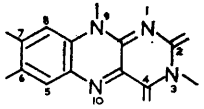
FIG. 1. *Riboflavine spectra,* ——— *absorption;* - - - - - *fluorescence;* . . . . *polarisation of fluorescence*

The effect of substituents on the electronic transitions of the isoalloxazine chromophore has not been systematically studied except for the work of Dudley, Ehrenberg, Hemmerich, and Müller.[14] There are, however, examples in the literature and some of these are summarised in Table 1. Methyl substitution in the 6-position results in a bathochromic shift in the two long-wavelength absorption bands although it apparently only affects the 375 mμ band in the 5- and 7-positions. It has been suggested that the 5-methyl group forces the N(10) = C–C = N(1)– group out-of-plane producing the bathochromic shift observed.[15] Since a 7-methyl group has a similar effect this explanation seems unlikely. In general, as expected, extension of conjugation (*e.g.*, compound XIV Table 1) causes a displacement of the absorption bands to longer wavelengths. Substituents on nitrogen atoms 9 and 3 have only a small influence on the absorption spectra.

Loss of the proton from the 3-position causes a small bathochromic shift. Similar effects may be present in some flavoproteins. The protonated form of riboflavine has a spectrum nearly identical with compound (XVI) but different from those of (XVII) and (XVIII) (Table 1) indicating that the most basic position in N(1) in the isoalloxazine nucleus.

[14] K. H. Dudley, A. Ehrenberg, P. Hemmerich, and F. Müller, *Helv. Chim. Acta*, 1964, **47**, 1354.
[15] V. M. Berezovskii and E. P. Rodinova, *J. Gen. Chem.* (*U.S.S.R.*), 1958, **28**, 1016.

Table 1 *The effect of substituents on isoalloxazine spectra*



Compound number	*Substituents:* 1	2	3	4	5	6	7	9	*Near U.V.* (λ_{max}, mμ)	*Visible* (λ_{max}, mμ)	*Ref.*
I	–	=O	H	=O	H	H	H	Me	348	433	*a*
II	–	=O	H	=O	H	Me	H	$(CH_2)_2$ NEt_2	356	447	*a*
III	–	=O	H	=O	H	Me	Me	ribityl	375	447	*a*
IV	–	=O	H	=O	H	Me	Me	Me	375	447	*a*
V	–	=O	H	=O	Me	Me	H	ribityl	382	448	*b*
VI	–	=O	H	=O	Me	Me	Me	ribityl	394	446	*b*
VII	–	=O	Me	=O	H	Me	Me	Me	369	444	*a*
VIII	–	=O	H	=O	H	Cl	H	$(CH_2)_3$ NEt_2	338	446	*a*
IX	–	=O	H	=O	H	H	Cl	$(CH_2)_3$ NEt_2	361	431	*a*
X	–	=S	H	=O	H	Me	Me	Me	364,400	490	*a*
XI	–	=O	H	=S	H	Me	Me	Me	367	452	*a*
XII	–	=NH	H	=O	H	Me	Me	Me	384	443	*c*
XIII	–	$=N-CH_2-CH_2OH$	H	=O	H	Me	Me	Me	366	454	*a*
XIV	–	=N–N=CHPh	H	=O	H	Me	Me	Me	not recorded	510	*d*
XV	H	=O	H	=O	H	Me	Me	Me+	264,390	—	*d*
XVI	CH_2	=O	H	=O	H	Me	Me	CH_2+	262,390	—	*d*
XVII	–	–OEt	–	–OEt	H	Me	Me	Me+	262,402	—	*d*
XVIII	–	–OEt	Et	=O	H	Me	Me	Me+	262,394	—	*d*

[a] These spectra, although mentioned in the literature, have been rerecorded to be included in this Review. The compounds were given by the original authors: Drs. R. M. Acheson, P. Hemmerich, and G. Tollin. [b] V. M. Berezovskii and E. P. Radinova, *J. Gen. Chem., U.S.S.R.*, 1958, **28**, 1016. [c] R. M. Cresswell, A. C. Hill, and H. C. S. Wood, *J. Chem. Soc.*, 1959, 698. [d] K. H. Dudley, A. Ehrenberg, P. Hemmerich, and F. Müller. *Helv. Chim. Acta*, 1964, **47**, 1354.

A plausible interpretation of the spectrum may be as follows. The two longer wavelength transitions are both of π–π^* type (the high extinction coefficients are consistent with this), the 375 mμ band having some contribution from an intramolecular charge-transfer process leading to a polar excited state. This would account for the solvent-dependence of its position. The inflections in the long-wavelength band observed in non-polar solvents correspond to vibrational levels within a single electronic transition.[16] This may be shown by a study of the polarisation spectrum of fluorescence (Figure 1). This polarisation is constant across each of the last two absorption bands[16] (0·43 and 0·28 for the 447 and 375 mμ bands respectively with glycerol as solvent and linearly polarised light for excitation) indicating that in this region there are only two independent electronic transitions. The transition moments are probably at an angle of about 30° although molecular orbital calculations[17] give this angle as 80°. It would be interesting to measure these moments from the spectra of single crystals.

Fluorescence.—Flavines show an intense fluorescence with maximal emission at 530 mμ (Figure 1). Riboflavine has a quantum yield of fluorescence of 0·25 in aqueous solutions,[18] 0·52 in 90% dioxan–water, and 0·71 in dioxan.[12] Other organic solvents (*e.g.*, dimethyl sulphoxide) on the other hand decrease the quantum yield.[19] The fluorescence of flavine nucleotides is weaker than that of riboflavine, FAD being considerably weaker.[20] Only the uncharged forms of flavines are fluorescent and not their anions or cations.

The flavine fluorescence is generally quenched when the chromophore is bound to a protein. Studies of the fluorescence of flavines are therefore mostly concerned with the effect of quenchers and quenching mechanisms. In the case of riboflavine quenching is by a variety of substances: electrolytes (*e.g.*, KI), metal ions, aromatic substances (such as phenols, purines, and pyrimidines), or thiols (*e.g.*, lipoic acid and thioglycolic acid).[20,21]

Two types of quenching mechanism have been observed. The first, brought about by electrolytes including most metal ions, is due to collisions in which the electronic excitation energy is transformed into kinetic energy. The second (discussed in detail in Section 2(*b*) involves the formation of non-fluorescent complexes in the ground state. These two mechanisms can be easily distinguished. It is known that if the quenching process is competitive with the emission the relation $F_0/F = \tau_0/\tau$ holds.[22] (F_0 is the fluorescence efficiency and τ_0 the life-time of the excited state in the absence of quencher, and F and τ are the corresponding quantities in the presence of quencher.) Thus collisional quenching causes a decrease in the life-time of the excited state but quenching brought about by the formation of a non-fluorescent complex in the ground state causes

[16] G. Weber, 'Flavines and Flavoproteins' ed. E. C. Slater and C. Veeger, Elsevier, Amsterdam, 1966.
[17] B. Grabe, personal communication.
[18] G. Weber and F. W. J. Teale, *Trans. Faraday Soc.*, 1957, **53**, 646.
[19] I. M. Gascoigne and G. K. Radda, *Chem. Com.*, 1965, 534.
[20] G. Weber, *Biochem. J.*, 1950, **47**, 114.
[21] W. J. Rutter, *Acta. Chem. Scand.*, 1958, **12**, 438; K. Sakai, *Nagoya J. Med. Sci.*, 1956, **18**, 245.
[22] F. Perrin, *Ann. Physique*, 1929, **12**, 169.

no change in the life-time of the excited state, *i.e.*, $\tau_0/\tau = 1$. In a situation where both processes may contribute to quenching $F_0/F > \tau_0/\tau$. F_0/F can be directly measured while τ_0/τ can be calculated from measurements of fluorescence polarisation according to the equation:[20,23]

$$\frac{\tau_0}{\tau} = \frac{\frac{1}{P_i} - \frac{1}{P_0}}{\frac{1}{P} - \frac{1}{P_0}}$$

where P_0 refers to the value of polarisation at 'infinite' rigidity of the chromophore during the life-time of the first excited state and P_i and P are the measured polarisations in the absence and presence of quencher respectively.

Quenching by collisions has two other characteristics that can be used to identify it. First, it is more efficient at higher temperatures owing to the increase in the number of collisions and the decrease in the viscosity of the solvent. Secondly, quenching is not linearly related to the concentration of the quencher at high concentrations, for quenching efficiency increases steadily.[20]

Competitive quenching of flavine fluorescence by substances such as KI and thioglycolic acid is observed while with aromatic systems normally a mixed type of quenching with a major contribution from the static mechanism occurs. On the other hand even for aromatic substances quenching is entirely collisional in non-aqueous solvents (*e.g.*, for caffeine in chloroform and naphthalenesulphonate ions in formamide; see Table 2).

Detailed studies on fluorescence quenching may provide further information. For instance, from the concentration-dependence of competitive quenching the life-time of the excited state can be calculated according to the equation derived by Wawilov.[24] For riboflavine this is approximately 10^{-8} sec. From this life-time, and measurements of polarisation of fluorescence in media of different viscosities, the molecular volumes in solution can be calculated from the relation:

$$\frac{1}{P} = \frac{1}{P_0} + \left(\frac{1}{P_0} - \frac{1}{3}\right)\frac{RT}{\eta V}\tau_0$$

(η = viscosity, V = molecular volume). The molar volumes for riboflavine and FAD are 550 and 756 ml. respectively. These might be useful in understanding protein specificity for the different flavine nucleotides in terms of size.

Infrared.—In principle the infrared spectra of flavines should be useful in elucidating their mode of binding to proteins. In practice such measurements have so far been limited because of the difficulties in using aqueous solutions and low concentrations of flavines and proteins. For the unbound flavine it has been shown by infrared spectroscopy that the 4-oxygen is in the keto-form.[25]

[23] B. Sveshnikoff, *Acta. Physicochim. U.S.S.R.*, 1936, **4**, 453.
[24] S. I. Wawilov, *Z. Physik*, 1929, **53**, 665.
[25] J. T. Spence and E. R. Peterson, *J. Inorg. Nuclear. Chem.*, 1962, **24**, 601.

Table 2 *The quenching of flavine fluorescence**

Quencher (*ca.* 3 × 10^{-3}M)	*Quenching* $\left(\frac{I_0-I}{I_0}\right) \times 100$	*Ref.*
Phthalic acid	0	*a*
Nicotinamide	0	*a*
DL-Phenylalanine	0	*b*
NAD^+	0	*b*
Citric acid	<1	*a*
Allantoin	<1	*c*
Urea	1	*a*
Benzoic acid	2	*a*
Phenol-2,4-disulphonic acid	2	*a*
Methionine	4	*a*
Thioglycolic acid	5	*a*
L-Ascorbic acid	5	*a*
L-Tyrosine	6	*b*
Sulphanilic acid	8	*a*
p-Hydroxybenzoic acid	9	*a*
p-Aminobenzoic acid	9	*a*
Cytosine	9	*c*
Thiourea	10	*a*
Phenol	11	*a*, *e*
Pyrogallol	11	*a*
Thymine	11	*c*
Salicylic acid	12	*a*
Adenosine-5′ monophosphate	12, 15	*c*, *f*
Deoxyadenosine-5′ monophosphate	12	*f*
Resorcinol	13, 14	*a*, *e*
Catechol	15, 14, 19	*a*, *e*, *c*
Quinol	14, 16	*c*, *e*
Phloroglucinol	17, 16	*a*, *e*
Adenosine	23, 17	*c*, *f*
Deoxyadenosine	17	*f*
DL-Lipoic acid	18	*b*
Adenine	23, 19	*c*, *f*
Adenine in formamide	2	*f*
Caffeine	21	*c*, *d*
Caffeine in $CHCl_3$	1	*d*
p-Aminosalicylic acid	21, 22	*a*, *e*
DL-Tryptophan	22	*b*
Hypoxanthine	23	*c*
Naphthalene-β-sulphonic acid	29	*d*
Naphthalene-β-sulphonic acid in formamide	2	*d*

Quencher (*ca.* 3 × 10^{-3}M)	*Quenching* $\left(\frac{I_0-I}{I_0}\right) \times 100$	*Ref.*
Serotonine creatinine sulphate	36	*b*
β-Hydroxynaphthoic acid	100	*a*
FAD compared to FMN	80	*b*
0·5 M-Phenobarbital in FMN	33	*g*
0·5 M-Phenobarbital in FAD	−25	*g*
Sodium hydrosulphite	6	*a*
Sodium iodide	9	*a*
Potassium iodide	11	*c*
Silver nitrate	91	*c*
Silver acetate	91	*c*

* In order to get comparable figures, values are corrected to a quencher concentration of *ca.* 3 × 10^{-3}M on the assumption that I_0/I^{∞} concentration (see ref. *f*). Concentrations of riboflavine, FMN or in ref. *d* tetra-O-acetyl-3-*N*-methylriboflavine, where quoted, range from 6·25 × 10^{-6} to 2·6 × 10^{-5}M. Temperatures ranged from 15° to 30°, the temperature coefficient of fluorescence depending on the nature of the quenching involved.

[a] K. Sakai, *Nagoya J. Med. Sci.*, 1956, **18**, 245. [b] G. K. Radda, *Biochim. Biophys. Acta*, 1966, **112**, 448. [c] G. Weber, *Biochem. J.*, 1950, **47**, 114. [d] G. Weber in ref. 16. [e] K. Yagi and Y. Matsuoka, *Biochem. Z.*, 1956, **328**, 138. [f] J. C. M. Tsibris, D. B. McCormick, and L. D. Wright, *Biochemistry*, 1965, **4**, 504. [g] A. Giuditta and L. Casola, *Biochim. Biophys. Acta*, 1965, **110**, 17.

B. Intra- and Inter-molecular Complexes.—The interactions of flavines with a number of organic substances (mostly aromatic) have been known for many years. Many of these substances have been used to increase the solubility of riboflavine in pharmacological preparations. Their biochemical significance lies in two observations, that the flavine and adenine parts of FAD are in close intramolecular association and that aromatic amino-acids are among the substances capable of interacting with FMN or riboflavine. The former observation may be relevant to the coenzymic activity of FAD while the latter may partly explain the mode of interaction of flavines with proteins.

Evidence for the formation of inter- and intra-molecular complexes will be discussed first. The *absorption spectrum* of FAD is different from those of riboflavine and FMN. The molar extinction coefficient of FAD at 260 mμ is lower than the sum of molar extinction coefficients of riboflavine and adenosine at this wavelength.[20,26] There is also a decrease in absorption at 375 and 450 mμ, the latter being accompanied by a broadening of the band.[20] Similar spectral changes are caused by amino-acids such as tryptophan and tyrosine[27] or by purines and pyrimidines (*e.g.*, caffeine, adenosine monophosphate).[20] These spectral shifts

[26] O. Warburg and W. Christian, *Biochem. Z.*, 1948, **296**, 294; 1948, **298**, 150; L. G. Whitby, *Biochem. J.*, 1953, **54**, 437.

[27] I. Isenberg and A. Szent-Györgyi, *Proc. Nat. Acad. Sci.*, 1958, **44**, 857; M. A. Slifkin, *Nature*, 1963, **197**, 277.

have often been recorded by 'difference spectroscopy' between free and interaction solutions when the broadening at long wavelengths has been mistakenly identified as a new absorption band.[27]

The *fluorescence* of FAD is only one fifth that of FMN. Similarly FMN fluorescence is quenched by many other aromatic compounds (Table 2). Quenching in these instances is probably mainly by the static mechanism. This has been clearly shown for purines.[20]

The *optical rotatory dispersion* of FAD differs significantly from that of FMN and from that of an equimolar mixture of FMN and adenosine monophosphate. In particular a pronounced Cotton effect is observed in the visible region for FAD under conditions when fluorescence indicates complex formation.[19]

Further corroborative evidence for complex formation has been obtained from measurements of oxidation–reduction potentials of flavines. For instance caffeine shifts the redox potential of FMN by + 14 mv while L-tryptophan shifts it by − 15 mv.[13] Although the redox potential of FAD is very nearly the same as that of FMN, the lower reactivity of FAD in the reaction with reduced nicotinamide-adenine dinucleotide (NADH)[28] and in photochemical reactions[29] has also been attributed to complexing.

The nature of the complexes.—It is reasonable to assume that the planar aromatic systems in these complexes will best interact in a sandwich arrangement although there is no direct experimental evidence to support this. Equally uncertain is the nature of the forces responsible for the stability of the complexes. Szent-Györgyi and his associates assume that the FMN–indole derivative and FMN–NADH complexes are of the charge-transfer type.[27,30] For the former the spectral shifts produced a red colour. In the latter an electron spin resonance (e.s.r.) signal attributable to the flavine semiquinone was observed. This evidence is insufficient since no new absorption band was observed and electron transfer leading to unpairing should not be confused with charge-transfer.[30,31] Similar criticisms apply to the work of Slifkin on amino-acid-riboflavine complexes[27] and of Harbury and his co-workers.[12] By varying the structure of FAD analogues in the purine nucleotide McCormick and his co-workers concluded that these complexes are not of the classic charge-transfer type but 'may be characterised as of the donor–acceptor type as broadly defined. Such a classification involves mutual polarisability or partial charge-transfer effects'.[32] An additional rôle is attributed to hydrogen bonding which may become particularly important with 6-aminopurines. Molecular orbital calculations suggest the possibility of regions of complementary charge between the isoalloxazine and adenine rings.[33]

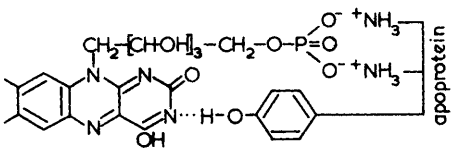
It is, however, important that in no solvent other than water have these complexes been detected in spite of a careful search.[16] In aqueous organic

[28] C. H. Suelter and D. E. Metzler, *Biochim. Biophys. Acta*, 1960, **44**, 23.
[29] W. R. Frisell, C. W. Chung, and C. G. Mackenzie, *J. Biol. Chem.*, 1959, **234**, 1297; G. K. Radda and M. Calvin, *Nature*, 1963, **200**, 464.
[30] I. Isenberg, S. L. Baird, jun., and A. Szent-Györgyi, *Proc. Nat. Acad. Sci.*, 1961, **47**, 245.
[31] E. M. Kosower, ref. 16.
[32] J. C. M. Tsibris, D. B. McCormick, and L. D. Wright, *Biochemistry*, 1965, **4**, 504.
[33] B. Pullman and A. Pullman, 'Quantum Biochemistry', Interscience, New York, 1963.

solvents (*e.g.*, methyl carbitol, dimethyl sulphoxide) fluorescence of FAD compared to FMN indicates unfolding as the water concentration is decreased.[19,34] Even in formamide, which has a dielectric constant higher than that of water, complexing is not significant.[16,5] These observations strongly underline the unique position of water in stabilising complexes. It is likely that here we are dealing with the same phenomenon of hydrophobic interactions as is observed for proteins and polypeptides.[35] We believe that in the flavine complexes contribution of charge-transfer at best can only be very small compared to the stabilisation by hydrogen bonding, hydrophobic interactions, and other forces such as dipole–dipole and induced dipole–dipole attractions.

Molecular complexes between protonated flavines and phenols do show new absorptions that can be correlated with the donor ability of the phenol. Large single crystals of 1 : 1 complexes show an electrical conductivity that is increased on illumination. The photocurrent is composed essentially equally of positive and negative carriers and e.s.r. shows that complete electron-transfer can occur at lattice imperfection sites. Here we are probably dealing with a true charge-transfer phenomenon.[36]

Interactions of flavines with biopolymers.—In spite of numerous studies on model systems little is known about the nature of binding of flavine prosthetic groups to proteins, except that binding is usually non-covalent. In general the two long-wavelength absorption peaks are altered on binding, the 375 mμ peak being shifted to shorter wavelengths and the 447 mμ band to longer. In about one third of the known flavoproteins, however, the general shape of the absorptions remain the same while in the others shoulders around 430 and 480 mμ appear in the long-wavelength peak.[37] It is likely that, just as in model systems, the splitting of the last band is a result of the partial restoration of the vibrational structure in a non-polar environment. It is interesting that in many cases the 375 mμ band is shifted to longer wavelengths in contrast to expectations from models. We feel that this apparently anomalous behaviour is due to specific interactions of the isoalloxazine ring with dipolar groups on the protein. This may mean that while overall the chromophore is surrounded by non-polar groups, it still interacts at a particular point with the dipole, producing an effect



(5)

[34] S. F. Velick, 'Light and Life', ed. W. D. McElroy and B. Glass, Johns Hopkins Press, Baltimore, 1961, p. 108.
[35] W. Kauzmann, 'Advances in Protein Chemistry', ed. C. B. Anfinsen jun., M. L. Ansen, K. Bailey, and J. T. Edsall, Academic Press, New York, 1959, vol. XIV, p. 1.
[36] D. E. Fleischman and G. Tollin, *Biochim. Biophys. Acta*, 1965, **94**, 248; A. Ray, A. V. Guzzo, and G. Tollin, *Biochim. Biophys. Acta*, 1965, **94**, 258.
[37] V. Massey and H. Ganther, *Biochemistry*, 1965, **4**, 1161.

similar to that of a substituent. For instance, Theorell and his co-workers[38] on the basis of fluorescence studies arrived at a picture for FMN–apoprotein interaction (5).

Protein tyrosyl group–flavine interactions have been implicated in other instances[39] and the interaction of riboflavine with tryptophan groups in proteins has been used to characterise the availability of this amino-acid residue to the solvent.[40]

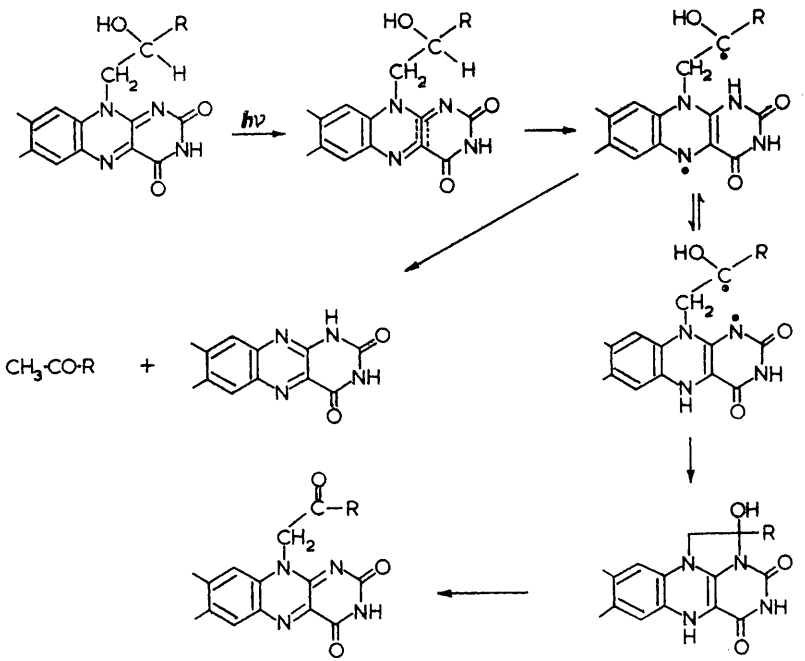
3 Properties Related to the Catalytic Function of Flavines

On the basis of potentiometric titrations and magnetic measurements several authors concluded about thirty years ago that the reduction of riboflavine occurs in two distinct one-electron steps through the semiquinoid intermediate (cf. Scheme 1).[41] The colour changes noted by early workers were spectroscopically investigated by Beinert[42] who observed two bands at 570 and 900 mμ during the reduction. E.s.r. showed the presence of radicals in solutions of partially reduced FMN.[43] The extinction at 570 mμ is related to the amplitude of the e.s.r. signal and is due to absorption by the free radical.[44] The broad absorption band in the 900 mμ region has been attributed on the basis of temperature- and concentration-dependence to a bimolecular (or higher molecular) complex of semiquinones with each other or with other components of the solution.[42] Gibson, Massey, and Atherton,[44] on the other hand, believe it to be a charge-transfer absorption of an FMN–$FMNH_2$ complex. They also observed that the radical concentration was maximal at about 70% reduction of FMN (and not at 50% as might be expected from the disproportionation shown in Scheme 1). It is likely that this asymmetry is due to the presence of another radical species, a complex between the semiquinone and $FMNH_2$. Other dimers are present in these equilibria and the system is further complicated by additional acid–base dissociations. The various interactions based on acid–base titrations and spectral measurements are summarised in Scheme 1.

The rates of several of the processes in Scheme 1 have been measured by fast-reaction techniques, flash photolysis,[45] and stopped flow.[46] Temperature-jump relaxation[47] gave values of $k_{12} = 4 \times 10^8$ M^{-1} sec.$^{-1}$, $k_{21} = 2 \times 10^5$ sec.$^{-1}$, $k_{23} = 8 \times 10^{-1}$ sec.$^{-1}$ and $k_{32} = 4 \times 10^7$ M^{-1} sec.$^{-1}$.

The interpretations of the equilibria in Scheme 1 are confirmed by e.s.r. studies. The hyperfine structure of the e.s.r. absorption of FMNH· varies with pH in a

[38] H. Theorell 'Proceedings of the Fourth International Congress on Biochemistry', ed. O. Hoffmann-Ostenhof, Pergamon Press, London, 1960, vol. VIII, p. 167.
[39] P. Strittmatter, *J. Biol. Chem.*, 1961, **236**, 2329.
[40] J. H. Swineheart and G. P. Hess, *Biochim. Biophys. Acta*, 1965, **104**, 205.
[41] Ref. 4, p. 372.
[42] H. Beinert, *J. Amer. Chem. Soc.*, 1956, **78**, 5323.
[43] H. Beinert and R. H. Sands, 'Free Radicals in Biological Systems', ed. M. S. Blois, jun., H. W. Brown, R. M. Lemmon, R. O. Lindblom and M. Weissbluth, Academic Press, New York, 1961, ch. 2.
[44] Q. H. Gibson, V. Massey, and N. M. Atherton, *Biochem. J.*, 1962, **85**, 369.
[45] B, Holmström, *Photochem. Photobiol.*, 1964, **3**, 97.
[46] Q. H. Gibson and J. W. Hastings, *Biochem. J.*, 1962, **83**, 368.
[47] J. H. Swineheart, *J. Amer. Chem. Soc.*, 1965, **87**, 904.



SCHEME 1. *Redox and acid–base equilibria in flavines*

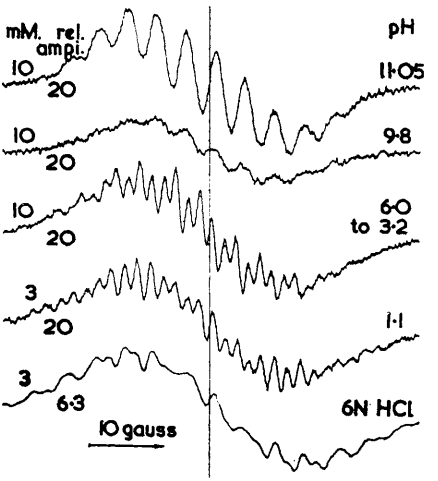


FIG. 2. *E.s.r. spectra of flavine semiquinones as a function of* pH *(from ref.* 48)

characteristic manner (Figure 2). Three forms of the free radical can be clearly distinguished: the radical anion at pH higher than 8, the neutral radical between pH 6 and 2, and the protonated form.[48]

The best resolved spectrum [that of the anion radical of lumiflavine (6,7,9-trimethylisoalloxazine)] has been interpreted in detail with the aid of isotopic substitution. The following assignments were possible. (1) By preparing [9-methyl-$^2H_3$]lumiflavine it was shown that the three methyl protons in lumiflavine participate in the main hyperfine structure interaction and are equivalent. (2) ^{15}N-Substitution in positions 1 and 3 did not alter the main hyperfine structure and therefore the unpaired spin is virtually isolated from the pyrimidinoid ring. (3) ^{15}N-Substitution in the 10 position alters the spectrum. This proves that N(10) has a considerable spin density on it. (4) By substituting deuterium in the benzenoid ring it was shown that H(5) but not H(8) is involved in the hyperfine interaction. Spin densities on the 6- and 7-positions are not clear as only derivatives with different substituents were studied and not deuterated forms. But from the ethyl- and chloro-derivatives it appears that spin density is very low on C(6) but is high on C(7).[48]

Free radicals are produced by partial reduction of flavoproteins by substrate or some other reducing agent. The hyperfine structures of these radicals are very poorly resolved compared with the unbound flavine radicals in solution. This is a consequence of the slow thermal rotational relaxation of the protein molecule. Free radicals in flavoproteins therefore have to be identified by correlating kinetic and spectroscopic results with e.s.r. measurements and the radical signals have to be characterised by properties such as their widths and power saturation characteristics.

Oxidation–Reduction Potentials.—The positions at which flavines participate in biochemical electron transfer depends on their oxidation–reduction potentials. Free FMN, FAD, riboflavine, and lumiflavine have very nearly identical potentials, the experimental range varying from $-0{\cdot}186$ v to $-0{\cdot}218$ v at pH 7. The potential depends very largely on the nature of the substituents in the benzenoid ring and to a lesser extent on the 9-substituent.[49] The observation that on complex-formation the redox potentials of flavines are slightly displaced (cf. Section 2*b*) is of interest because they can be changed in either direction on binding to proteins. A shift of $+0{\cdot}067$ v is observed for the redox potential of FMN on binding to the apoprotein of the old yellow enzyme, indicating a firmer binding of the reduced form, and in butyrylcoenzyme A dehydrogenase the shift is $+0{\cdot}374$ v. A large negative shift of $-0{\cdot}153$ v is observed for lipoyl dehydrogenase. This indicates that flavoproteins, just like the cytochromes, may occupy several positions in the electron-transport chain.

Metals in Flavine Catalysis.—Many flavoproteins contain metal ions (*e.g.*, iron, molybdenum, copper) which are functionally important in the catalytic processes.

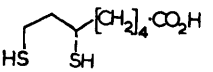
[48] A. Ehrenberg, 'Electronic Aspects of Biochemistry', ed. B. Pullman, Academic Press, New York, 1964; A. Ehrenberg, L. E. G. Eriksson, and F. Müller in ref. 16.
[49] W. M. Clark, 'Oxidation–Reduction Potentials of Organic Systems', Williams and Wilkins Co., Baltimore, 1960, p. 441.

The interaction of metals with free flavines is, therefore, an important part of flavine chemistry and has recently been reviewed in detail.[5] The similarity of flavines to 8-hydroxyquinoline prompted Albert to investigate the stability of transition-metal–flavine complexes,[50] and Foye and Lange claimed to have isolated some of them.[51] Hemmerich and his co-workers, however, have clearly shown that the oxidised forms of flavines do not have any marked affinity for metals in water because of the low basicity of the 10-nitrogen and the unfavourable energetics of 3,4-prototropy, although in acetonitrile interactions do occur.[52] Silver, cuprous, and mercuric ions, however, form strong complexes in water, probably stabilised through charge-transfer. Reduced flavines have no metal affinity. The flavine semiquinone on the other hand is readily chelated by metal ions having *d* electrons which leads to a shift towards the radical in the FMN–$FMNH_2$ disproportionation.[5] E.s.r. studies of the radical paramagnetic relaxation show that the semiquinone can interact with metals in flavorproteins as well.[53]

Reactions with reducing agents.—Flavines are reduced by sodium dithionite, catalytic hydrogenation, zinc and hydrochloric acid etc. The reduced flavine is rapidly autoxidised and so reduction has to be carried out in anærobic conditions.

The kinetics of the reduction of riboflavine by dithionite at pH 9 have been studied using a rapid mixing apparatus. Because of the high pH only the 900 mμ absorption was observed. The kinetics of its appearance suggested that it was formed from two molecules of the semiquinone (cf. ref. 44),[54] but it is still doubtful whether the semiquinone is an intermediate in this reaction or whether it is formed by a reaction between oxidised and reduced flavine.

The mechanisms of the reduction by NADH and by dihydrolipoic acid, which may serve as models for flavoprotein systems, have been studied. In the case of NADH the reaction is thought to proceed by a hydride-ion transfer. The evidence is derived from the effects of substituents in both flavine and nicotinamide, solvent effects, and the deuterium isotope effect.[28,55,56,57] The alternative, a fast reversible electron transfer followed by a rate-determining hydrogen abstraction, has not been entirely excluded. The reaction of dihydrolipoic acid (6) is also believed to be a two-electron process on the basis of similar substituent



(6)

effects, but a fast dissociation of one of the thiol groups of lipoic acid precedes the reduction.[56]

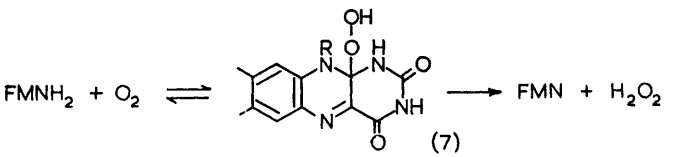
[50] A. Albert, *Biochem. J.*, 1953, **54**, 646.
[51] W. O. Foye and W. E. Lange, *J. Amer. Chem. Soc.*, 1954, **76**, 2199.
[52] P. Hemmerich, ref. 16.
[53] H. Beinert and P. Hemmerich, *Biochem. Biophys. Res. Comm.*, 1965, **18**, 212.
[54] G. P. Burn and J. R. P. O'Brien, *Biochim. Biophys. Acta*, 1959, **31**, 328.
[55] T. P. Singer and E. B. Kearney, *J. Biol. Chem.*, 1950, **183**, 409.
[56] I. M. Gascoigne and G. K. Radda, *Chem. Com.*, 1965, **211**.
[57] G. K. Radda and M. Calvin, *Biochemistry*, 1964, **3**, 384.

Reactions with oxygen.—The hydrogen atoms in the 7-methyl group of riboflavine are active. They are exchanged in D_2O,[58] condense with aromatic aldehydes, and undergo oxidative dimerisation[5] under non-solvolytic basic conditions. The activity of this methyl group in flavines is similar to its activity in *p*-nitrotoluene.

The kinetics of the autoxidation of reduced FMN followed by rapid reaction techniques are consistent with the occurrence of two simultaneous mechanisms.[46] In the first the semiquinone of the flavine reacts with oxygen by a free-radical path:

$$FMN + FMNH_2 \rightleftharpoons 2FMNH\cdot$$
$$FMNH\cdot + \tfrac{1}{2}O_2 \rightarrow FMN + \tfrac{1}{2}H_2O_2$$

while the competitive process involves a direct two-electron oxidation through a flavine peroxide:



(7)

Chemical evidence for the hydroperoxide intermediate in the reoxidation of reduced isoalloxazines and the analogous tetrahydropyrazines is that N(10)-substituted dihydroalloxazines are resistant to autoxidation while N(9)-substituted ones are very sensitive.[59]

Reduced flavines are not easily autoxidised when bound to proteins of the mitochondrial electron-transport chain but they are reactive in many other flavoproteins (*e.g.*, amino-acid oxidases). There is no satisfactory explanation yet.

Hydroxylation by reduced flavines and oxygen.—Aromatic compounds foreign to an organism often undergo non-specific hydroxylation. Two chemical models for this reaction have been investigated. Hydroxyl radicals produced by Fenton's reagent give a different pattern of aromatic substitution from the radical produced by a mixture of ascorbic acid, ferrous ions, EDTA and oxygen. In the latter system perhydroxyl radicals may be involved.[60] Recently it was found that reduced FMN and oxygen together also hydroxylate aromatic compounds. There is disagreement about the nature of the hydroxylating radical in this system. The perhydroxyl radical has again been implicated but we feel that a flavine hydroperoxide [cf. (7)] is more likely.[60]

[58] F. J. Bullock and O. Jardetzky, *J. Org. Chem.*, 1965, **30**, 2056.
[59] W. Berends, J. Posthuma, J. S. Sussenbach, and M. I. X. Mager, ref. 16.
[60] H. Staudinger, B. Kerekjarto, V. Ullrich, and Z. Zubrzycki, 'Oxidases and Related Redox Systems', ed. T. E. King, H. S. Mason, and M. Morrison, John Wiley and Sons Inc., New York, 1965, vol. II; R. O. C. Norman and G. K. Radda, *Proc. Chem. Soc.*, 1962, 138.

4 Photochemistry

A. Anærobic Photochemistry.—When considering the photoreduction of flavines in the absence of oxygen a clear distinction must be made between two possible situations: reaction in the presence of external electron donors (photoreduction) and that in their absence (photobleaching). The two situations, though closely related, show some basic differences.

An aqueous anærobic solution of riboflavine containing an external electron donor is reduced on illumination with ultraviolet or visible light. There is a concomitant loss of colour as the reduced form of the flavine has no visible absorption in a dilute solution. On admitting oxygen to the reduced solution riboflavine is quantitatively restored.[61] When riboflavine is illuminated in the absence of external donors fading is again observed, but in this case colour is only partially restored when oxygen is admitted. The products include lumiflavine and lumichrome (6,7-dimethylalloxazine), of which only lumiflavine has a spectrum similar to that of the initial reactant.[61,62] Photobleaching, therefore, is not even partially reversible, in complete contrast to photoreduction. In addition the quantum yield (ϕ) of photobleaching is much lower ($\phi = 0{\cdot}006$)[63] than those observed for photoreductions by EDTA ($\phi = 0{\cdot}06$) and NADH ($\phi = 0{\cdot}25$).[57]

The spectra of both photobleached and photoreduced riboflavine solutions resemble those obtained by the dark reduction of riboflavine by dithionite. This has led to the suggestion that the mechanism of photobleaching is reduction by water-splitting[64,65] providing a chemical analogue for the water-splitting reaction in photosynthesis:

$$\mathrm{FMN} + \mathrm{H_2O} \xrightarrow{h\nu} \mathrm{FMNH}\cdot + \mathrm{OH}\cdot$$

The evidence quoted by Nickerson and his co-workers and by Vernon in support of the mechanism is: (*a*) hydrogen peroxide is produced during anærobic photobleaching. (*b*) Catalase (a specific enzyme which destroys hydrogen peroxide) inhibits the reaction. (*c*) Methionine sulphoxide is produced when methionine is added to the reaction mixture. (*d*) The riboflavine spectrum is nearly restored on reoxygenation. (*e*) The reaction does not occur in a non-aqueous solvent like ethanol. They also thought that photoreduction is faster than photobleaching because the added 'activators' (*e.g.*, EDTA, NADH, Methionine) reduce the energy required to break the O–H bonds of water by forming some kind of complex. It was suggested that some degradation of riboflavine occurred during reoxygenation in the absence of activators which can somehow protect the side-chain.

[61] G. Oster, J. S. Bellin, and B. Holmström, *Experientia*, 1962, **18**, 249.
[62] W. M. Moore, J. T. Spence, F. A. Raymond, and S. D. Colson, *J. Amer. Chem. Soc.*, 1963, **85**, 3367.
[63] B. Holmström and G. Oster, *J. Amer. Chem. Soc.*, 1961, **83**, 1867.
[64] G. Strauss and W. J. Nickerson, *J. Amer. Chem. Soc.*, 1961, **83**, 3187.
[65] L. P. Vernon, *Biochim. Biophys. Acta*, 1959, **36**, 177.

This evidence is unsatisfactory. (*a*) The methods used to detect hydrogen peroxide are not unequivocal.[57] (*b*) The effect of catalase is uncertain; some authors have found it to accelerate photobleaching rather than to inhibit it, and in any case it is difficult to visualise how decomposition of a reaction product can retard the reaction in which it is produced. (*c*) The production of methionine sulphoxide can be explained by a mechanism in which methionine acts as an electron donor; its oxidation product is subsequently hydrated to the sulphoxide. In fact more recent studies have shown that the primary product of anærobic photoreduction by methionine is 3-(methylthio)propionaldehyde, not methionine sulphoxide.[66] (*d*) The near restoration of the riboflavine spectrum only shows that the isoalloxazine chromophore is not completely destroyed. (*e*) It has now been shown that photobleaching *does* occur on illumination of ethanolic riboflavine solutions.[57]

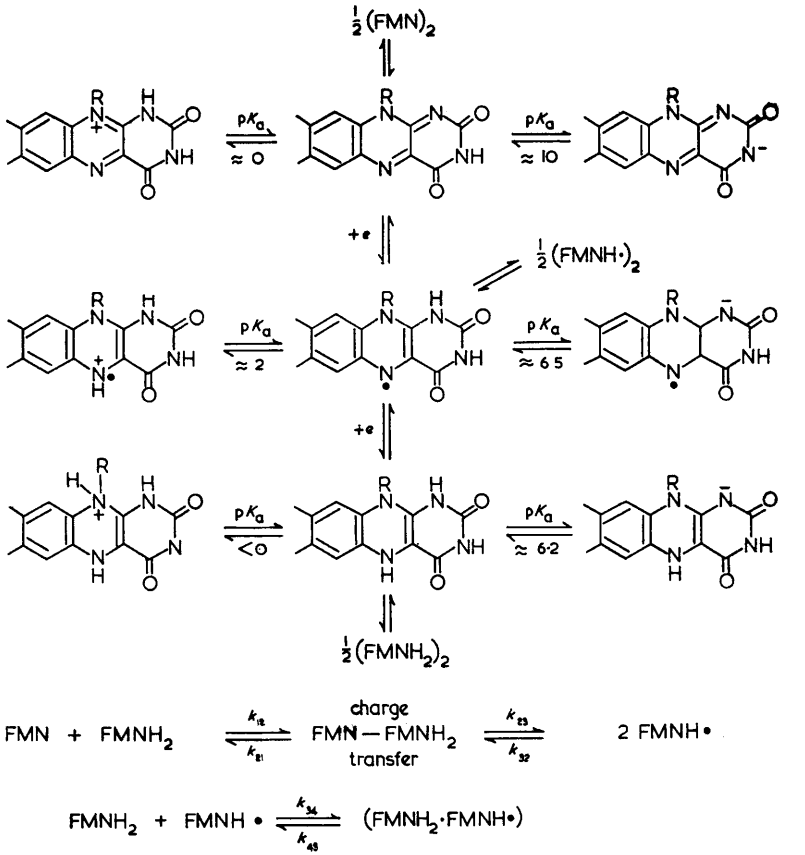
A more satisfactory mechanism has been suggested by Holmström, Oster, and others.[57,62,63] The suggestion is that during photobleaching an intramolecular disproportionation occurs in which the ribityl side-chain is oxidised and the isoalloxazine nucleus is reduced.

There is a great deal of evidence for side-chain participation. Smith and Metzler have identified the products of photobleaching as the reduced forms of lumiflavine, lumichrome, and 6,7-dimethyl-9-formylisoalloxazine, so the side-chain has certainly reacted.[67] Moore and his co-workers[62] observed a kinetic isotope effect ($k_H/k_D = 2{\cdot}5$) for the photobleaching of 9-(2′-hydroxyethyl)-isoalloxazine and [2′,2′-$^2H_2$]9-(2′-hydroxyethyl) isoalloxazine, which shows conclusively that hydrogen abstraction from the side-chain occurs during the rate-determining step. The lack of isotope effect in D_2O compared with water suggests that hydrogen is not abstracted from water or the hydroxyl groups of the side-chain. Lumiflavine in the presence of ribitol is photobleached much more slowly than riboflavine indicating that the reaction is intramolecular.[57]

The suggested mechanism for photobleaching is shown in Scheme 2. Although hydrogen at the 2′-position is by far the most favourable for abstraction it has been shown that ω-hydroxyl chains of 3—6 carbon atoms will allow some reaction in the order $3 > 5 > 6 > 4$.[68] Radical stabilisation in the side-chain can be affected by nitrogen as well as oxygen.[69]

The mechanism of photoreduction is similar to that of photobleaching. It probably involves hydrogen abstraction from the electron donor, not from the side-chain. The reactions of EDTA, NADH, and methionine have been most throughly studied, though probably many amines can fulfil their rôle. The stoicheiometry of the EDTA reaction is unknown, but it has been shown that one mole of glyoxylic acid is produced for each mole of flavine reduced.[66] With NADH the major product is NAD^+ (ref. 57), and with methionine, methional.[66]

[66] K. Enns and W. H. Burgess, *J. Amer. Chem. Soc.*, 1965, **87**, 5766.
[67] E. C. Smith and D. E. Metzler, *J. Amer. Chem. Soc.*, 1963, **85**, 3285.
[68] C. S. Yang and D. B. McCormick, *J. Amer. Chem. Soc.*, 1965, **87**, 5763.
[69] G. K. Radda, *Biochim. Biophys. Acta*, 1966, **112**, 448.



SCHEME 2. *Mechanism of photobleaching of riboflavine*

Finer mechanistic details are similar for both photoreduction and photobleaching. The most studied of these is the nature of the active flavine excited state. Small amounts (*ca.* 10^{-6}M) of KI significantly decrease the quantum yield of photoreduction whereas the concentration required for significant fluorescence quenching is *ca.* 10^{-4}M.[63] Collision-theory calculations suggest that the chemically active species has a minimum life-time of 10^{-3}—10^{-4} sec.[57,63] Paramagnetic metal ions inhibit photobleaching whereas diamagnetic ones do not. It is clear that excited singlet flavine is too short-lived to be the reactive species, so an excited triplet probably participates in the rate-determining step of the reaction. It is conceivable, though unlikely, that flavine radicals participate in the slow step because their life-time is also of the order of 10^{-3} sec.,[70] and they are known to form complexes with metals.[5] In flash experiments it is possible to observe

[70] B. Holmström, *Arkiv Kemi*, 1964, **22**, 329.

the spectrum of the flavine radical and to show that it occurs as a reaction intermediate of both photobleaching and photoreduction.[70] An e.s.r. signal has also been detected, though the time resolution of the method is too low to permit the study of the radical as an intermediate.[57] Addition of KI inhibits the rate of radical formation as well as that of the whole reaction. This indicates that the triplet is involved in the rate-determining process, the reaction proceeding by two one-electron steps. The rate of photoreduction has a small positive temperature coefficient, and depends comparatively little on donor concentration. This is consistent with the slow step's involving an excited flavine–reducing agent complex.

Although under conditions of reaction the triplet is too short-lived to be detected it has been observed at low temperatures. It gives rise to phosphorescence at 630 mμ,[71] and an e.s.r. signal at 1560 gauss. This corresponds to a forbidden $\Delta m = 2$ transition. The half-life of the FMN triplet in N-HCl is 15 msec.[72] Measurements of the anistotropy of the spin-spin coupling tensor for several isoalloxazines show that the electron distribution in the triplet states is not very sensitive to the substituent in the 9-position but does depend on the substituents at C(6) and C(7).[73] Photochemical reactivity is also much more dependent on 6- and 7- than on 9-substituents.[69]

Riboflavine can sensitise some other photochemical reactions. For example, on illuminating an anærobic mixture of riboflavine and ergosterol with visible light the steroid is dehydrogenated to give, quantitatively, the 6,6′-dimer bisergostadienol.[74] The ease of reaction decreases FMN > riboflavine > lumiflavine = FAD = 0 in contrast to the flavine reactions so far discussed. Triplet involvement is indicated by the action of phosphorescence quenchers, and rate-independence of ionic strength suggests the participation of flavine radicals. The reactivity order was attributed to more favoured singlet–triplet conversion in the molecules with large side-chains. Perhaps, however, the difference between this reaction and that with EDTA is that ergosterol can only react with the flavine radical, which is only produced when the side-chain is oxidisable. The inactivity of FAD is common, and results from very efficient quenching by the adenine moiety.

Illuminated riboflavine solutions can also, apparently, act as reducing agents. Rutter has observed the riboflavine-catalysed photoreduction of Fe^{III} to Fe^{II}. A better explanation than his suggested water-splitting mechanism is that riboflavine is bleached in the usual way, and the reduced flavines so formed achieve the reduction of Fe^{III}. A similar mechanism may explain the photoreduction of nitrate to nitrite in the presence of FMN and Mo^{VI} (ref. 75) and a likely sequence seems to be: FMN $\xrightarrow{h\nu}$ reduced flavine $\xrightarrow{Mo^{VI}}$ Mo^{V} + FMN + lumichrome, $Mo^{V} + NO_3^- \longrightarrow Mo^{VI} + NO_2^-$.

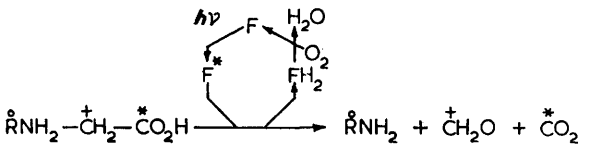
Flavines can photosensitise *cis–trans* isomerisation of conjugated double

[71] C. Dhere and V. Castelli, *Compt. rend.*, 1938, **206**, 2003.
[72] T. Shiga and L. H. Piette, *Photochem. Photobiol.*, 1964, **3**, 213.
[73] J. M. Lhoste, A. Haug, and P. Hemmerich, *Biochemistry*, 1966, **5**, 3290.
[74] L. R. Tether and J. H. Turnbull, *Biochem. J.*, 1962, **85**, 517.
[75] J. T. Spence and J. A. Frank, *J. Amer. Chem. Soc.*, 1963, **85**, 116.

bonds, *e.g.*, stilbenes and pimaricin, an olefinic fungicide. When either *cis*- or *trans*-stilbene-4-carboxylic acid is illuminated in the presence of riboflavine an equilibrium mixture of 40% *cis*- and 60% *trans*-isomers is rapidly produced. Lumichrome is as effective as riboflavine, so neither the side-chain nor the isoalloxazine nucleus is vital here.[59,76] Oxygen and paramagnetic ions quench the reaction, so again triplet involvement seems likely. A triplet–triplet energy transfer is suggested for the mechanism, isomerisation occurring in the excited olefin. This can be induced by other molecules with triplet energies greater than that of riboflavine. Greater certainty about energy transfer will be possible when the triplet energy of stilbene-4-carboxylic acid is measured. Singlet-singlet energy transfer between *p*-terphenyl and riboflavine, on the other hand, has certainly been observed.[77]

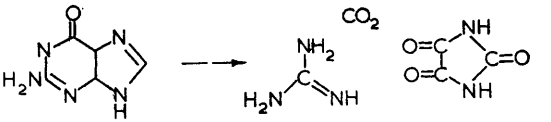
B. Aerobic Photochemistry.—With more intense light than is required anærobically, riboflavine is photobleached in the presence of oxygen. Halwer found that in alkaline solution the main product is lumiflavine, whereas below pH 7 it is lumichrome.[78] Aerobic photobleaching is subject to general acid–base catalysis and so an ionic mechanism is probable with a contribution from competing radical processes such as the anærobic one.

Flavines sensitise the photo-oxidation of amino-acids. Labelling experiments show the way the acids generally break up, and a suggested mechanism, with its obvious correlation to the anærobic reaction, is shown in Scheme 3.[79]



Scheme 3

Lumichrome as well as riboflavine photosensitises the destruction of biological activity of DNA by destroying the guanine bases. Several products are formed but ^{14}C labelling shows that about 50% of the observed degradation can be expressed by Scheme 4.



Scheme 4

The reaction, which shows an induction period, is inhibited by paramagnetic ions. The peroxides produced are not intermediates. Guanine itself is under no condi-

[76] W. Berends and J. Posthuma, *J. Phys. Chem.*, 1962, **66**, 2547.
[77] C. L. Gemmill, *Radiation Res.*, 1956, **5**, 216.
[78] M. Halwer, *J. Amer. Chem. Soc.*, 1951, **73**, 4870.
[79] W. R. Frisell, C. W. Chung, and C. G. MacKenzie, *J. Biol. Chem.*, 1959, **234**, 1297.

tions photosensitive, so energy transfer is unlikely. It has been suggested that triplet flavine interacts with oxygen to give ground-state flavine and singlet oxygen, a reactive species known to degrade guanine. This mechanism does not readily explain why degradation is specific to guanine of all the heterocyclic bases in DNA.[76] Hydroperoxides produced during the oxidation of reduced flavines may participate in this reaction (see Section 3).

In addition to the reactions with nucleotides, nucleic acids, and amino-acids, flavines can sensitise the photo-oxidation of some indole derivatives and proteins. Photoinactivation of enzymes like α-amylase, urease, and trypsin has also been observed although the mechanisms of these processes are not clearly understood.[80] The photo-oxidation of indoleacetic acid, a plant hormone, may be responsible for the phototropic curvature of plants as the growth hormone concentrations on the light and dark sides of the stem are different. Evidence for this is tenuous. The action spectrum of phototropic movement resembles the visible region of a flavine absorption spectrum, but could equally well be explained by the photosensitising action of carotenoids.[11]

C. Chemiluminescence Involving Flavines.—The emission of light by the bacterium *Achromobacter fischeri* depends on the presence of FMN. Although the spectrum of the emitted radiation corresponds to neither the fluorescence nor the phosphorescence of free flavines it is still possible that the isoalloxazine chromophore is directly involved in the light process through some kind of complex.[9]

A possible model for this is provided by the chemiluminescence of riboflavine caused by addition of hydrogen peroxide. The radiation emitted has the spectrum of riboflavine fluorescence, and its intensity is enhanced by the presence of some metals, notably Fe^{II} and Cu^{II}. The delayed light emission can also be enhanced by illumination of the reaction by visible light. This increase is reduced by small amounts of KI, NADH, benzene, and nitrobenzene, which suggests that the primary photochemical event is again mediated through a flavine triplet. Added aromatic molecules are hydroxylated during the reactions and this, along with the enhancement by metals ions, has led to the suggestion that light emission is a consequence of interaction between riboflavine and hydroxyl radicals. The fact that the energy of the riboflavine triplet (47 kcal./mole) is the same as that of the O–O-bond in hydrogen peroxide fits in with this general scheme of sensitisation.[81] Recently we found that the pattern of aromatic hydroxylation does not correspond to hydroxyl radicals, and flavine hydroperoxide may again be involved.[82]

D. Inhibition and Enhancement of Photoprocesses.—The herbicides monuron (3-*p*-chlorophenyl-1,1-dimethylurea) and diuron [3-(2,4-dichlorophenyl)-1,1-

[80] A. W. Galston, *Science*, 1950, **111**, 619; M. G. Ferri, *Arch. Biochem. Biophys.*, 1951, **31**, 127; C. A. Ghiron and J. D. Spiles, *Photochem. Photobiol.*, 1965, **4**, 13.
[81] R. H. Steele, *Biochemistry*, 1963, **2**, 529; J. R. Williams and R. H. Steele, *Biochemistry*, 1965, **4**, 814.
[82] G. K. Radda and T. N. Young, unpublished observations.

dimethylurea] are among the most active inhibitors of photosynthesis. The effect of monuron on cyclic photophosphorylation with FMN as cofactor suggests that it blocks photosynthesis close to the site of the FMN electron-transport system. It is interesting, therefore, to find that monuron affects several flavine photoreactions. The nature of the response varies with the reaction but in general inhibition results. This is the case for the riboflavine-sensitised ærobic photo-oxidations of EDTA and triethylenetetramine as well as the anærobic photobleaching and photoreduction by EDTA.[69] The inhibition by high oxygen concentration of flavine-sensitised photo-oxidation of ascorbic acid and 2,3-dioxogulinic acid is itself reversed by monuron.[83] The fact that similar effects are not observed when other dyes like methylene blue and the phthalein dyes replace riboflavine has led to the attractive hypothesis that the flavine–monuron interaction is specific to this pair of substances.

More generally[69] it is found that compounds expected to be good electron donors towards riboflavine and which form ground-state complexes with it (see Table 2) are powerful inhibitors of photoreduction. Good electron acceptors (*e.g.*, methyl viologen) and substances which do not form a complex with riboflavine have no effect. The concentrations of ground-state complexes are insufficient to explain the extent of inhibition. Interaction between flavine triplet and inhibitor offers an answer. This is supported by the observed temperature effects.

These inhibitions are potentially important to the understanding of photosynthesis, though more work is required completely to elucidate their mechanisms. We do not believe that the specificity of herbicides in model systems is the result of a different mechanism. The kinetic characteristics of all the inhibition reactions are very similar. Chemically there seems no reason why monuron inhibition should involve different types of interactions from that of, *e.g.*, tryptophan. The substituted ureas are comparatively weak inhibitors of even flavine reactions and their lack of effect on other dyes could be the result of molecular geometry.[83]

It is interesting to note that good inhibitors of anærobic flavine photoreduction, including monuron, are easily photo-oxidised aerobically using riboflavine as sensitiser.

A different kind of interaction is observed in the presence of some micellar macromolecules (polyvinylpyrrolidone, sodium decyl sulphate, etc.) which enhance the photosensitivity of many dyes. It is thought that the excited flavine interacts with the macromolecule resulting in increased triplet life-time.[84]

In the final analysis the full value of chemical model systems discussed throughout this Review is only realised when they can be related to biological functions.

Addendum—Since this review was written several relevant papers have appeared. It is now likely that the flavine-indole and flavine-purine complexes are of different kinds. In the former charge-transfer forces may play a rôle but they

[83] P. Homann and H. Gaffron, *Photochem. Photobiol.*, 1964, **3**, 499.
[84] H. B. Kostenbauder, P. P. Deluca, and C. R. Kowarski, *J. Pharm. Sci.*, 1965, **54**, 1243.

are unlikely to be significant in flavine-purine complexes.[85] In contrast $FMNH_2$ acts as an electron donor in forming distinct charge-transfer complexes with nicotinamide adenine dinucleotide.[86]

In some work the optical rotatory dispersion curves for FAD appear to have been distorted by instrumental factors[19] and only the 370 mμ absorption band of the flavine is optically active,[87] although in circular dichroism a small activity is associated with the 447 mμ band too.[88]

Further evidence shows that the reaction of NADH with FMN does involve an initial two-electron reduction and not a single electron transfer.[89]

In a new approach it was suggested that the photoreduction of flavines by phenylacetic acid involves the formation of a benzyl flavohydroquinone.[90] The photochemical behaviour and fluorescence properties of flavines in a solid matrix of methyl cellulose have also been examined.[91]

[85] J. E. Wilson, *Biochemistry*, 1966, **5**, 1351.
[86] T. Sakurai and H. Hosoya, *Biochim. Biophis. Acta*, 1966, **112**, 459.
[87] R. T. Simpson and B. L. Vallee, *Biochem. Biophys. Res. Comm.*, 1966, **22**, 712; D. Wellner, *Biochemistry*, 1966, **5**, 1585; I. Listowsky, S. Englard, J. J. Betheil and S. Seifter, *Biochemistry*, 1966, **5**, 2548.
[88] T. Takagi, K. Aki, T. Isemura and T. Yamano, *Biochem. Biophys. Res. Comm.*, 1966, **24**, 501.
[89] J. L. Fox and G. Tollin, *Biochemistry*, 1966, **5**, 3865.
[90] P. Hammerich, V. Massey and G. Weber, *Nature*, in the press.
[91] G. R. Penzer and G. K. Radda, *Nature*, 1967, **213**, 251.