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The Chemistry of Flavins and Flavoproteins:
AEROBIC PHOTOCHEMISTRY

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1. When a mixture of FMN and a reducing substrate (e.g. unprotonated amine) is illuminated oxygen is consumed. 2. The rate of oxygen uptake increases as oxygen concentration falls with some substrates (type I reaction), but with other substrates (typically aromatic compounds) the rate falls as the oxygen concentration falls (type II reaction). 3. The kinetics of type I reactions with EDTA, DL-α-phenylglycine and diethanolamine are all consistent with a mechanism in which the rate-determining step, hydrogen abstraction by the FMN triplet, is followed by rapid reoxidation of reduced FMN by oxygen. The reaction is faster at low oxygen concentrations because oxygen quenches the triplet. 4. The sensitivity of reaction rates to substituents in DL-α-phenylglycine can be described by a Hammett ρ value of −0.6. 5. Individual rate constants for quenching and reaction of the FMN triplet with substrate were calculated (2.4×10^8 and 2.1×10^7 $M^{-1}s^{-1}$ respectively for EDTA) on the assumption that oxygen quenches the triplet in a diffusion-controlled reaction. 6. The pH-dependences of oxygen uptake rates with six natural amino acids as substrates were measured. 7. Photoinactivations of L-glutamate dehydrogenase and D-amino acid oxidase by FMN were demonstrated.

Riboflavin is photobleached more slowly in the presence of oxygen than under anaerobic conditions (Halwer, 1951; Yang & McCormick, 1965). Reactions in which the flavin acts as a photosensitizer are often faster than the aerobic bleaching. Some substrates (typically amines, amino acids and some aromatic compounds) are apparently oxidized (e.g. Frisell, Chung & Mackenzie, 1959), but flavins can also sensitize the polymerization of olefins (Oster, 1954) and the deiodination of aromatic iodo compounds (Lissitzky, Benevent & Rogues, 1961). The mechanism of the sensitized oxidation of sarcosine probably involves reduction of the flavin followed by its rapid reoxidation by air (Frisell *et al.* 1959), whereas the reaction of aromatic compounds probably involves the production of singlet oxygen that is then the reactive species (Berends, Posthuma, Sussenbach & Mager, 1966; Kearns, Hollins, Kahn & Radlick, 1967).

Photosensitized destruction of amino acids and nucleotide bases has been used to induce chemical modification in proteins and nucleic acids (Simon, 1967). Different dyes vary in specificity towards the individual subunits and the specificity of a given dye depends critically on the reaction conditions. Although some model studies on the reactions of flavins with amino acids have been reported (Bellin & Yankus, 1968), there are insufficient data for an interpretation of flavin-photosensitized destruction of proteins.

The results in the present paper mainly concern the sensitized photo-oxidation of amino acids, their reactivities and the mechanisms of reaction. Various other compounds (including two enzymes) have also been tested for their susceptibility to photosensitized reaction with flavins.

MATERIALS

FMN and ox liver L-glutamate–NAD(P) oxidoreductase (deaminating) (EC 1.4.1.3) were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The benzoate complex of hog kidney D-amino acid oxidase (EC 1.4.3.3) was prepared from fresh kidneys by the method of Yagi *et al.* (1967) except that the second calcium phosphate-gel column filtration was omitted. All other reagents were commercial products in their purest forms.

METHODS

Oxygen concentrations were measured with an oxygen electrode (Clark, 1956). The electrode (E.I.L., Richmond, Surrey U.K.; model SOH33), was connected to a polarograph (Radiometer PO4b) that could apply a steady potential of −0.6 V and record currents continuously from 0.01 μA upwards. The membrane used was polythene

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supplied by E.I.L., who quoted its response time as 2–4min; this was about the time taken for the electrode to give a steady current after a large change in oxygen concentration. Small concentration changes were registered almost instantaneously. The electrode response fell by less than 3% as a result of consumption of oxygen after immersion in the same solution for 30min and remained constant to ±5% for air-saturated water during a day's use of the instrument. The reaction vessel used for photochemical studies was a glass cyclinder kept at 25°C by circulating water. The oxygen electrode fitted the top of this with an airtight seal and care was taken to exclude all air from the vessel before study of a reaction by filling it completely with solution. The solutions were stirred magnetically sufficiently fast for small changes in stirring speed to have an undetectable effect on the electrode response. To avoid the effects of stray light the outside of the reaction vessel was covered with black tape except for a small window through which the exciting beam could pass. The electrode position was such that the light (from a tungsten lamp with a filter to remove wavelengths less than 400nm; see Penzer & Radda, 1968) did not shine directly on it.

Oxygen-electrode response is proportional to oxygen activity (Dixon & Kleppe, 1965) so only two points are needed for calibration. The standard solutions used here were air-saturated water ($[O_2]$ 0.257mM at standard barometric pressure) and a solution containing reduced FMN, which was produced by photochemical reduction of FMN with EDTA ($[O_2]$ 0).

Glutamate dehydrogenase, which was stored as an $(NH_4)_2SO_4$ suspension, was dialysed against 0.1M-sodium phosphate buffer, pH7.8, before use. It was assayed spectrophotometrically by the method of Dodd & Radda (1969). D-Amino acid oxidase was also stored as an $(NH_4)_2SO_4$ suspension and was dialysed against 16.7mM-sodium pyrophosphate buffer containing 8mM-benzoic acid, pH 8.3, before use. It was assayed spectrophotometrically by the method of Fonda & Anderson (1967). Both assays gave activities that were reproducible to ±6%.

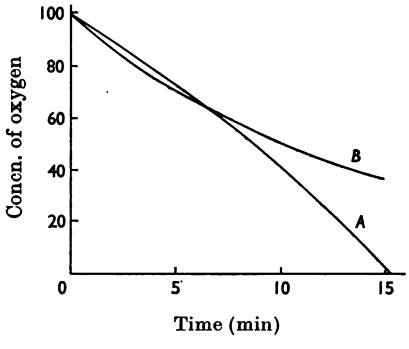

Fig. 1. FMN-photosensitized oxygen uptake. Reactions were at 25°C in 0.1M-phosphate buffer; $[O_2]$ 100 for a solution in equilibrium with the atmosphere. Curve *A*, type I reaction (0.2mM-FMN+5mM-EDTA, pH7.0); curve *B*, type II reaction (0.1mM-FMN+1.0mM-tryptophan, pH8.0).

RESULTS

When a flavin-sensitized photo-oxidation is studied by measuring the rate of oxygen consumption, two forms of kinetic behaviour are observed. In the first (type I) the rate of oxygen uptake

Table 1. *Rates of FMN-photosensitized oxygen uptake in the presence of different substrates*

All reactions were at 25°C, starting with solutions in equilibrium with the atmosphere, containing 0.1M-phosphate buffer at the pH stated. Substrate concentrations were about 5mM; FMN concentrations were 0.2mM. ++++ denotes a reaction that consumes all oxygen in a solution initially in equilibrium with the atmosphere in about 15min: + denotes a reaction 1–3% of this rate.

Substrate	pH	Type of kinetic behaviour	Approximate initial rate
None	7	I	+
Uracil	7	Intermediate	++++
Thymine	7	I	+
Cytosine	7	I	+
Guanosine	8	II	++
Adenosine	8	II	+
Benzoic acid	7	I	+
NN-Dimethylaniline	7	II	+
Benzaldehyde	7	I	+
p-Toluidine	7	II	+++
p-Toluidine	8	II	++++
m-Toluidine	8	II	++
p-Cresol	8	II	++
m-Cresol	8	II	++
o-Cresol	8	II	++
p-Aminobenzoic acid	8	I	+
Phenylacetic acid	7	Intermediate	++
EDTA	7	I	++++
DL-α-Phenylglycine	8	I	++
Ethanolamine	7	I	+
Diethanolamine	7	I	++
Triethanolamine	7	I	++++
3-(*p*-Chlorophenyl)-1,1-dimethylurea	8	II	+++
L-Tryptophan	8	II	++++
L-Tyrosine	8	II	+++
L-Alanine	8	I	+
L-Phenylalanine	8	I	+
L-Cysteine	8	I	++
L-Methionine	8	I	++
L-Histidine	8	I	+++
Ethylene glycol	7	I	+
Ethylamine	7	I	+
Diethylamine	7	I	+
Triethylamine	7	I	+
Benzylamine	8	I	++
Serotonin	8	II	++
Phenol	8	I	+
Nicotinamide	8	I	+

increases as the reaction proceeds, whereas in the second (type II) it falls (Fig. 1). Various photooxidizable substrates were studied and distinguished according to the shape of the oxygen uptake curve (Table 1). Those substrates whose reaction character is described as intermediate have a sigmoidal uptake curve, type I at high oxygen concentrations and type II at low ones.

Type I reactions: pH *effects*. The effects of pH on the initial rate of oxygen uptake when aqueous solutions of FMN and ethanolamine, or FMN and benzylamine, are illuminated are shown in Fig. 2. In both cases the rate of reaction is greatest at about pH 9.5. These profiles suggest that reaction only occurs between the unprotonated form of the amine [pK_a(ethanolamine) 9.495, pK_a(benzylamine) 9.37 (Sober, 1968)] and an excited neutral isoalloxazine nucleus (pK for loss of a proton from the ground state about 10; Hemmerich, Veeger & Wood, 1965). The pH-dependence of anaerobic photoreduction is similar at least up to pH 8.5 (Penzer & Radda, 1968). These results alone could be explained by a reaction mechanism in which protonated amine and deprotonated FMN are the reactive species, but this possibility is unlikely for the following reasons. Deprotonated flavins do not fluoresce (Penzer & Radda, 1967) and are not photochemically reactive above pH 10 with compounds (e.g. phenylacetic acid) without an acid–base pK close to this value (A. Gordon-Walker & G. K. Radda, unpublished work). At pH 7 those type I substrates that contain unprotonated amino groups at neutral pH values (e.g. EDTA) photoreact fastest with FMN (Table 1).

The relative rates of oxygen uptake during reaction of the unprotonated forms of ethanolamine, diethanolamine and triethanolamine are in the order tertiary amine>secondary amine>primary amine (Table 2). This agrees with the predicted ease of abstraction of hydrogen from the carbon atom α to the amino group.

When a solution of FMN is illuminated alone oxygen is consumed more slowly than in the presence of unprotonated amines. The reaction rate increases slightly at pH values less than 5, with a larger enhancement at pH values above 8. This agrees with previous observations (Halwer, 1951; Yang & McCormick, 1965).

Type I reactions: *dependence on substrate*. The pH effects are consistent with a reaction mechanism involving reduction of the flavin by substrate followed by reoxidation of the reduced flavin by air. We have previously used the effects of substituents in α-phenylglycines on reaction rate to identify the rate-determining step of anaerobic photoreduction (Penzer & Radda, 1968). Similar experiments were carried out for the aerobic reaction to see whether the rate-determining step is again the same.

The rates of reaction of the substituted α-phenylglycines are given in Table 3. The pK values are changed for different substituents and as the reaction rate is pH-dependent corrections must be made before the results can be analysed in terms of the Hammett (1940) equation. The corrections are made on the assumption that reaction rate is directly proportional to the concentration of unprotonated α-phenylglycine. This is not strictly true (see below),

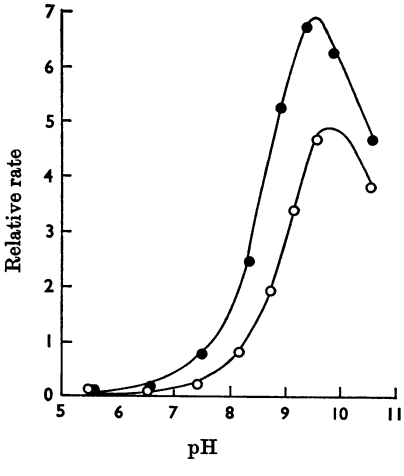

Fig. 2. pH-dependence of FMN-photosensitized oxygen uptake with benzylamine (●) and ethanolamine (○). Reactions were at 25°C in 0.1 M-phosphate buffer below pH 8.0 and in 0.1 M-Na_2HPO_4 adjusted with NaOH above pH 8.0. [FMN] 0.2 mM; [amine] 10 mM.

Table 2. *Rates of oxygen uptake with mono-, di- and tri-ethanolamine as substrates*

All reactions were at 25°C in 0.1 M-phosphate buffer at the pH stated. FMN concentrations were 0.4 mM; amine concentrations were 10 mM. Initial rates were measured for solutions in equilibrium with air. pK_a values were measured by titration with M-HCl in 0.1 M-NaCl. The correction for pK_a assumes that reaction rate is proportional to concentration of neutral amine. This is a reasonable approximation for the concentrations involved here (see below). No correction is made for the reaction of FMN in the absence of amine.

Amine	pK_a	pH of reaction	Relative rate of oxygen uptake	Relative rate corrected for pK_a
Ethanolamine	9.56	7.02	1.4	40
Diethanolamine	9.13	7.08	8.8	82
Triethanolamine	8.10	7.02	100	100
FMN alone	—	7.00	0.4	

Table 3. *Rates of FMN-photosensitized oxygen uptake with substituted* DL-α-*phenylglycines*

All reactions were at 25°C in 0.1M-phosphate buffer, pH7.61. FMN concentrations were 0.2mM; acid concentrations were 5mM. pK_a values ($14.00-pK_b$ at 25°C) are from Penzer & Radda (1968); Hammett σ values are from Wells (1963). The correction assumes that reaction rate is proportional to concentration of acid anion (see the Discussion section).

Acid	pK_a	Rate of oxygen uptake (relative to DL-α-phenylglycine = 100)	Corrected rate (k) (relative to DL-α-phenylglycine = 100[k_0])	$\log\left(\frac{k}{k_0}\right)$	(σ)
DL-α-Phenylglycine	8.91	100	100	0.000	0.000
DL-α-*m*-Fluorophenylglycine	8.55	133	58	$\bar{1}$.763	0.337
DL-α-*m*-Chlorophenylglycine	8.48	176	65	$\bar{1}$.813	0.373
DL-α-*p*-Methylphenylglycine	8.96	108	121	0.083	−0.170
DL-α-*p*-Fluorophenylglycine	8.84	104	88	$\bar{1}$.945	0.062
DL-α-*p*-Methoxyphenylglycine	9.06	432	609	0.785	−0.268

Table 4. *Rates of oxygen uptake by EDTA and illuminated FMN at different oxygen and hydrogen peroxide concentrations*

All reactions were at 25°C in 0.1M-phosphate buffer, pH7.0. FMN concentrations were 0.2mM; EDTA concentrations were 5mM. Oxygen concentrations are relative to air-saturated buffer = 100.

Concn. of oxygen	Initial rate of oxygen uptake (units/min)	Rate (units/min) during the run starting at $[O_2] = 167$
45	6.2	6.5
50	5.8	6.1
73	5.1	5.1
98	4.8	4.5
100	4.6	4.5
148	3.4	3.1
167	3.1	3.1
*100 (no H_2O_2)	9.8	
*100 (+0.1mM-H_2O_2)	9.9	

* These runs had a different light-intensity from the others.

but is reasonably accurate for the very low concentrations involved in these measurements. By plotting the corrected rates against Hammett σ values (Table 3) the Hammett ρ value and correlation coefficient (c) for the aerobic reaction are ρ −1.2, c 0.83 (ρ −0.6, c 0.98, excluding *p*-methoxy-DL-α-phenylglycine). For the anaerobic reaction ρ is −1.1 and c 0.94 (Penzer & Radda, 1968). When the two reactions are compared directly the gradient is 1.1 with c 0.89. In both cases *p*-methoxy-DL-α-phenylglycine reacts faster than it should to fit well with the rest of the Hammett plot; this may be because the methoxy group provides a second oxidizable site on the substrate.

Type I reactions: oxygen quenching. The rate of oxygen uptake in the FMN-sensitized photo-oxidation of EDTA increases as the concentration of oxygen falls (Table 4). The rate of reaction is the same at a given oxygen concentration whether this concentration is the initial one or is achieved after falling from a higher value during the course of reaction. Hydrogen peroxide, a likely reaction product, has no effect on the rate of oxygen uptake (Table 4). These results mean that product inhibition is unimportant, and the effect of a range of oxygen concentrations on the rate of reaction can be found from a single kinetic run.

It is convenient to consider the results of oxygen-quenching experiments in terms of the following scheme, which resembles that proposed by Penzer & Radda (1968) with extra terms to describe the effects of oxygen.

$$F + h\nu \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} F^* \quad (1)$$

$$F^* + \text{solv.} \xrightarrow{k_{+2a}} F + \text{solv.} \quad (2a)$$

$$F^* + RH_2 \xrightarrow{k_{+2b}} F + RH_2 \quad (2b)$$

$$F^* + O_2 \xrightarrow{k_{+2c}} F + O_2 \quad (2c)$$

$$F^* \xrightarrow{k_{+3a}} F^t \quad (3a)$$

$$F^* + RH_2 \xrightarrow{k_{+3b}} F^t + RH_2 \quad (3b)$$

$$F^* + \text{solv.} \xrightarrow{k_{+3c}} F^t + \text{solv.} \quad (3c)$$

$$F^* + FH^\cdot \xrightarrow{k_{+3d}} F^t + FH^\cdot \quad (3d)$$

$$F^* + O_2 \xrightarrow{k_{+3e}} F^t + O_2 \quad (3e)$$

$$F^t \xrightarrow{k_{+4a}} F \quad (4a)$$

$$F^t + RH_2 \xrightarrow{k_{+4b}} F + RH_2 \quad (4b)$$

$$F^t + solv. \xrightarrow{k_{+4c}} F + solv. \quad (4c)$$

$$F^t + FH^\cdot \xrightarrow{k_{+4d}} F + FH^\cdot \quad (4d)$$

$$F^t + F^t \xrightarrow{k_{+4e}} F + F^\star \quad (4e)$$

$$F^t + O_2 \xrightarrow{k_{+4f}} F + O_2 \quad (4f)$$

$$F^t + RH_2 \xrightarrow{k_{+5}} FH^\cdot + RH^\cdot \quad (5)$$

even when considering rates other than initial ones steps (3d) and (4d) should be unimportant. Step (4e) must be insignificant compared with step (+1), because no delayed fluorescence can be detected from aerobic aqueous solutions of flavins.

When step (9) is very fast compared with step (5) the rate of oxygen consumption, $R = k_{+5}[F^t][RH_2]$. Applying a steady-state treatment to $[F^\star]$ and $[F^t]$ it is found that:

$$R = \frac{k_{+1}k_{+5}(k_{+3a} + k_{+3c}[\text{solv.}])I_0[RH_2][F]}{\{k_{-1} + (k_{+2a} + k_{+3c})[\text{solv.}] + k_{+3a}\}\{k_{+4a} + (k_{+4b} + k_{+5})[RH_2] + k_{+4c}[\text{solv.}] + k_{+4e}[F^t] + k_{+4f}[O_2]\}}$$

$$FH^\cdot + RH^\cdot \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} FH_2 + R \quad (6)$$

$$2FH^\cdot \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} FH_2 + F \quad (7)$$

$$FH^\cdot + RH_2 \underset{k_{-8}}{\overset{k_{+8}}{\rightleftharpoons}} FH_2 + RH^\cdot \quad (8)$$

$$FH_2 + O_2 \xrightarrow{k_{+9}} F + H_2O_2 \quad (9)$$

F, $FH^\cdot$ and FH_2 are oxidized, half-reduced and fully reduced flavin respectively, and RH_2, $RH^\cdot$ and R are reduced, half-oxidized and fully oxidized reducing agent. $F^\star$ is excited-singlet flavin and F^t is the flavin triplet. Assuming a type I reaction is photoreduction of the flavin followed by rapid oxidation of the reduced flavin by oxygen, the rate-determining step is reaction (5) at high oxygen concentrations, as in anaerobic photoreduction (Penzer & Radda, 1968), and the forward reaction sequence is +1, +3, +5, +6 or +7, +9. The overall reaction is:

$$RH_2 + O_2 \xrightarrow[h\nu]{\text{FMN}} R + H_2O_2$$

When DL-α-phenylglycine was the substrate the chief reaction product detected by vapour-phase chromatography (Penzer & Radda, 1968) was benzaldehyde. This is also the major product of the anaerobic reaction. At lower oxygen concentrations the bimolecular step (reaction 9) must eventually be rate-determining. In these experiments, with solutions initially in equilibrium with the atmosphere, the rate of reaction continued to increase until over 99% of the oxygen had been consumed, which indicates that reaction (5) was the slowest step throughout.

Some of the steps denoted in the scheme are negligible. k_{+2b}, k_{+3b} and k_{+8} can be neglected for most type I substrates (Penzer & Radda, 1968). The fluorescence of FMN is not quenched in aerobic solution, so that k_{+2c} and k_{+3e} can also be neglected. As long as step (5) determines the rate no large concentration of reduced flavin can build up and so

where I_0 is the intensity of the incident light, and $k_{+1}I_0[F]$ gives the rate at which light is absorbed (only strictly true for low values of [F]). Let:

$$A = \frac{k_{+1}(k_{+3a} + k_{+3c}[\text{solv.}])I_0[F]}{k_{-1} + (k_{+2a} + k_{+3c})[\text{solv.}] + k_{+3a}}$$

This is constant for a given intensity of illumination and flavin concentration. Let:

$$B = k_{+4a} + k_{+4c}[\text{solv.}] + k_{+4e}[F^t]$$

This is not constant because $[F^t]$ varies with $[O_2]$. $k_{+4e}[F^t]$ is probably small compared with other triplet-deactivation processes, and so to a first approximation the variations in B can be neglected. Taking the reciprocal of R and dividing up the terms:

$$\frac{1}{R} = \frac{B}{k_{+5}[RH_2]A} + \frac{(k_{+4b} + k_{+5})}{k_{+5}A} + \frac{k_{+4f}[O_2]}{k_{+5}[RH_2]A}$$

If the Mechanism proposed for type I reactions is correct their kinetics should fit this expression.

When $1/R$ is plotted against $[O_2]$ for the reactions of FMN with EDTA, DL-α-phenylglycine and

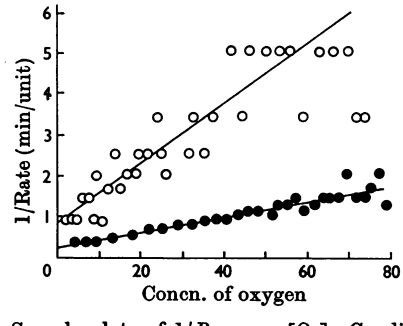

Fig. 3. Sample plots of $1/R$ versus $[O_2]$. Conditions of reaction are given in Table 5. $[O_2]$ 80 for a solution in equilibrium with the atmosphere: ○, [α-phenylglycine] 0.18 mM (correlation coefficient 0.79); ●, [α-phenylglycine] 0.90 mM (correlation coefficient 0.92).

Table 5. *Composite rate constants for the aerobic photoreduction of FMN by EDTA,* DL-α-*phenylglycine and diethanolamine: G_1 and J_1*

All reactions were at 25°C under the same intensity of illumination. Solutions contained 0.1 M-phosphate buffer at the pH stated and 0.1 mM-FMN. G_1 and J_1 are defined in the text.

(*a*) EDTA: reactions at pH 7.3

Concn. of EDTA (mM)	No. of points plotted	$10^{-9}\ G_1$ (l^2 min mol^{-2})	$10^{-5}\ J_1$ (l min mol^{-1})	Least-squares coefficient of correlation
23	21	0.084	0.35	0.91
11.5	25	0.083	0.44	0.92
9.2	21	0.098	0.47	0.64
6.9	23	0.069	0.45	0.71
4.6	23	0.159	0.41	0.90
2.3	20	0.441	0.54	0.88

(*b*) DL-α-Phenylglycine: reactions at pH 7.8

Concn. of phenylglycine anion (mM)	No. of points plotted	$10^{-9}\ G_1$ (l^2 min mol^{-2})	$10^{-5}\ J_1$ (l min mol^{-1})	Least-squares coefficient of correlation
1.8	26	1.0	0.75	0.96
0.9	31	1.8	0.80	0.92
0.36	37	5.9	0.50	0.92
0.18	37	7.0	2.77	0.79

(*c*) Diethanolamine: reactions at pH 7.8

Concn. of neutral diethanolamine (mM)	No. of points plotted	$10^{-9}\ G_1$ (l^2 min mol^{-2})	$10^{-5}\ J_1$ (l min mol^{-1})	Least-squares coefficient of correlation
1.27	26	0.8	0.56	0.97
0.64	17	1.1	0.67	0.89
0.25	28	3.5	1.18	0.95
0.13	31	3.9	0.88	0.95

Table 6. *Composite rate constants for the aerobic photoreduction of FMN by EDTA,* DL-*phenylglycine and diethanolamine: G_2, G_3 and J_2*

Reaction conditions are as described in Table 5. G_2, G_3 and J_2 are defined in the text.

Substrate	$10^{-5}\ G_2$ (l min mol^{-1})	G_3 (min)	$10^{-4}\ J_2$ (l min mol^{-1})	$10^7/G_2$ ($mol\ l^{-1}$ min^{-1})
EDTA	9.3	22	3.7	10.8
DL-α-Phenylglycine	15.7	26.5	4.8	6.4
Diethanolamine	8.7	13	4.7	11.7

diethanolamine, reasonable straight lines are obtained (Fig. 3 and Table 5). The gradients (G_1) of these plots vary inversely with reducing-agent concentration when reducing agent is always in large excess over both oxygen and flavin (Table 6). The intercepts (J_1) of the plots at zero $[O_2]$ give poor straight lines with a positive slope when plotted against $1/[RH_2]$ (Table 6). Inspection of the kinetic scheme shows that the gradients (G_2) of the plots of G_1 versus $1/[RH_2]$ are given by $k_{+4f}/k_{+5}A$. Under constant illumination conditions and at constant flavin concentration k_{+5} is the only term in this expression that varies when the substrate is changed. Thus the ratios of $1/G_2$ for the different substrates give the ratios of the k_{+5} values for these compounds (Table 6).

The gradient of a plot of J_1 versus $1/[RH_2]$ (G_3) is given by $B/k_{+5}A$, and its intercept when $1/[RH_2]$ is zero (J_2) is given by $(k_{+4b}+k_{+5})/k_{+5}A$. Thus the proportions $G_2:G_3:J_2$ give $k_{+4f}:B:(k_{+4b}+k_{+5})$ for a given reducing substrate. k_{+4f} and B are independent of the nature of the substrate, so that it is possible to deduce the ratio of $k_{+4b}+k_{+5}$ terms for different substrates (Table 6). The values of B are subject to

Table 7. *Rate constants for photoreduction of FMN*

Substrate	$10^{-9}\,k_{+4f}$ ($M^{-1}s^{-1}$)	$10^{-5}B$ ($M^{-1}s^{-1}$)	$10^{-7}k_{+5}$ ($M^{-1}s^{-1}$)	$10^{-8}k_{+4b}$ ($M^{-1}s^{-1}$)
EDTA	6.6	1.5	2.1	2.4
DL-α-Phenylglycine (anion)	6.6	1.1	1.2	1.9
Diethanolamine (neutral)	6.6	1.0	2.2	3.3

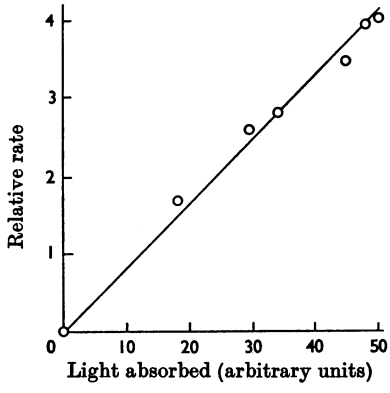

Fig. 4. Dependence of FMN-photosensitized oxygen uptake on the amount of light absorbed. Reactions were at 25°C in 0.1 M-phosphate buffer, pH 7.0, initially in equilibrium with the atmosphere. The substrate was diethanolamine (10 mM); [FMN] 0.02–0.20 mM.

large errors because the data are not good enough to give accurate G_3 values.

In all cases k_{+4f} is about 25 times $(k_{+4b}+k_{+5})$. Triplet quenching by oxygen is often a diffusion-controlled reaction (Chien, 1967), and on this assumption k_{+4f} (and hence $k_{+4b}+k_{+5}$ and B) can be calculated. The rate constant of a diffusion-controlled reaction is given by $8RT/3\times10^3\eta\,M^{-1}s^{-1}$ (Umberger & LaMer, 1945), which equals $6.6\times10^9\,M^{-1}s^{-1}$ for an aqueous solution at 25°C.

It is now possible to calculate k_{+4b} and k_{+5} separately for each reducing agent by using the known quantum yield (Q) of anaerobic photoreduction of FMN by EDTA (Radda & Calvin, 1964). Q equals 0.06 at high EDTA concentrations, where the rate is independent of $[RH_2]$. In this situation $Q=Q_0k_{+5}/(k_{+4b}+k_{+5})$, where Q_0 is the quantum yield of triplet production. The maximum value for Q_0 is $1-Q_f$, where Q_f is the quantum yield of fluorescence (0.26 for riboflavin; Weber & Teale, 1957). It has been shown for 9-phenylanthracene that in pure solvents and under non-reactive conditions the quantum yields of fluorescence and phosphorescence add up to 1 (Horrocks, Medinger & Wilkinson, 1967), which means that in this case $Q_0=1-Q_f$. Assuming the same to be true for flavins, $k_{+5}/(k_{+4b}+k_{+5})$ for the EDTA reaction is 0.081. Taking the $(k_{+4b}+k_{+5})$ values calculated from comparison with k_{+4f}, k_{+4b} and k_{+5} were calculated separately for EDTA and hence for the other substrates (Table 7).

Type I reactions: dependence on FMN concentrations. The effect of varying the FMN concentration on the rate of oxygen uptake with diethanolamine was measured. The rate of reaction is proportional to the amount of light absorbed up to 0.2 mM-FMN (Fig. 4). At higher flavin concentrations dimer formation becomes more important (Radda & Calvin, 1964) and under these conditions a fall in the rate of oxygen uptake is observed.

Type II reactions. Substrates that typically undergo type II photo-oxidation usually contain oxidizable aromatic residues (Table 1). Two kinds of mechanism can be envisaged: (i) production of singlet oxygen, which reacts with the substrates; (ii) formation of an active adduct 'moloxide' between flavin and oxygen (Gollinck & Schenck, 1964). The kinetics of these routes are expected to be similar, so they are indistinguishable by steady-state kinetics. In practice the sensitized photo-oxidation of tryptophan by FMN does not fit a simple rate expression at all, so that perhaps type II photo-oxidation is a complex reaction with several competing mechanisms for oxygen uptake. This interpretation is consistent with the finding that more than 1 mol of oxygen is consumed for each mol of tryptophan destroyed in the Methylene Blue-sensitized reaction (Weil, Gordon & Buchert, 1951). The reaction is subject to product inhibition (Table 8).

Some compounds have intermediate oxygen-uptake curves, type I at high oxygen concentrations and type II at low ones. The reasons for this behaviour vary, but with phenylacetic acid at least it is probably a result of the formation of a photo-addition product. This does not react quickly with oxygen, but the reaction is accelerated in the light (Hemmerich, Massey & Weber, 1967).

Sensitized photo-oxidation of natural amino acids. The behaviour of FMN in the reactions described above suggests that it may prove a useful reagent for the chemical modification of proteins. There is

also the possibility that the prosthetic group in a flavoprotein might act as a photosensitizer, causing reaction of nearby residues and hence helping to characterize the environment of the binding site. The photoreactivities of FMN with the more reactive amino acids were studied in some detail, with both pH and temperature being varied, to see how selective the method could be.

Table 8. *Rates of oxygen uptake by tryptophan and illuminated FMN at different oxygen concentrations*

All reactions were at 25°C in 0.1M-phosphate buffer, pH6.9. FMN concentrations were 0.2mM; tryptophan concentrations were 10mM. Oxygen concentrations are relative to air-saturated buffer = 100.

Concn. of oxygen	Initial rate of oxygen uptake (units/min)	Rate of oxygen uptake after 3min reaction (units/min)
71	6.1	3.6
79	6.4	3.6
87	6.3	3.6
100	6.5	4.0
107	6.6	4.2
125	6.7	4.3
181	8.2	5.6
204	8.0	—

The pH profiles for reactivity are given in Fig. 5. They do not correspond to the acid titration curve for tryptophan, tyrosine or methionine, and all the acids tested were significantly different from each other both in pH profile and in overall reactivity. The behaviour of tryptophan and tyrosine might be expected to be complex because they are both type II substrates, but the other acids (including methionine) all react in the type I mode. The reason for methionine's unusual behaviour is not certain, but it should be noted that the divergence from normal acid titration behaviour at acid pH resembles that of phenylacetic acid (Penzer & Radda, 1968).

Observations were made on two enzymes (L-glutamate dehydrogenase and D-amino acid oxidase) to see whether photosensitized destruction of activity occurs. In each case it does (Fig. 6 and Table 9).

DISCUSSION

The effects of pH, substrate structure and oxygen concentration on type I reaction rates support the view that reaction occurs by reduction of the flavin followed by rapid reoxidation by oxygen. The kinetic scheme proposed for the reaction is based on this assumption. Experimental results are consistent (within their accuracy) with the scheme,

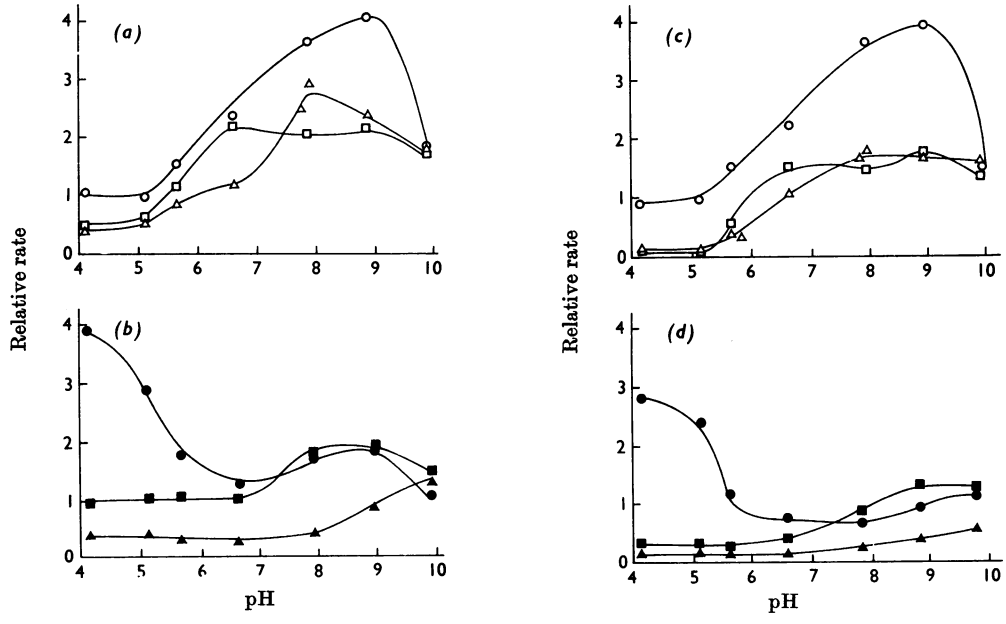

Fig. 5. pH-dependence of FMN-photosensitized oxygen uptake with amino acids. Reactions were at 45°C in (*a*) and (*b*) and at 25°C in (*c*) and (*d*). [FMN] 0.1mM; [acid] 1.0mM: buffers were 0.1M-acetate below pH5.5, 0.1M-phosphate in the range pH5.5–7.5, and 0.1M-borate above pH7.5. ○, Tryptophan; □, histidine; △, tyrosine; ●, methionine; ■, cysteine; ▲, phenylalanine.

though they are inadequate to rule out all possible alternatives (Penzer & Radda, 1968). The following comments assume that the proposed mechanism is correct.

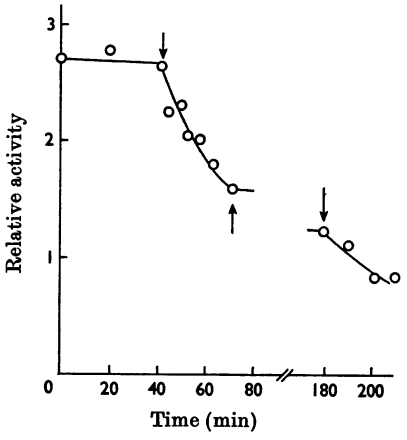

Fig. 6. FMN-photosensitized inactivation of L-glutamate dehydrogenase. Reactions were at 25°C in 0.1 M-phosphate buffer, pH 7.6. [FMN] 0.1 mM. ↓, light on; ↑, light off.

Reaction rates at particular light-intensities and flavin concentrations are proportional to:

$$\frac{k_{+5}[RH_2]}{\{B+k_{+4f}[O_2]+(k_{+4b}+k_{+5})[RH_2]\}}$$

so comparison of rates for different substrates by use of the Hammett equation is not rigorous unless $(k_{+4b}+k_{+5})[RH_2]$ is negligible compared with $(B+k_{+4f}[O_2])$. The experiments described here used mixtures in which:

$$\frac{(k_{+4b}+k_{+5})[RH_2]}{(B+k_{+4f}[O_2])}$$

ranged from 0.08 to 0.02. These ratios are all sufficiently small for the sign and approximate value of the Hammett ρ value to retain their significance.

The values of individual rate constants derived on the assumption that triplet quenching by oxygen is diffusion-controlled will not be numerically accurate if the quenching is less efficient. The values are also subject to large experimental error (±25%) because they are all obtained from second-order plots of the data. This does not affect the order of magnitude of the ratio k_{+5}/k_{+4b}, which is always less than 1:10. Three mechanisms for the quenching can be envisaged: (i) collisional quenching;

Table 9. *FMN-photosensitized inactivations of* L-*glutamate dehydrogenase and* D-*amino acid oxidase*

All reactions with glutamate dehydrogenase (GDH) were at 25°C in 0.1 M-phosphate buffer, pH 7.8. Solutions contained 0.2 mM-FMN. All reactions with D-amino acid oxidase (D-AAO) were at 25°C in 16.7 mM-pyrophosphate buffer containing 8 mM-benzoic acid, pH 8.3.

Reaction mixture (total vol. 30 ml)	Relative activity before illumination	$\frac{-\text{d(log activity)}}{dt}$ (min^{-1})
1.8 mg of GDH	0.99*	0.0102
	1.08*	0.0080
	0.90	0.0130
1.8 mg of GDH + 10 mM-α-oxoglutarate	1.00*	0.0078
	1.18*	0.0080
	0.89	0.0115
1.2 mg of GDH	0.75	0.0138
0.6 mg of GDH	0.32	0.0191
0.3 mg of GDH	0.16	0.0270

Reaction mixture	Relative activity after various periods of illumination					
Time (min) ...	0	10	15	30	45	60
D-AAO	1.00	—	0.95	1.20	—	0.96
D-AAO + FAD	1.00	—	1.00	1.00	—	0.97
D-AAO + FMN	1.00	0.65	—	0.49	—	0.37
	1.00	—	0.71	0.60	—	0.41
*D-AAO + FMN (light-intensity about 650 lm/ft^2)	1.00	0.89	0.87	0.81	0.74	0.67
*D-AAO + FMN (light-intensity about 1000 lm/ft^2)	1.00	0.87	0.81	0.81	0.67	0.62

* The intensity of illumination was less in these experiments than in the rest.

(ii) quenching by complex-formation with the flavin triplet; (iii) rapidly reversed electron transfer (i.e. $F^t+RH_2 \rightarrow FH^{\cdot}+RH^{\cdot} \rightarrow F+RH_2$). Good quenchers by process (i) are ions with large diffuse electron clouds (e.g. I^-). None of the reducing agents fits this category. Quenching by process (ii) may be by formation of hydrophobic complexes between the chromophore and an aromatic quencher (Penzer & Radda, 1968). DL-α-Phenylglycine could quench by this route, in which case the neutral-pH form would probably have an effect as well as the anion. This was not observed for the anaerobic photoreduction of FMN by EDTA (Penzer & Radda, 1968). All three reducing agents could quench by process (iii), and this seems the most likely of the possibilities. The quenching rate constants increase in the same order as the k_{+5} terms for the different substrates, indicating that the better electron donors are also the better quenchers (cf. Radda, 1966).

The kinetic expression suggests that under conditions of constant illumination and constant flavin concentration the rate of anaerobic photoreduction varies with reducing-agent concentration in a way given by $k_{+5}[RH_2]/\{B+(k_{+4b}+k_{+5})[RH_2]\}$, whereas the rate of the aerobic reaction varies as:

$$\frac{k_{+5}[RH_2]}{\{B+(k_{+4b}+k_{+5})[RH_2]+k_{+4f}[O_2]\}}$$

These expressions are plotted for EDTA and DL-α-phenylglycine in Figs. 7 and 8. Experimental points (normalized round a single value) are shown as well.

B gives the rate constant for triplet decay in the absence of reaction or quenching by oxygen. The values obtained from experiments with different reducing agents are similar (Table 7) and give a lifetime of about 10 μs. This can be compared with a directly measured lifetime of 100 μs (Knowles & Roe, 1968); perfect agreement is unlikely because of the different compositions of the solutions used.

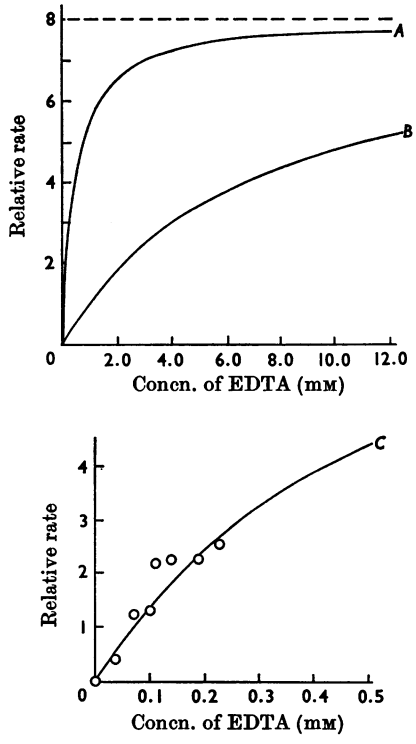

Fig. 7. Theoretical curves for the concentration-dependence of aerobic and anaerobic photo-oxidation of EDTA by FMN. ---, Limiting rate. Curve A, anaerobic reaction; curve B, aerobic reaction; curve C, anaerobic reaction for low concentrations showing experimental points (normalized round a single value) taken from Fig. 2(a) of Penzer & Radda (1968) (concentrations in this figure were labelled 100 times too high in error).

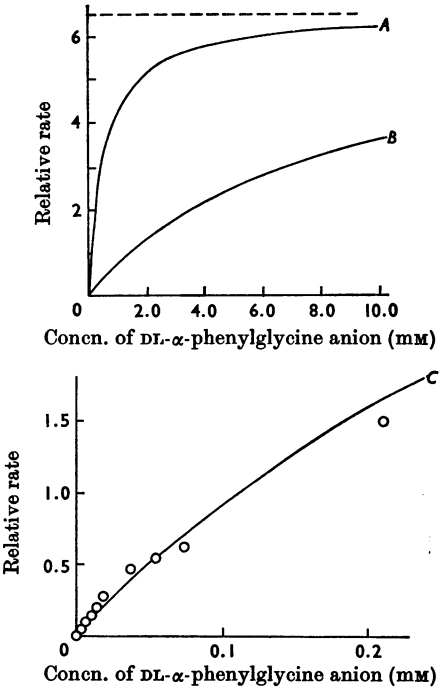

Fig. 8. Theoretical curves for the concentration-dependence of aerobic and anaerobic photo-oxidation of DL-α-phenylglycine by FMN. ---, Limiting rate. Curve A, anaerobic reaction; curve B, aerobic reaction; curve C, anaerobic reaction for low concentrations showing experimental points (normalized round a single value) taken from Fig. 2(b) of Penzer & Radda (1968) (concentrations in this figure were labelled 100 times too high in error).

The pH-dependence of reactivity of methionine requires comment. It shows type I kinetics, so that the divergence between reactivity and an acid titration curve cannot be dismissed as the result of a complex 'unclean' reaction, as with type II substrates like tyrosine and tryptophan. Another photochemical reaction of FMN, with phenylacetate, also shows rate enhancement at acid pH values (Penzer & Radda, 1968). Both reactions have pK_a values of 5–6. This may correspond to protonation of the flavin triplet to give a cationic nucleus, which is a better oxidizing agent than the uncharged species. A lower pK_a value has been suggested for the flavin triplet in low-temperature glasses (Lhoste, Haug & Hemmerich, 1966), but it is not necessarily correct for a fluid solution. Most amine reducing substrates are inactive at acid pH because of protonation, and so they show no rate enhancement, but methionine may react either at the carbon atoms α to the sulphur atom, or at the β carbon atom (by neighbouring-group participation) as well as at the carbon atom α to the carboxyl group. The first two positions are not greatly affected by the acid–base behaviour of the amino group.

The pH-dependence of photoreactivity of FMN with the amino acids shows some similarities with other photosensitizing dyes, as well as some differences from them (Weil *et al.* 1951; Sluyterman, 1962). Preliminary experiments show that photosensitized inactivation of some enzymes by FMN certainly occurs, but it is uncertain whether, by careful choice of conditions, the method can induce a single well-characterized reaction.

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