The Chemistry of Flavines and Flavoproteins

PHOTOREDUCTION OF FLAVINES BY AMINO ACIDS

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1. Flavines are photoreduced through their triplet states by amines and amino acids (e.g. EDTA and DL-phenylglycine). The anaerobic photoreduction of FMN and several other flavines with DL-phenylglycine was analysed in terms of a detailed kinetic scheme. 2. The reaction produces equimolar amounts of benzaldehyde, carbon dioxide and reduced flavine. 3. The sensitivity of the rates to substituents in the DL-phenylglycine can be described by a Hammett ρ -value of -1.1 . 4. Phenylacetic acid behaves differently from DL-phenylglycine or benzylamine towards a series of flavines. 5. The photoreductions are quenched by several aromatic compounds. From the effects of light-intensity and temperature, and by comparison with potassium iodide quenching, it is concluded that inhibition by the aromatic compounds is not simply a collisional process. 6. FAD reacts more slowly than FMN both in the photoreduction and in dark reduction by NADH. Urea and dimethyl sulphoxide decrease the intramolecular interaction in FAD, but they have no effect on the rate of dark reduction of FAD compared with FMN. In contrast, the photoreduction of FAD is quicker in urea.

The potential function of flavines in several photobiological processes has prompted many studies on the photochemistry of riboflavine, FMN and their analogues (for a summary see Penzer & Radda, 1967). Though no direct evidence has been put forward for the action of excited flavines in photosynthesis or vision, flavines participate in photosynthetic electron transport (Whatley, Allen & Arnon, 1959; Shin, Tagawa & Arnon, 1963), and riboflavine occurs in the eye of some fish and mammals (Pirie, 1960). Flavines photosensitize *cis-trans* isomerizations (Posthuma & Berends, 1966; Gordon-Walker & Radda, 1967) and aerobic photo-oxidations of amino acids and proteins (Galston, 1950; Frisell, Chung & MacKenzie, 1959). The flavine nucleus is anaerobically photoreduced by electron donors, e.g. NADH, EDTA (Vernon, 1959; Radda & Calvin, 1964), methionine (Strauss & Nickerson, 1961; Yang, Ku & Pratt, 1967) and the ribityl side chain of riboflavine (Holmström & Oster, 1961). Flash photolysis (Holmström, 1964) and isotope effect studies (Moore, Spence, Raymond & Colson, 1963) implicate hydrogen atom abstraction in these reactions.

Recently the photoreaction of several flavines with the phenylacetate anion was shown to follow a different route (Hemmerich, Massey & Weber, 1967). This reaction leads to the substitution of a benzyl group in the flavine nucleus, suggesting

that in addition to its redox function the chemical activity of the isoalloxazine ring may be important in flavoprotein catalysis. It is also possible that the redox function itself may involve group transfer rather than electron or hydrogen transfer (Hemmerich *et al.* 1967). One such suggestion has already been made (Coffey, Neims & Hellerman, 1965), and the lack of electron-spin-resonance signals during the reaction of several flavoproteins would be consistent with this interpretation.

The present paper is primarily concerned with the reaction of amino acids with excited flavines. We chose DL-phenylglycine and its derivatives for our investigations for two reasons. First, because their reaction with several amino acid oxidases has been studied (Neims, DeLuca & Hellerman, 1966; Radda, 1964), and, secondly, because phenylglycines are the amino acids closest in structure to phenylacetic acid. Thus we hoped that by comparing the several mechanisms of interaction of excited flavine with these substrates the biochemical significance of the model studies might be clarified.

MATERIALS

FMN was purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany), FAD from Sigma Chemical Co. (St Louis, Mo., U.S.A.), and riboflavine from British Drug Houses Ltd. (Poole, Dorset). Lumiflavine was

synthesized in this Laboratory by the method of Hemmerich, Fallab & Erlenmeyer (1956). The isalloxazine derivatives listed in Table 4 were gifts from the following: compounds (I) and (II), Dr P. Hemmerich; compounds (III) and (V), Professor T. Wagner-Jauregg; compound (IV), Dr R. M. Acheson. Substituted DL-phenylglycines were given by Dr J. H. C. Nayler, and 3-(*p*-chlorophenyl)-1,1-dimethylurea and 3-(2,4-dichlorophenyl)-1,1-dimethylurea were gifts from E. I. Du Pont de Nemours and Co. Inc. (Wilmington, Del., U.S.A.). All other reagents were commercial products in their purest form. Dimethyl sulphoxide was further purified by redistillation under vacuum.

METHODS

Reaction rates were measured by following flavine concentration spectrophotometrically at the adsorption maximum (for a Table of the maxima of flavine derivatives see Penzer & Radda, 1967) where reduced flavine does not absorb (Holmström & Oster, 1961). Reactants were mixed in a 1 cm. stoppered cuvette (concentrations are given in the Tables presented below). The solutions were deoxygenated by bubbling N₂ (passed through a solution of vanadous sulphate and H₂SO₄ containing amalgamated zinc to remove traces of O₂) for at least 20 min.

Solutions were illuminated by a 500 w tungsten lamp with a potentiometer to regulate light-intensity. A filter (OY 18; Chance Bros., Birmingham) was used to cut out wavelengths below 400 mμ, and a water bath absorbed the infrared. Light-intensity was quantitatively varied by neutral-density filters. The cuvette was supported in a thermostatically controlled water bath during illumination. Reaction rates were reproducible to ±5%.

Absorption spectra were recorded on a Cary 14 recording spectrophotometer, and fluorescence measurements were made on a Zeiss PMQII spectrofluorimeter.

pH measurements were made with an Electronic Instruments Ltd. type 23A pH-meter. *pK*_a values were measured by titration, with a Radiometer Titrator type TTT 1b. The amino acid was dissolved in 0.1M-NaCl and titrated with 2N-NaOH solution made up in CO₂-free water.

Benzaldehyde was estimated by vapour-phase chromatography. The instrument was a Perkin-Elmer F11, with a 2m. column packed with diethylene glycol adipate, pentaerythritol and phosphoric acid in the proportions 25:2:73 (by wt.). The method was as follows. A 40 ml. sample of the reaction mixture containing lumiflavine (0.10–0.15 mm in different runs) and DL-phenylglycine (10 mm) was adjusted to pH 8.3 with Na₂HPO₄ and placed in a vessel thermostatically controlled at 25°. This was thoroughly deoxygenated by bubbling N₂ for 2 hr. with stirring. The anaerobic mixture was illuminated until the extinction showed that lumiflavine concentration had fallen below 0.01 mm (typically 2 hr.). Then 1 μl. of hexan-1-ol was added to 25 ml. of this solution to act as an internal standard for the vapour-phase chromatography. The aqueous solution was extracted with 10 ml. of ether, which was subsequently dried with anhydrous Na₂SO₄. Samples (2 μl.) of the ethereal solution were injected into the chromatograph with a Hamilton micro-syringe. With N₂ carrier gas at 15 lb. in.⁻² pressure and a column temperature of 100°, the retention times were: hexan-1-ol, 4.1 min.; benzaldehyde, 13.0 min. The detector response was calibrated by standard mixtures of benzal-

dehyde and hexan-1-ol in ether. The extraction procedure was standardized with known aqueous solutions of benzaldehyde and hexan-1-ol at pH 8. Area ratios for a given solution and extraction were consistent to ±5%, and for separately made and extracted solutions to ±10%. Unilluminated blanks gave no benzaldehyde.

For estimation of benzoic acid and phenylglyoxylic acid the method was modified. The reaction mixture was acidified to pH 2 with conc. HCl before extraction. The same column was used at 190°. The retention times were: benzaldehyde, 1.6 min.; benzoic acid, 12.2 min.; phenylacetic acid, 19.5 min.; phenylglyoxylic acid, 36.0 min. The ethereal extracts were evaporated to a very small volume before injection to give maximum response. Blanks showed that the method should have detected 0.01 mm-acid in the aqueous solution.

Phenylglyoxylic acid was also estimated spectrophotometrically by the reaction with *o*-phenylenediamine, by the method of Bruce & Topping (1963).

CO₂ was determined by acidifying an anaerobic reaction mixture (identical with that described for the benzaldehyde experiments) with conc. HCl. The CO₂ released was then blown by a stream of N₂ into a solution of 0.5 mm-Ba(OH)₂ for 1 hr. Both these operations were performed without allowing the reaction mixture to contact the atmosphere. The Ba(OH)₂ solution was now titrated with standard HCl in the automatic titrator, and the titre was subtracted from that obtained by using an identical procedure with an unilluminated reaction mixture. The quantities of CO₂ involved were insufficient to precipitate BaCO₃, so the reaction occurring was:

Thus the change in concentration of OH⁻ ions is equivalent to the amount of CO₂ absorbed.

This technique gave results accurate to ±15%. The inaccuracies were a result of the extreme sensitivity of the method to absorption of atmospheric CO₂ and the irreproducibility of the bubbling procedure, which was 70% efficient (±10%) in runs with solutions of known CO₂ concentration.

RESULTS AND DISCUSSION

Kinetic studies. It is convenient to present our results in terms of the scheme outlined below, which is more detailed than that given by Radda & Calvin (1964):

The rate of production of F* is proportional to the amount of light adsorbed according to the Beer-Lambert law. This may be simplified to:

$$I_0 - I = I_0(1 - e^{-\epsilon[\text{F}]l})$$

$$\simeq I_0\epsilon[\text{F}]l \text{ when } \epsilon[\text{F}]l < 0.25$$

F , $\dot{F}H$ and FH_2 are oxidized, half-reduced and fully reduced flavine respectively, and RH_2 , $\dot{R}H$ and R are reduced, half oxidized and fully oxidized reducing agent. F^* is excited singlet flavine and F^t the flavine triplet. ϵ is the extinction coefficient of the flavine, l is the cell path length and I_0 is the incident light-intensity. This scheme supposes that the reactive species in photoreduction is the flavine triplet. This has already been demonstrated for photobleaching (Radde & Calvin, 1964), and was confirmed by observing that paramagnetic ions lower the rate of photoreduction (Table 1).

The rates of some of the processes listed are negligible. Neither EDTA nor DL-phenylglycine quenches flavine fluorescence, so k_{2b} and k_{3b} are unimportant. Second-order triplet deactivation (reaction 4e) is not always negligible, because the rate of photoreduction of FMN does not depend linearly on light-intensity (Fig. 1). In contrast, fluorescence intensity is directly proportional to excitation light-intensity. By restricting our considerations to initial reaction rates deactivations by the flavine radical (reactions 3d and 4d) can be neglected.

Reaction (8) has been independently studied by investigating the behaviour of the flavine radical produced in solution by catalytic hydrogenation of FMN. Following the radical concentration spectrophotometrically at 600m μ (Gibson, Massey &

Table 1. *Effect of metal ions on the photoreduction of FMN by EDTA*

All measurements were made at 40° on solutions containing 0.1 M-NaCl, 0.1 M-EDTA, 0.04 mM-FMN and 1 mM-metal chloride. Under these conditions fluorescence quenching by the metal chlorides was less than 1%.

Metal chloride added	Initial rate (fall in $E_{447}/\text{min.}$)	Percentage inhibition $\left[\left(1 - \frac{k_{\text{inhib.}}}{k_{\text{uninhib.}}} \right) \times 100 \right]$
None	0.078	0
ZnCl ₂	0.079	0
MgCl ₂	0.080	0
NiCl ₂	0.067	14
MnCl ₂	0.055	29

Fig. 1. Effects of temperature and light-intensity on the rate of photoreduction of FMN by EDTA. Reaction mixtures contained 0.1 M-phosphate buffer, pH 7.0, 1 mM-EDTA and 0.04 mM-FMN. ○, 40°; ●, 10°.

Atherton, 1962), we observed no reaction with EDTA in the dark, or on illumination by light with wavelengths greater than 550m μ . Light of wavelengths lower than 500m μ caused rapid reaction. This was caused by excitation of oxidized flavine followed by a change in radical concentration described by the equilibrium:

The radical concentration reached its maximum at half reaction. These observations suggest that k_{+8} is negligible.

Considering initial rates only, reactions (2b), (3b), (3d), (6), (7) and (8) are unimportant. Under these

conditions, and assuming that flavine concentration is sufficiently low for the rate of excited singlet production to be given by $k_{+1}I_0[F]$, it can be shown that:

$$-\left(\frac{d[F]}{dt}\right)_{\text{init.}} = \frac{k_{+1}k_{+5}(k_{+3a} + k_{+3c}[\text{solv.}])}{(k_{+2a}[\text{solv.}] + k_{+3a} + k_{+3c}[\text{solv.}] + k_{-1})} \cdot \frac{I_0[\text{RH}_2][F]}{(k_{+4a} + (k_{+4b} + k_{+5})[\text{RH}_2] + k_{+4c}[\text{solv.}] + k_{+4e}[F^t])}$$

This expression is obtained by applying steady-state approximations to the concentrations of flavine excited states, and is independent of the relative magnitudes of the k_{+6} and k_{+7} , and of whether route (5) or (5') is followed. It can account for the apparently contradictory reports that the rate of photoreduction is proportional to the concentration of reducing agent (Holmström, 1962) and that the two are independent (Radda & Calvin, 1964). At low reducing agent concentrations the $(k_{+4b} + k_{+5})[\text{RH}_2]$ term is unimportant relative to the other quenching terms in the denominator, so linear concentration-dependence should be found. At high concentrations of reducing agent solvent quenching becomes relatively unimportant as reaction with RH_2 reduces triplet lifetime. The anaerobic photoreductions of lumiflavine by EDTA

Fig. 2. Effects of concentration of (a) EDTA and (b) DL-phenylglycine on the rate of photoreduction of flavines. Reaction mixtures contained 0.1M-phosphate buffer, pH 7.0 (a) or pH 8.3 (b), and 0.02mM-FMN (a) or 0.035mM-lumiflavine (b). Temperature was 25°.

and DL-phenylglycine both show this behaviour (Fig. 2).

Reaction products. The photoreaction of DL-phenylglycine with a variety of flavines is completely

reversible in the dark by oxygen, indicating that reduced flavine is a reaction product. The reaction of phenylacetic acid is not reversible in this way. DL-Phenylglycine is oxidized to benzaldehyde and carbon dioxide, which are produced in a mole-for-mole ratio to lumiflavine reduced (Table 2). Neither benzoic acid nor phenylglyoxylic acid was detected. Our analytical methods were capable of detecting a yield of 10% or more phenylglyoxylic acid in spite of its slow photosensitized decomposition.

Effect of reducing agent structure on photoreduction. As benzaldehyde is the major product of reduction by DL-phenylglycine the primary reaction centre involves the α -carbon atom. Thus the effect of *meta* and *para* substituents in the benzene ring should indicate the nature of the rate process. The same substituents also alter the dissociation constants of the amino groups, and hence a knowledge of the pK_b values and of the influence of pH on reaction rate is essential for understanding the substituent effects. The pH-dependences of the reaction rates of a lumiflavine derivative with DL-phenylglycine and phenylacetic acid are recorded in Fig. 3. Whereas the phenylacetic acid curve does not correspond to the titration curves of the reactants, the DL-phenylglycine result is readily explained if only the anion, $\text{Ph}\cdot\text{CH}(\text{NH}_2)\cdot\text{CO}_2^-$, but not its zwitterion, reacts. The dissociation constants measured by titration, and the reaction rates at pH 7.0, are recorded in Table 3. The substituent effects may be analysed in terms of the Hammett (1940) equation. The photoreduction

Table 2. Reaction products of the photoreduction of lumiflavine by phenylglycine

All reactions were at 25° and pH 8.2. For details of the analyses see the Methods section.

Amount of lumiflavine reacting (μ moles)	Amount of benzaldehyde formed (μ moles)	Amount of CO_2 formed (μ moles)
4.8	5.2	
4.8	4.8	
4.8	5.6	
5.2		5.0
5.0		4.8
2.8		3.0

Fig. 3. pH-dependence of the rate of photoreaction of 9-acetoxyethyl-3-methyl-lumiflavine with DL-phenylglycine and with phenylacetic acid. Reaction mixtures contained 0.1M-phosphate buffer of appropriate pH, 0.025mM-flavine and 5mM-acid at 25°. ○, DL-Phenylglycine; ●, phenylacetic acid.

Table 3. Dissociation constants and corrected rates for the photoreduction of lumiflavine by substituted phenylglycines

pK_b values were measured in 0.1M-NaCl at 25° ($\pm 0.2^\circ$). Reaction rates were measured in solutions containing 0.1M-phosphate buffer, pH 7.0, 0.015mM-lumiflavine and 5mM-phenylglycine at 30°.

Acid	pK_b	Corrected rate [Initial rate $\times$ anti- log(pK_b (phenyl- glycine) - pK_b (substituted acid))]
DL-Phenylglycine	5.09	0.78
DL- <i>m</i> -Fluorophenylglycine	5.45	0.36
DL- <i>m</i> -Chlorophenylglycine	5.52	0.28
DL- <i>p</i> -Methylphenylglycine	5.04	0.86
DL- <i>p</i> -Fluorophenylglycine	5.16	0.67
DL- <i>p</i> -Methoxyphenylglycine	4.94	2.10

rates give a ρ -value of -1.1 , with a correlation coefficient of 0.94. This relatively small ρ -value is consistent with a radical mechanism. Its negative sign suggests a transition state with an electron-deficient α -carbon atom on the amino acid. This is possible if the flavine triplet behaves like an electrophilic radical (the extraction of benzylic hydrogens by bromine atoms may be an analogous reaction; Kooyman, van Helden & Bickel, 1953). In contrast, the photoreduction of lumiflavine by substituted phenylacetic acids gives a very different pattern (J. A. Taylor & G. K. Radda, unpublished work).

Influence of flavine structure on reaction rates. We have observed that flavines are photoreduced by the unprotonated form of a variety of amines (e.g. diethanolamine, benzylamine, triethylamine). If decarboxylation is in the rate-determining step for

the acids (as expected for phenylacetic acid) a comparison of relative photoreaction rates of different flavines with amines, amino acids and phenylacetic acid might distinguish between different mechanisms. Such a comparison is shown in Table 4. It is apparent that, whereas the ratio of reactivity of DL-phenylglycine to benzylamine remains constant within experimental error, the reactivity of phenylacetic acid ranges from the slowest to the fastest of the three substrates with different flavines. This behaviour suggests a dissimilarity of mechanism.

It was also observed that the DL-phenylglycine and benzylamine reactions were invariably rapidly reversed by air, but the phenylacetic acid one was not.

Inhibition of photoreductions. The interaction of some aromatic amines with excited flavines leads to inhibition of reactivity rather than photoreduction (Radda & Calvin, 1963; Homann & Gaffron, 1964; Radda, 1966). The elucidation of these interactions (cf. step 4b in our scheme) is important for an understanding of the mechanism of reduction. Some of the inhibitors are tryptophan, tyrosine, serotonin, 3-(*p*-chlorophenyl)-1,1-dimethylurea and 3-(2,4-dichlorophenyl)-1,1-dimethylurea. The first three inhibit the photoreduction of other dyes, but the substituted ureas do not. This has provoked the suggestion that there is a special type of interaction between the ureas and flavines (Homann & Gaffron, 1963). We have therefore compared the photoreductions of flavines and methylene blue in their sensitivity towards inhibitors.

The inhibition must involve quenching of the flavine triplet, because, though some of the inhibitors form ground-state complexes with flavines and are also fluorescence quenchers (Penzer & Radda, 1967), these effects are too small to explain the magnitude of the effect on reactivity [e.g. 3-(*p*-chlorophenyl)-1,1-dimethylurea quenches fluorescence by less than 1%; for the others see Radda (1966)]. The triplet may be quenched by either collision or complex-formation with the inhibitor (Radda, 1966). These are potentially distinguishable on the basis of the temperature-dependence of reaction rate. The effects of temperature on the inhibited and uninhibited photobleaching and photoreduction of FMN by EDTA are summarized in Table 5. The uninhibited reactions both have small positive temperature coefficients, in spite of the increase in collisional quenching by the solvent. This means that there is a small activation energy for the reaction. Photoreduction of FMN by NADH has a small negative temperature coefficient (Radda & Calvin, 1964), and the increase in coefficient in the order NADH, EDTA and ribityl side chain corresponds to decreasing reducing power.

Potassium iodide is generally assumed to be a

Table 4. *Relative rates of photoreduction of flavine derivatives by phenylglycine, phenylacetic acid and benzylamine*

Flavine concentrations were all approx. 0.02 mM. Solutions contained 5 mM-reducing agent in 0.1 M-phosphate buffer, pH 8.00 (for DL-phenylglycine), pH 7.89 (for phenylacetic acid) or pH 7.93 (for benzylamine).

Flavine				Rate of reduction by DL-phenylglycine	Rate of reduction by DL-phenylglycine
R ₁	R ₁	R ₃	R ₄	Rate of reduction by phenylacetic acid	Rate of reduction by benzylamine
H	Me	Me	Ribityl phosphate (FMN)	8.25	5.5
H	Me	Me	Me (lumiflavine)	7.0	5.5
H	Me	Me	Me (N ³ -Me) (3-methyl-lumi-flavine, compound I)	8.0	6.0
Me	Me	H	Ribityl (isoribo-flavine, compound II)	10.0	5.3
H	Cl	MeO	$\begin{array}{c} \text{CH}_2\text{-CH}_2 \\ \\ [\text{CH}_2]_2\text{-N} \\ \\ \text{CH}_2\text{-CH}_2 \end{array}$ (compound III)	2.7	4.0
H	Cl	H	$[\text{CH}_2]_3\text{-NEt}_2$ (compound IV)	3.5	6.4
H	H	H	$[\text{CH}_2]_2\text{-NMe}_2$ (compound V)	0.4	5.0

collisional quencher, yet its inhibition of photobleaching falls with temperature. Triplet lifetime is decreased as a result of increased reactivity at the higher temperature, so that changes in potassium iodide quenching are not dominated by variations in collision frequency alone. For the other inhibitors the $k_{\text{inhb.}}/k_{\text{uninhb.}}$ ratio values at the two temperatures are greater than or equal to [for 3-(*p*-chlorophenyl)-1,1-dimethylurea] that found for potassium iodide (Table 5). This suggests that inhibition by serotonin and tryptophan has at least a component from complex-formation.

A given concentration of inhibitor affects photoreduction less than photobleaching. The temperature effects are smaller too. These can be explained as follows. The relative contribution of an inhibition term to the denominator of the rate equation is less when $k_{+5}[\text{RH}_2]$ is large. The rate constants for those processes that involve an activation energy increase more with temperature than for simple collision processes. Now, the activation energy for photobleaching is higher than for photoreduction, so the increase of the k_{+5} term relative to the quenching term (k_{+4b}) is more important for photobleaching.

The variation of inhibition with light-intensity (Fig. 1 shows this dependence for uninhibited reaction) depends on second-order triplet deactivation (k_{+4e}). This is greater for uninhibited reactions at a particular light-intensity because of the higher $[\text{F}^t]$. It is not possible to detect significant changes of inhibition over the light-intensity range covered, because $[\text{F}^t]$ is never large. At higher intensities the uninhibited reaction rate is too fast for accurate measurement, but approximate measurements indicate that inhibition falls under intense illumination, as expected (Table 5).

Neither DL-phenylglycine nor its derivatives inhibit the photoreduction of FMN by EDTA at pH 7.0 (where the contribution due to reduction by the DL-phenylglycine is negligible), showing that for phenylglycines k_{+4b} is very small.

Photoreduction of methylene blue by EDTA has qualitatively similar temperature- and light-intensity-dependences to the FMN reaction (Table 6). The temperature coefficient, however, is higher ($k_{41^\circ}/k_{15^\circ}$ ratio 2.4) and inhibition by a given concentration of inhibitor is lower. Tryptophan and serotonin inhibit, but 3-(*p*-chlorophenyl)-1,1-dimethylurea and 3-(2,4-dichlorophenyl)-1,1-

Table 5. *Temperature and light-intensity effects on the rates of inhibited and uninhibited photobleaching and photoreduction of FMN*

Photobleaching: unless stated to the contrary all solutions contained 0.1M-phosphate buffer, pH 7.0, 0.4mM-inhibitor and 0.06mM-FMN:

$$k_{\text{uninhb. at } 38^\circ} / k_{\text{uninhb. at } 17^\circ} = 1.44$$

Photoreduction: unless stated to the contrary all solutions contained 0.1M-phosphate buffer, pH 7.0, 1mM-EDTA, 0.5mM-inhibitor and 0.04mM-FMN:

$$k_{\text{uninhb. at } 40^\circ} / k_{\text{uninhb. at } 10^\circ} = 1.20$$

Abbreviation: CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea.

Photobleaching				$\frac{k_{\text{inhb.}}}{k_{\text{uninhb.}}}$ at 38°
Inhibitor	Temp.		$\frac{k_{\text{inhb.}}}{k_{\text{uninhb.}}}$	$\frac{k_{\text{inhb.}}}{k_{\text{uninhb.}}}$ at 17°
CMU	17°		0.18	1.33
CMU	38		0.24	
Tryptophan	17		0.04	2.00
Tryptophan	38		0.08	
Serotonin (0.1 mm)	17		0.04*	~ 14
Serotonin (0.1 mm)	43		0.57*	
KI (0.01 mm)	17		0.40	1.30
KI (0.01 mm)	38		0.52	
Photoreduction				$\frac{k_{\text{inhb.}}}{k_{\text{uninhb.}}}$ at 40°
Inhibitor	Temp.	Light-intensity (relative units)	$\frac{k_{\text{inhb.}}}{k_{\text{uninhb.}}}$	$\frac{k_{\text{inhb.}}}{k_{\text{uninhb.}}}$ at 10°
CMU	40°	100	0.35	1.00
CMU	10	100	0.35	
CMU	40	60	0.39	1.05
CMU	10	60	0.37	
CMU	40	40	0.40	1.08
CMU	10	40	0.37	
CMU	40	20	0.41	1.11
CMU	10	20	0.37	
Tryptophan	40	100	0.32	0.89
Tryptophan	10	100	0.36	
Tryptophan	40	20	0.32	0.86
Tryptophan	10	20	0.37	
Serotonin (0.1 mm)	40	100	0.29	> 1.32
Serotonin (0.1 mm)	20	100	0.22	
CMU (FMN conc. 0.08 mm)	20	> 200	0.55	
Tryptophan (FMN conc. 0.08 mm)	20	> 200	0.40	

* Taken from Radda (1966).

dimethylurea do not. Inhibition by tryptophan increases slightly as the temperature is raised.

These differences between the indole and urea derivatives may be a result of the ability of the former to form stronger complexes with the dyes. The methylene blue complexes are generally weaker than flavine ones, so that 3-(*p*-chlorophenyl)-1,1-dimethylurea may just fall outside the range of significant interaction.

Effects of urea on inhibitions. Both substituted

ureas (Hansch & Deutsch, 1966) and flavines (Weber, 1966) participate in hydrophobic interactions in their ground states. This is shown by the effect of urea and non-aqueous solvents on flavine-aromatic molecule complexes (Weber, 1966; Gascoigne & Radda, 1965). If similar interactions occur with flavine triplets they could explain the photoinhibitions. Table 7 shows that urea slightly inhibits photobleaching, but it accelerates the reaction in the presence of other inhibitors. This

Table 6. *Temperature and light-intensity effects on the rates of inhibited and uninhibited photoreduction of methylene blue by EDTA*

All solutions contained 0.1 M-phosphate buffer, pH 7.0, 0.5 mM-inhibitor and 0.02 mM-methylene blue:

$$k_{\text{uninhb. at } 41^\circ} / k_{\text{uninhb. at } 15^\circ} = 2.4$$

Abbreviations: CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea; DCMU, 3-(2,4-dichlorophenyl)-1,1-dimethylurea.

Inhibitor	Temp.	Light-intensity (relative units)	$\frac{k_{\text{inhb.}}}{k_{\text{uninhb.}}}$	$\frac{k_{\text{inhb.}}}{k_{\text{uninhb.}}}$ at 41°
				$\frac{k_{\text{inhb.}}}{k_{\text{uninhb.}}}$ at 15°
Tryptophan	41°	100	0.68	0.79
Tryptophan	15	100	0.86	
Tryptophan	41	60	0.73	0.88
Tryptophan	15	60	0.83	
Tryptophan	41	40	0.72	0.84
Tryptophan	15	40	0.86	
Tryptophan	41	20	0.73	0.97
Tryptophan	15	20	0.75	
CMU	41	100	1.0	
CMU	15	100	1.0	
DCMU	20	100	1.0	
Serotonin	20	100	0.05	
Tryptophan	20	> 200	0.95–1.0	

Table 7. *Effects of 6.5 M-urea on the rates of inhibited and uninhibited photobleaching, photoreduction and fluorescence of FMN*

Solutions contained 0.1 M-phosphate buffer, pH 7.0, and 0.015 mM-FMN. Abbreviation: CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea.

Reactants	Reaction rate in absence of urea	Reaction rate in 6.5 M-urea	Fluorescence intensity	Fluorescence intensity
	Reaction rate of FMN alone	Reaction rate in absence of urea	Fluorescence intensity of FMN alone	Fluorescence intensity in 6.5 M-urea in absence of urea
FMN	1.00	0.84	1.00	0.98
FMN + KI (0.01 mM)	0.34	1.16	—	—
FMN + KI (20 mM)	—	—	0.67	1.07
FMN + tryptophan (0.5 mM)	0.06	2.00	—	—
FMN + tryptophan (2 mM)	—	—	0.89	1.07
FMN + CMU (0.5 mM)	0.18	1.52	—	—
FMN + serotonin (2 mM)	—	—	0.59	1.41
FMN + EDTA (1 mM)	—	1.01	—	—

Table 8. *Effect of urea on the rates of photobleaching and photoreduction of FAD by EDTA*

All solutions contained 0.1 M-phosphate buffer, pH 7.0, 0.025 mM-FAD and 1 mM-EDTA.

Reactants	Conc. of urea (M)	Reaction rate in urea
		Reaction rate in absence of urea
FAD	6.5	1.00
FAD + EDTA	1.6	1.04
FAD + EDTA	3.2	1.15
FAD + EDTA	4.9	1.24
FAD + EDTA	6.5	1.30

suggests that complex-formation, partly stabilized by hydrophobic interaction, is a likely contributor to the inhibition (cf. Radda, 1966). The small acceleration that urea induces in the potassium iodide-inhibited reaction is probably caused by a change in viscosity. This is also reflected in a slight fall in fluorescence quenching of FMN by potassium iodide in urea. Urea removes the ability of tryptophan to quench flavine fluorescence, and decreases quenching by serotonin by 60% (Table 7). The negligible change in the rate of photoreduction of FMN by EDTA in urea solutions corresponds to

Fig. 4. Effect of urea on the second-order rate constant (k_2) for dark reduction of FAD and FMN by NADH. Reaction mixtures contained 0.02M-phosphate buffer, 0.06mM-FAD or -FMN and 2mM-NADH at 25°. ○, FMN; ●, FAD; *italicized numbers* give $k_2(\text{FMN})/k_2(\text{FAD})$.

Fig. 5. Effect of dimethyl sulphoxide on the second-order rate constant (k_2) for dark reduction of FAD and FMN by NADH. Reaction mixtures contained 0.02M-phosphate buffer, 0.06mM-FAD or -FMN and 2mM-NADH at 25°. ○, FMN; ●, FAD; *italicized numbers* give $k_2(\text{FMN})/k_2(\text{FAD})$.

Fig. 6. Dependence of the FAD/FMN relative fluorescence intensity on urea concentration. Flavine concentrations were 0.015mM.

Fig. 7. Dependence of the FAD/FMN relative fluorescence intensity on dimethyl sulphoxide concentration. Flavine concentrations were 0.015mM.

the lower inhibitions generally observed for photoreduction in accordance with our kinetic scheme.

The low rates of photoreduction and photobleaching of FAD compared with those of FMN (Radda, 1966; McCormick, Koster & Veeger, 1967) have been attributed to the formation of an intramolecular complex in the former (Weber, 1950). When urea is added there is no change in the rate of photobleaching (Table 8), though there is a large increase in fluorescence, indicating unfolding (Gascoigne & Radda, 1965). With the inhibitory effect of urea on the photobleaching of FMN being taken into account, unfolding must result in a faster rate. The effect is more clearly shown for photoreduction by EDTA, where inhibition by urea is less important. Even so, in 6.5M-urea the increase in reactivity (1.30-fold) is less than the increase in fluorescence (2.13-fold).

The dark reactions of FMN and FAD with NADH (cf. Radda & Calvin, 1964) are not significantly altered on unfolding. This has been shown by comparing the rates of reaction of FMN and FAD in solvents containing urea or dimethyl sulphoxide (Figs. 4 and 5). The relative fluorescence intensities change markedly under these conditions (Figs. 6 and 7). This shows that in the dark reaction steric hindrance is about the same in the folded and unfolded forms. We would expect this to apply equally to photoreactions, so we attribute the increase in rate here to decreased triplet quenching (cf. the effect of urea on intermolecular inhibitions).

In conclusion, we consider that all experimental results on the photoreduction of flavines by amino acids are most readily understood in terms of the steps presented in our scheme. It is likely that the first step is a one-electron process between the amino acid and the excited flavine. This is probably not the case for the reaction between excited flavines and phenylacetic acid, which shows several marked differences from the other photoreductions: (1) it is not completely reversed by air in the dark when the others are; (2) the pH-dependence of reaction rate is not simply explicable in terms of the pK_a values of the reactants; (3) the order of photo-reactivity with various flavine derivatives is widely different.

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