Flavin-Photosensitized Reactions of Retinol and Stilbene

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1. Retinol and stilbene are both isomerized when they are illuminated anaerobically in the presence of flavins. 2. Triplet quenchers (e.g. oxygen, potassium iodide and paramagnetic ions) inhibit the reaction more efficiently than they quench flavin fluorescence. At 77°C in a diethyl ether–isopentane–ethanol (5:5:2) glass retinol quenches flavin phosphorescence, but not its fluorescence. 3. For the stilbene reaction *cis/trans* photostationary-state mixtures are obtained with different flavins and these are linearly related to the phosphorescence transition energies of the flavins used. 4. The reaction involves the triplet state of flavin and a scheme for the reaction is suggested. 5. The dependence of the rate of reaction on substrate concentration is explicable in terms of this scheme. 6. The photobleaching of rhodopsin sensitized by flavin is also demonstrated.

Flavins and carotenoids are found together in many photoreceptors (Beinert, 1960; Burnett, 1965; Whittingham, 1965); this suggests that these compounds may interact with one another in some photobiological processes.

Flavins are found in the eyes of fishes (von Euler & Adler, 1934) and some nocturnal mammals (Pirie, 1959; Elliot & Futterman, 1963; Matsui, 1965), and are involved in photosynthesis (Whatley, Allen & Arnon, 1959) and phototropism in plants (Thimann & Curry, 1960). The action of flavins *in vivo* is not yet understood, but their photo-reactions *in vitro* have been extensively studied (e.g. Penzer & Radda, 1967). They are photo-reduced in the presence of electron donors (Suelter & Metzler, 1960; Radda & Calvin, 1964; Hemmerich, Massey & Weber, 1967) and will photosensitize both oxidations of amino acids (Frisell, Chung & MacKenzie, 1959) and *cis-trans* isomerizations (Posthuma & Berends, 1966; Gordon-Walker & Radda, 1967). A mechanism involving the excited flavin triplet has been implicated in all these reactions.

Carotenoids are also important in biological light-processes, e.g. photosynthesis (Whittingham, 1965) and phototropism and phototaxis (Burnett, 1965). Retinal is the chromophore in rhodopsin and its isomerization is the first light-process in vision (Hubbard & Kropf, 1958). Thus the flavin and retinol model system may be considered as a model for various photobiological processes.

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The photosensitized isomerizations of stilbene have been studied (Hammond *et al.* 1964; Posthuma & Berends, 1966) and are postulated to proceed by a triplet mechanism perhaps involving ‘phantom triplets’ (Hammond *et al.* 1964). These are defined as triplets with twisted configurations that have a lower energy than the classical triplets (Hammond *et al.* 1964). A further suggestion is that non-vertical excitation can occur from the ground state to the ‘phantom triplet’ (Hammond *et al.* 1964).

In this paper the photosensitized isomerizations of retinols and stilbenes are investigated as model systems for the more complex biological processes. An experiment to show the possible application of these model systems to rhodopsin bleaching is also included.

MATERIALS

The flavins (see Table 2) were obtained from the following sources: FMN (1) from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); FAD (2) from Sigma Chemical Co. (St Louis, Mo., U.S.A.); riboflavin (3) from BDH Chemicals Ltd. (Poole, Dorset, U.K.); lumiflavin (4) was synthesized in this laboratory by the method of Hemmerich, Fallab & Erlenmeyer (1956); 3-methyl-lumiflavin (5), 2-thiolumiflavin (6), 2-morpholinolumiflavin (7), 4-morpholinolumiflavin (8) and isoperacetylriboflavin (9) were gifts from Dr P. Hemmerich; 9-methylisoalloxazine (10) was a gift from Dr G. Tollin; lumichrome (11) was a gift from Dr D. E. Metzler.

13-*cis*- and all-*trans*-Retinol were obtained from Sigma Chemical Co. *trans*-Stilbene was obtained from BDH Chemicals Ltd., and the *cis* isomer was obtained from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.)

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METHODS

The reactants were mixed in a 1 cm stoppered quartz spectrophotometer cell. For deoxygenation, nitrogen that was especially low in oxygen content was passed through a solution of vanadous sulphate and H_2SO_4 containing zinc amalgam, and then through water, to remove any traces of oxygen. When organic solvents were used the nitrogen gas was usually passed through the solvent to avoid evaporation from the solution in the cell. The nitrogen was bubbled through the solution in the cell in darkness for 20–30 min by using syringe needles as inlet and outlet tubes. With organic solvents polystyrene stoppers with two small holes bored in them were used. In the rhodopsin experiments deoxygenation was achieved by passing the nitrogen over the top of the solution for 2–3 h. The presence of digitonin in the solution would have caused frothing if the nitrogen had been bubbled into the solution.

A 500W tungsten projection lamp was used to illuminate the solution. The light-beam was passed through 5 cm of water to cut out i.r. light and through a yellow filter (OY18; Chance Bros., Smethwick, Staffs., U.K.) to exclude light of wavelength below 400 nm. In the rhodopsin experiments these measures were inadequate to prevent reaction in the absence of flavin, presumably because the rhodopsin absorbed light of wavelength above 400 nm. In this case illumination was carried out with light of wavelength 370 nm (which was absorbed by the flavin in its near-u.v. peak) from a 250W xenon lamp (operated with a Bellingham and Stanley stabilized d.c. power supply) passed through a Hilger and Watts prism monochromator. Another light-source was 370 nm light from a Unicam SP.500 spectrophotometer with 2 mm slits.

Spectrophotometry. The reactions were followed spectrophotometrically by using either a Unicam SP.500 or a Cary 14 recording spectrophotometer. The photosensitized reactions were monitored at the substrate absorption peak (325 nm for retinol, 321 nm for stilbene and 500 nm for rhodopsin). At 0.01 mm-stilbene the spectral method was used, although the extinctions of solutions containing 1.0 mm-stilbene were also measured after 100-fold dilutions. Values of *cis/trans* ratios were calculated from these extinctions by using the equation:

$$E = \epsilon_c x + \epsilon_t (a - x)$$

where ϵ_c and ϵ_t are the extinction coefficients of the *cis*- and *trans*-stilbene isomers respectively, measured at 321 nm and 295 nm on standard solutions of the two isomers; their values are: $\epsilon_{c,321} = 0.12 \times 10^4 \text{ l. mol}^{-1} \text{ cm}^{-1}$, $\epsilon_{t,321} = 1.68 \times 10^4 \text{ l. mol}^{-1} \text{ cm}^{-1}$, $\epsilon_{c,295} = 0.76 \times 10^4 \text{ l. mol}^{-1} \text{ cm}^{-1}$, $\epsilon_{t,295} = 2.82 \times 10^4 \text{ l. mol}^{-1} \text{ cm}^{-1}$; a is the original total concentration of stilbene and x is the concentration of the *cis* isomer in the mixture. This formula assumes that the only reaction taking place is the interconversion of the two isomers. At this concentration of stilbene (1.0 mm) each point on the reaction curve was determined in a separate experiment because of the dilution required before the spectrum was taken.

Fluorescence measurements were made on a Zeiss PMQII spectrofluorimeter.

Gas-liquid chromatography. At 1.0 mm-stilbene concentration g.l.c. was also used to measure these ratios

and the two methods agreed to within 10%. A Perkin-Elmer F11 gas chromatograph containing a 2 m column with 2 mm internal diameter and fitted with a flame ionization detector was used. The column was packed with Apiezon L/Chromosorb P (80–100 mesh) (3:17, w/w) and gave a good separation of the stilbene isomers. The conditions used were: column temperature 220°C; injection temperature 405°C; nitrogen at 15 l./in² as carrier gas. Under these conditions good symmetrical peaks were obtained with retention times of 15–20 min for the *cis* isomer and 40–50 min for the *trans* isomer. The illumination mixture containing flavin and stilbene was sampled at intervals during the reaction. A Hamilton syringe (Hamilton Co. Inc., Whittier, Calif., U.S.A.) was inserted through a deoxygenation needle into the cuvette and 1–2 μl of the mixture was withdrawn and injected into the chromatograph. The g.l.c. was standardized with known mixtures of pure stilbene isomers. The method of triangulation was used to measure the area under the peaks and was accurate to within 5%. The standardization showed that the ratios of peak areas had to be multiplied by 0.82 ± 0.04 to give correct weight ratios.

With some of the more unstable flavins long illumination times could not be used to obtain the final *cis/trans* ratios from pure isomers. For these flavins an approximate final ratio was obtained and then mixtures of the two stilbenes near this ratio (total concentration 10–15 mm) were made up and illuminated for short times to give the exact photostationary-state ratio of isomers (Hammond *et al.* 1964). The photostationary-state mixture was always approached from both sides to give an accurate value and the final ratios were averages of two or three experiments for each flavin. The error between experiments was less than 5%.

Thin-layer chromatography. T.l.c. was used to follow sensitized isomerization reactions of retinols (1.0 mm). The method was that of von Planta *et al.* (1962). The plates were of Kieselgel G (E. Merck A.-G., Darmstadt, Germany) (25 g + 75 ml of water) and the solvent system was light petroleum (b.p. 40–60°C)–6-methylhept-5-en-2-one (11:2, v/v). The solvent took about 35 min to run 10 cm up the plate. The spots were observed under u.v. light. The average R_F values for the all-*trans* and 13-*cis* isomers were 0.52 and 0.64 respectively.

Extraction of rhodopsin. The rhodopsin was extracted from freshly obtained ox eyes by a modification of Saito's sugar flotation method (Collins & Morton, 1950; Collins, Love & Morton, 1952). Ten to 25 retinas were used in each experiment. The retinas were shaken with 45% (w/v) sucrose (15 ml/24 retinas) for 30 s and then centrifuged at 1600 g for 15 min in a Spinco model L centrifuge (Beckman Instruments Inc., Palo Alto, Calif., U.S.A.) with a no. 40 rotor. The supernatant containing the rods was taken and diluted with 2–3 vol. (approx. 30 ml) of 0.9% NaCl and recentrifuged. The scarlet precipitate was hardened in 4% (w/v) potassium alum [$\text{K}_2\text{SO}_4 \cdot \text{Al}_2(\text{SO}_4)_3 \cdot 24\text{H}_2\text{O}$] for 30 min to 24 h and the rods were centrifuged down. The precipitate was washed in a 1:1 (v/v) mixture of 0.9% NaCl and neutral buffer (16.4 ml of 0.2 M- Na_2HPO_4 + 3.5 ml of 0.1 M-citric acid, pH 7) and then extracted with 1 ml of 2% (w/v) digitonin for 1 h. If the extraction were continued after 1 h progressively more impurities would be leached out (Collins *et al.* 1952). The mixture was again centrifuged,

and the clear supernatant was mixed with an equal volume of 50 mM-sodium borate buffer, pH 9.3, and recentrifuged. The spectrum was measured and the E_{280}/E_{500} and E_{400}/E_{500} ratios varied in the ranges 2.6–5.0 and 0.3–0.8 respectively in the preparations used. The whole extraction was carried out under a red safety lamp and the solution was kept dark during the experiments.

RESULTS

Flavin-sensitized retinol isomerization. (a) Reaction products. Lumiflavin and FMN photosensitized an isomerization of all-*trans*- and 13-*cis*-retinol that was accompanied by destruction of the retinol. Similar destruction of retinals in ethanol has been observed during illumination with visible light (Hubbard, Gregerman & Wald, 1952–53). The isomerization was shown by t.l.c. A mixture of lumiflavin (0.1 mM) and either 13-*cis*- or all-*trans*-retinol (1.5 mM) was illuminated with light of wavelengths greater than 400 nm. When the 13-*cis*-retinol was illuminated for about 20 min with flavin present a second spot developed on the chromato-

gram, while the original one remained visible. The second spot had an R_F value similar to that of the all-*trans*-retinol. Similarly the all-*trans*-retinol on illumination in the presence of flavin gave a second spot, which had an R_F value similar to that of the 13-*cis*-retinol, although as no other retinol was available for comparison this could be some other *cis* isomer (for average R_F values see the Methods section).

To follow the reaction between lumiflavin and retinol spectrophotometrically lower concentrations of the reactants were necessary (0.01 mM-retinol, 0.02 mM-lumiflavin). The reaction was found to proceed more slowly at lower concentrations of both reagents and was followed by observing the decrease in extinction at the retinol peak (328 nm) (Fig. 1). Neither substrate alone showed any reaction. FMN can replace lumiflavin in this reaction, but here a blank reaction was obtained for the FMN alone, as a result of photobleaching (Holmström & Oster, 1961), and so further experiments were carried out with lumiflavin.

The quantum yield of the reaction (0.01 mM-retinol) was measured by comparison with the photoreduction of FMN by EDTA in phosphate buffer, pH 7.0 and I 0.1 (quantum yield 0.06) (Radda & Calvin, 1964), and was 0.002.

(b) Effect of quenchers. The reaction between lumiflavin (0.02 mM) and retinol (0.016 mM) was inhibited by oxygen, paramagnetic ions (e.g. Ni^{2+} at 1.0 mM) and potassium iodide (Table 1). All these molecules quench triplet states more efficiently than they quench singlets. Fig. 2 shows that potassium iodide inhibited this reaction much more effectively than it quenched flavin fluorescence, and this is indicative of the flavin triplets being involved in this reaction.

At room temperature lumiflavin fluorescence was quenched by retinol at concentrations greater than those used in the illumination experiments. The

Fig. 1. Lumiflavin-photosensitized decrease of all-*trans*-retinol extinction at 328 nm in methanol as a function of time of illumination by light of wavelengths greater than 400 nm. The lumiflavin concentration was 0.02 mM and that of retinol was 0.016 mM. The temperature was 25°C.

Table 1. Effect of oxygen, potassium iodide and paramagnetic ions on the rate of lumiflavin-photosensitized reaction of all-*trans*-retinol

The lumiflavin concentration was 0.02 mM and that of retinol was 0.016 mM. The temperature was 25°C. Results are given $\pm$ S.D. where appropriate.

Experiment	Average initial rate (decrease in E /min)
all- <i>trans</i> -Retinol + lumiflavin	0.12 $\pm$ 0.03
all- <i>trans</i> -Retinol + lumiflavin + oxygen	0.007 $\pm$ 0.003
all- <i>trans</i> -Retinol + lumiflavin + 0.004 mM-KI	0.07 $\pm$ 0.02
all- <i>trans</i> -Retinol + lumiflavin + 0.04 mM-KI	0.04
all- <i>trans</i> -Retinol + lumiflavin + 0.4 mM-KI	0.005
all- <i>trans</i> -Retinol + lumiflavin + 4.0 mM-KI	0.002
all- <i>trans</i> -Retinol + lumiflavin + approx. 40 mM-KI	0
all- <i>trans</i> -Retinol + lumiflavin + 1.0 mM-NiSO ₄	0.04
all- <i>trans</i> -Retinol + lumiflavin + 10 mM-NiSO ₄	0.002 $\pm$ 0.002

Fig. 2. Comparison between the inhibition by KI of the lumiflavin-photosensitized reaction of all-*trans*-retinol and the quenching by KI of the lumiflavin fluorescence in methanol. I and I_0 are fluorescence intensities with and without KI respectively and k and k_0 are the corresponding reaction rates. The lumiflavin concentration was 0.02 mM and that of retinol was 0.016 mM. The temperature was 25°C.

Fig. 3. Effect of temperature on the quenching of lumiflavin fluorescence by all-*trans*-retinol in methanol. I and I_0 are flavin fluorescence intensities with and without all-*trans*-retinol respectively. The lumiflavin concentration was 0.025 mM. ○, 5.5°C; ●, 35°C.

quenching was more significant at higher temperatures (Fig. 3). This suggested that complex-formation (which would cause quenching to decrease with increasing temperature) was not occurring and that a collisional mechanism was probably involved (Weber, 1950). At 77°K in a diethyl ether-isopentane-ethanol (5:5:2) glass the flavin fluorescence was not quenched by retinol, although the flavin phosphorescence was decreased. This again implicates a triplet mechanism.

The reaction of retinol and flavin is complicated by the instability of the retinol, its complex structure and the various isomers formed as

Fig. 4. Lumiflavin-photosensitized isomerization of *cis*- and *trans*-stilbene as a function of time of illumination by light of wavelengths greater than 400 nm. The temperature was 25°C. The lumiflavin concentration was 0.1 mM and that of stilbene was 1.5 mM. ●, *cis*-Stilbene; ○, *trans*-stilbene.

products. Therefore further characterization of the mechanism was carried out with stilbene, which is a simpler molecule structurally related to retinol.

Flavin-sensitized stilbene isomerization. (a) Reaction products. Either stilbene isomer (1.0–10 mM) when illuminated in the presence of flavin (0.05–0.10 mM) yielded a photostationary-state mixture of isomers (Fig. 4) that depended on the flavin used. No other product was detected by g.l.c. No change was found by either g.l.c. or spectral studies when stilbene and lumiflavin were left in the dark together for 1 h. The different *cis/trans* ratios are shown in Table 2, and Fig. 5 shows the variation of these ratios with the energy of the phosphorescence transition of the flavins concerned.

(b) Concentration-dependence of the rates. The rates of the reaction were measured spectrally and were found to increase with increasing stilbene concentration until they reached a saturation value at about 0.50 mM (Fig. 6). This concentration-dependence is similar to that found for other flavin photoreactions (Penzer & Radda, 1968; Penzer, Radda, Taylor & Taylor, 1970) (see the Discussion section). The quantum yield of the reaction between lumiflavin (0.10 mM) and either stilbene isomer (1.0 mM) after 30 s was found, by comparison with riboflavin photobleaching in 0.12 M-phosphate buffer, pH 6.8 (quantum yield 0.024) (Holmström & Oster, 1961), to be 0.05.

(c) Effect of quenchers. Both oxygen and potassium iodide inhibit the reaction between flavin and stilbene (Table 3). No photo-oxidation product was seen by g.l.c. in the photosensitized reaction. However, when stilbene was illuminated with u.v. light in the presence of oxygen a new product was formed

Table 2. *cis/trans* Photostationary-state weight ratios of stilbene after reaction photosensitized by flavins, together with the phosphorescence maxima of the flavins concerned

The ratios were measured by g.l.c. The flavin concentrations were 0.05–0.10 mM and those of stilbene were 0.10–0.15 mM. The temperature was 25°C. Phosphorescence maxima were from Gordon-Walker, Penzer & Radda (1970). Photostationary-state weight ratios are given $\pm$ s.d.

Substituents ...	Flavin										Photostationary-state weight ratio of <i>cis/trans</i> -stilbene	Phosphorescence maxima	
	R ¹	R ²	R ³	R ⁴	R ⁵	R ⁶	R ⁷	R ⁸	R ⁹	(nm)		(kcal/mol)	
(1)	—	=O	H	=O	H	Me	Me		Ribityl phosphate	—	—	598	47.8
(2)	—	=O	H	=O	H	Me	Me		Ribityl diphosphate	—	—	—	—
(3)	—	=O	H	=O	H	Me	Me		adenosine	—	—	498	47.8
(4)	—	=O	H	=O	H	Me	Me		Ribityl	0.31 $\pm$ 0.01	—	600	47.7
(5)	—	=O	Me	=O	H	Me	Me		Me	0.32 $\pm$ 0.02	—	597	47.9
(6)	—	=S	H	=O	H	Me	Me		Me	0.37 $\pm$ 0.02	—	—	—
(7)	—		—	=O	H	Me	Me		Me	0.22 $\pm$ 0.01	—	608	47.0
(8)	—	=O	—		H	Me	Me		Me	0.06 $\pm$ 0.01	—	605	47.3
(9)	—	=O	H	=O	Me	Me	H		Acetylated ribityl	0.33 $\pm$ 0.02	—	590	48.5
(10)	—	=O	H	=O	H	H	H		Me	0.85 $\pm$ 0.05	—	580	49.3
(11)	H	=O	H	=O	H	Me	Me		—	1.8 $\pm$ 0.2	—	550	52.0

with a lower retention time. This peak, which may represent a photo-oxidized product, was the same for either stilbene isomer.

Potassium iodide also quenched the fluorescence

Fig. 5. Dependence of the *cis/trans*-stilbene photo-stationary-state ratio on the phosphorescence transition energies of the flavins. Phosphorescence energies were from Gordon-Walker *et al.* (1970). The flavin concentrations were 0.05–0.10 mM and those of stilbene were 10–15 mM. The temperature was 25°C.

Fig. 6. Dependence of the rate of lumiflavin-photo-sensitized reaction of stilbene in methanol on the stilbene concentration. The lumiflavin concentration was 0.02 mM. The temperature was 25°C. ●, *cis*-Stilbene; ○, *trans*-stilbene.

of lumiflavin, but less efficiently than it inhibited the reaction. This quenching occurred by a collisional mechanism, as shown by the Stern–Volmer plots (Weber, 1950) (Fig. 7), which curved off at high concentrations of stilbene and showed more efficient quenching at higher temperatures.

Flavin-photo-sensitized reaction of rhodopsin. When rhodopsin (0.001–0.01 mM) was illuminated with 370 nm light in the presence of lumiflavin (0.05 mM) the rhodopsin absorption peak at 500 nm decreased with time (Fig. 8). The large blank reaction of rhodopsin alone complicated the original experiments when the normal illumination set-up or monochromatic 440 nm light was used. This is understandable because rhodopsin had its absorption peak at 500 nm, and therefore absorbed the visible light efficiently and became bleached. Only when 370 nm light was used was it found possible to cut down this blank reaction. Under these conditions the reaction in the presence of lumiflavin went faster than the blank reaction of rhodopsin alone.

DISCUSSION

The flavin-sensitized photoreaction of retinol and stilbene caused isomerization of the substrates, as shown by chromatography of the products. Some destruction also occurred in the case of retinol. Evidence for the involvement of the triplet excited state of flavin is given below.

Neither the trivial process of emission and reabsorption of light nor singlet–singlet energy transfer can account for these sensitization reactions, because the energy of flavin fluorescence is lower than that of retinol absorption. Triplet quenchers such as oxygen, potassium iodide and paramagnetic ions quenched the reactions more efficiently than they quenched flavin fluorescence.

No complexes were detected in either system at the concentrations of substrates used in the reaction. Also, flavin phosphorescence, but not fluorescence, was quenched by retinol at 77°K in a glass. The linear relationship between the *cis/trans*-stilbene photostationary-state ratios and the triplet

Table 3. *Effect of oxygen and potassium iodide on the rate of the lumiflavin-photo-sensitized reaction of stilbene*

The lumiflavin concentration was 0.1 mM and that of stilbene was 1.5 mM. The temperature was 25°C. Rates are given $\pm$ s.d. where appropriate.

Experiment	Rate after 5 min (decrease in E/min)
<i>cis</i> -Stilbene + lumiflavin	2.3 $\pm$ 0.2
<i>cis</i> -Stilbene + lumiflavin + 0.1 mM-KI	1.4
<i>cis</i> -Stilbene + lumiflavin + oxygen	1.0
<i>trans</i> -Stilbene + lumiflavin	0.75 $\pm$ 0.09
<i>trans</i> -Stilbene + lumiflavin + 0.1 mM-KI	0.54
<i>trans</i> -Stilbene + lumiflavin + oxygen	0.20

Fig. 7. Effect of temperature on the quenching of lumiflavin fluorescence by *trans*-stilbene in methanol. I and I_0 are the flavin fluorescence intensities with and without *trans*-stilbene respectively. The lumiflavin concentration was 0.033 mM. $\circ$, 5°C; $\bullet$, 36°C.

Fig. 8. Lumiflavin-photosensitized decrease of rhodopsin extinction at 500 nm as a function of time of illumination by light of 370 nm. The lumiflavin concentration was 0.03 mM and that of rhodopsin was approx. 0.003 mM. The solution was in 1% digitonin in 50 mM-sodium borate buffer, pH 9.3. The temperature was 25°C. $\bullet$, Rhodopsin + lumiflavin; $\circ$, rhodopsin alone; —, initial rate.

energies of the flavins was also suggestive of a triplet mechanism.

Hammond *et al.* (1964) postulated triplet energy transfer for similar photosensitized isomerizations of stilbene and obtained a complex correlation between the photostationary-state ratios and the triplet energies. The simpler relationship found in this work can be explained because flavins are a series of compounds with triplets formed between similar electronic states. The triplets have similar lifetimes and reactivities but different energies, and

there is therefore a systematic variation of the triplet properties. Some of the complexity of the relationship found by Hammond *et al.* (1964) may be caused by the diverse properties of the sensitizers used. Another possible explanation is that the triplet energies we used were for the phosphorescence transition and not for the 0-0 band.

From the phosphorescence spectra of the *cis*-stilbene ($\lambda_{\max}$, 500 nm, 57 kcal/mol) (Evans, 1957) and the flavin ($\lambda_{\max}$, approx. 605 nm, 46.5 kcal/mol) (Gordon-Walker *et al.* 1970) it can be seen that classical energy transfer from the flavin triplet to the *cis*-stilbene is unlikely to occur. To explain the sensitized isomerization of the *cis* isomer one must postulate non-classical excitation and 'phantom triplets' (Hammond *et al.* 1964). If one also postulated that the main decay to the ground state is from the 'phantom triplet' then the following scheme for the mechanism can be suggested, where F^* is the flavin excited singlet, F , c and t are the flavin, *cis*-stilbene and *trans*-stilbene ground states, F^t , c^t and t^t are the corresponding triplet states and p is the 'phantom triplet', Q is quencher and I_{abs} is the amount of light absorbed:

Excitation:

Classical energy transfer:

Non-classical energy transfer:

Interconversion of excited states:

Decay processes:

The quenching of flavin fluorescence by potassium iodide, oxygen and substrate showed that the inclusion of eqns. (2)–(4) was necessary in a detailed scheme; in the steady-state approximations below the rate of production of F^t was taken to be proportional to KI_{abs} , where KI_{abs} represents the rate of production of the singlet.

The phosphorescence spectrum of the *trans*-stilbene shows vibrational structure with a maximum at 577nm (Evans, 1957). Hammond *et al.* (1964) assigned this maximum to the 0-1 band and deduced that the 0-0 band would be at about 610nm (47kcal/mol). If this were the case classical energy transfer could probably occur between the flavin and the *trans* isomer, as the two energies would be similar.

The dependence of the rate of reaction on substrate concentration is very similar to that found for other flavin reactions where triplets have been implicated (Penzer & Radda, 1968; Penzer *et al.* 1970). This type of curve can be explained by the above scheme. Thus, taking the reaction from the *cis* isomer only, reactions (7), (9) and (11) can be ignored. Assuming that the rate is proportional to the rate of producing the 'phantom triplet' (which applies for low-energy sensitizers) and defining R as the fraction ('phantom triplet' decaying to the *trans* isomer) divided by ('phantom triplet' decaying to both the *cis* and *trans* isomers), then the rate of reaction is $(k_8 + k_{10})[F^*][c]R$. From a steady-state approximation on the flavin triplet concentration the rate is:

$$\frac{KI_{\text{abs.}}(k_8 + k_{10})[c]R}{(k_8 + k_{10})[c] + k_q[Q]} \quad (15)$$

and similarly for the reaction from the *trans* isomer [reactions (8), (10) and (12) ignored] the rate is:

$$\frac{KI_{\text{abs.}}(k_7 + k_9)[t](1 - R)}{(k_7 + k_9)[t] + k_q[Q]} \quad (16)$$

Thus when the substrate concentration is large k_q is negligible and the rate will be independent of the substrate concentration. When the substrate concentration is small, k_q is large and $(k_7 + k_9)[t]$ will be negligible and the rate will be:

$$\frac{KI_{\text{abs.}}(k_7 + k_9)(1 - R)[\text{substrate}]}{k_q[Q]}$$

i.e. a linear dependence of the rate on concentration will be observed. The observed form of the dependence of the rate on the substrate concentration fits the general features of this scheme.

It is possible that a complex between the flavin triplet and the substrate is formed. This type of complex has been suggested for other reactions of triplet flavin (Penzer & Radda, 1968; Penzer *et al.* 1970; Radda, 1966) and would not alter the concentration-dependence of the reaction.

The photoreaction of retinol with flavin can also be explained by a similar, but more complicated, scheme. The triplet energy of the retinol is not known and so 'phantom triplets' are not necessarily involved. However, if they are, at least three 'phan-

tom triplets' have to be postulated: a separate one between the all-*trans* isomer and each *cis* isomer, because the twist in the configuration of the 'phantom triplet' would hinder delocalization of the electrons in the excited state. Thus both these model systems proceed by a triplet mechanism, in which 'phantom triplets' may be implicated. For further elucidation of the mechanism it would be necessary to investigate the short-lived intermediates by direct methods such as flash photolysis.

The first process in the photobleaching of rhodopsin is known to be the isomerization of the retinal chromophore (Hubbard & Kropf, 1958). Some reaction of rhodopsin was found to be sensitized by lumiflavin. This destruction may be accompanied by a similar mechanism to that of the model compounds. However, rhodopsin has its main absorption peak at 500nm and thus has a lower energy than the flavin. This means that neither singlet energy transfer nor the direct process of emission from the flavin and re-excitation of the rhodopsin can be dismissed. The model experiments showed that the triplet energy transfer is also a possibility for this sensitization.

However, this sensitization, whatever its mechanism, supports the view (Pirie, 1959) that the role of flavin in vision in specific cases may be to convert the energy of near-u.v. light, which is inefficient in rhodopsin bleaching, into energy that can initiate the bleaching of rhodopsin more efficiently.

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