Biochem. J. (1979) **181**, 95–105
Printed in Great Britain

Flavin-Dependent Substrate Photo-oxidation as a Chemical Model of Dehydrogenase Action

By Wolfgang HAAS* and Peter HEMMERICH
Department of Biology, University of Konstanz, D-7750 Konstanz, Germany

(*Received* 2 *January* 1979)

As a model of flavin-dependent biological dehydrogenation, flavin-sensitized photodehydrogenation and photodecarboxylation were studied by variation of substrate, flavin, pH and solvent. Evidence for the following rules is given. (1) When the reactive site of a photosubstrate is an α-carbon atom of the type $>CH-CO_2^-$, decarboxylation is preferred over dehydrogenation, whereas the reverse is true for the neutral $>CH-CO_2H$. (2) Consequently these reactions do not exhibit a measurable isotope effect with $>C^2H-CO_2^-$, in contrast with the findings by Penzer, Radda, Taylor & Taylor [(1970) *Vitam. Horm.* (*N.Y.*) **28**, 441–466], which could not be reproduced. When the substrate does not contain a carboxylate group, isotope effects occur, in verification of previous reports, e.g. for benzyl alcohol $C_6H_5-C^2H_2OH$. (3) The mechanism of flavin-sensitized substrate photodecarboxylation is assumed to consist in a primary carbanion fixation at the flavin nucleus (position 4a, 5 or 8) with concomitant liberation of CO_2. This step is followed by rapid fragmentation of the adduct $>CH-Fl_{red.}^-$, provided that the substrate contains a functional and electron-donating group X, e.g. X = OH, OCH_3 or NH_2 (but not NH_3^+ !) in $_X>CH-CO_2^-$. (4) The minimal requirement for flavin-sensitized C–H dehydrogenation is the presence of a hydroxyl group. For example, methanol as substrate and solvent is dehydrogenated at pH sufficiently alkaline for detection of the presence of the active species CH_3O^-, whereas at more acidic pH substrate dehydrogenation is competing with flavin autophotolysis, which depends on the substituents in the flavin nucleus.

Flavin is biologically unique (Hemmerich, 1976) in its capacity to 'split' electron pairs coming from a hydride donor–acceptor system such as nicotinamide. Flavin as a chemical entity can thus deal with two electron equivalents as well as single electrons. It depends on the structure, or better the conformation, of the apoprotein and on the nature of the substrate which mode is preferred in each particular case.

The mechanism of the two-electron transfer is an unresolved question. Surprisingly, there is a far-reaching similarity between flavin-dependent substrate dehydrogenation, on the one hand, and flavin-sensitized substrate photo-oxidation, on the other hand, irrespective of the spin state of the excited species. Hence we have set out to find a structural relationship between the reactivities of protein-bound flavoquinone and its protein-free excited triplet state, since both are powerful dehydrogenating agents. The evidence comprises: (1) specification of the photoreactive species in the case of 'CH photosubstrates' such as α-hydroxy acids and α-amino acids, alcohols and amines, NAD(P)H and analogues; (2) determination of the product pattern in the case of competitive flavin-dependent photodehydrogenation and photodecarboxylation, i.e. C–H versus C–C bond photolysis; (3) reinvestigation of kinetic isotope effects claimed by Penzer *et al.* (1970), i.e. C–H versus C–^{2}H bond photolysis.

Some of these data have been communicated in a preliminary form (Haas & Hemmerich, 1972).

* Present address: Institute of Physiological Chemistry I, University of Düsseldorf, D-4000 Düsseldorf, Germany.

Experimental

Abbreviations and trivial names

The following abbreviations and trivial names are used: flavin, 10-substituted 7,8-dimethylisoalloxazine; lumiflavin, 7,8,10-trimethylisoalloxazine; lumichrome, 7,8-dimethylalloxazine; $Fl_{ox.}$, flavin in the neutral oxidized state; $\dot{F}l$, flavin radical; $Fl^*_{ox.}$, light-activated state of $Fl_{ox.}$; $^1Fl^*_{ox.}$ and $^3Fl^*_{ox.}$ singlet and triplet states of $Fl^*_{ox.}$; $Fl_{red.}H_2$, 1,5-dihydroflavin; $RFl_{red.}H$, alkylated dihydroflavin; 5-$dFl_{ox.}$, 5-deazaflavin.

Materials

Alloxazines and isoalloxazines were synthesized by common methods (Lambooy, 1967). 3-(3-Sulphopropyl)-lumiflavin was prepared from lumiflavin

and 3-hydroxypropane-1-sulphonic acid-γ-sultone in analogy to the method of Hemmerich (1964). [$\alpha\alpha$-$^2H_2$]Phenylacetic acid, [α-^{2}H]mandelic acid, 2-methyl-2-phenylpropanoic acid and 2-methoxy-2-methylpropanoic acid were synthesized by the methods of Clifford & Waters (1965), Kemp & Waters (1964), Brodhag & Hauser (1955) and Weizmann *et al.* (1948) respectively. [$\alpha\alpha$-$^2H_2$]Benzyl alcohol and [$\alpha\alpha$-$^2H_2$]benzylamine were prepared by reduction of benzoic acid ethyl ester and benzonitrile with $LiAlH_4$. By ^{1}H n.m.r. and mass spectroscopy chemical and isotope purity proved to be better than 97% for all deuterated compounds. Other reagents were commercially available in reagent-grade form and were used without further purification if not indicated otherwise.

Methods

Electronic spectra were recorded with a Cary 14R spectrophotometer, i.r.-absorption spectra with a Perkin–Elmer 621 spectrophotometer, ^{1}H n.m.r. spectra with a Varian A-60A spectrometer and mass spectra with a Varian CH7 mass spectrometer.

Measurements of pH were made with a Metrohm E 366 pH-meter before deoxygenation of the reaction solution and after aeration of the illuminated solution. The difference was less than 0.1 pH unit. The solutions were deoxygenated by bubbling argon ($O_2 \leqslant 2$ p.p.m.) for at least 15 min.

Reaction rates were determined spectrophotometrically in 1 cm quartz cuvettes; for reactant concentrations see the Figures and Tables. The cuvettes were supported in a thermostatically controlled water bath at 25.0±0.2°C and were illuminated by a 300 W tungsten lamp. If necessary, additional filters (K-2 or B-40/450/16; Balzers, Liechtenstein) were used to cut off wavelengths below 420 nm and above 500 nm. The light-intensity was varied by the distance between lamp and cuvette and was measured with a Gossen exposure meter at the front side of the cuvette. Reaction rates were determined spectrophotometrically by following the disappearance of the longest $\pi\pi^*$ absorption band of flavoquinone with $\lambda_{max.}$ 445 nm, where reduced flavin has less than 10% the absorbance of flavoquinone. Reaction rates were reproducible to ±3%.

Carbonate determination. Carbonate was determined with a Severinghaus CO_2 electrode connected to a Metrohm E 388 pH-meter. The reaction vessel was a 100 ml ground-glass cylinder kept at 20.80± 0.05°C by circulating water. The electrode response was calibrated under reaction conditions with solutions of known carbonate content and proved to be proportional within the experimental range 1–20 mM-carbonate corrected for volume change. At pH 7.09 the response time of the CO_2 electrode to ΔpH$\leqslant$0.001 pH units/3 min was 10–15 min. The vessel was irradiated by a Sylvania DNF-150W-21V halogen lamp through filter K-2. The pH of the solution was kept constant automatically by a Metrohm E 473 pH-stat at pH 10.90±0.05 during irradiation and at pH 7.09±0.01 during CO_2 measurement. Carbonate was independently determined by titration with standard $HClO_4$ (titration end point was at pH$\geqslant$8.2) as follows. An 80 ml volume of an aqueous solution 1.0 M in phenylacetate (80 mmol) and 21 mM in 3-(3-sulphopropyl)-lumiflavin (1.68 mmol) was deoxygenated and then irradiated at pH 10.9. These determinations were done at least in duplicate and mostly in triplicate (numbers given in parentheses). The reaction was monitored spectrophotometrically by 150-fold anaerobic dilution of 10 μl samples. After 2.5 h the absorption at 445 nm reached half the initial value. To keep a constant pH of 10.9, 1.61±0.04 (2) ml of 1.00 M-NaOH (1.61±0.04 mmol) was consumed. The half-reduced solution was then anaerobically titrated in the dark with 0.408 M-$HClO_4$ to pH 7.09±0.01 within 5 min or 1.5 h. The absorption spectrum remained unchanged during the fast as well as the slow titration and was identical with that at pH 10.9. After allowance for volume change, the carbonate concentration was determined to be 12.45±0.75 (3) mM (1.00±0.06 mmol) [11.77±0.80 (2) mM (0.94± 0.06 mmol)] by the CO_2 electrode and to be 11.9±0.75 (3) mM (0.96±0.06 mmol) [11.12±0.70 (2) mM (0.89± 0.06 mmol)] by acidimetry.

Benzaldehyde determination. A 2 ml volume of an aqueous solution 10 mM in mandelic acid (20 μmol) and 0.127 mM in 3-(3-sulphopropyl)-lumiflavin (0.254 μmol) was irradiated through filter B-40/450/16 under anaerobic conditions at pH 3.8, 8.0 and 10.9 until the flavoquinone absorption at 445 nm had disappeared. After aeration the solution was mixed with 2 ml of 0.08 M-H_2SO_4, 0.130 mM in 2,4-dinitrophenylhydrazine (0.26 μmol). After standing overnight, the pH of the resulting suspension was adjusted to pH 10.9 by adding solid Na_2CO_3. The hydrazone was extracted with 4 ml of chloroform and its concentration determined from the absorption at 375 nm to be 0.062±0.002 (3) mM, corresponding to 0.98±0.03 mol of benzaldehyde/mol of flavin reduced. With phenylglycine as photosubstrate at pH 10.9, 0.95±0.02 (3) mol of benzaldehyde was found/mol of flavin reduced. Within an experimental error of ±5% benzaldehyde can be quantitatively determined by this method, also at higher reactant concentrations [20 mM-3-(3-sulphopropyl)-lumiflavin and 1 M-mandelic acid at pH 8.0 and 10.9] after appropriate dilution of the irradiated solution. Phenylglyoxylic acid was also determined as the 2,4-dinitrophenylhydrazone by using the chloroform-extractable 3-methyl-lumiflavin and measuring the aqueous phase after chloroform extraction at pH 6.3. Phenylglyoxylic acid was also determined by the method of Bruice & Topping (1963) by

condensation with *o*-phenylenediamine. Blanks showed that these methods permit detection of phenylglyoxylate down to a few per cent per amount of flavin reduced.

Isotope retention. Isotope retention of substrate $C_{(\alpha)}H$ equivalents in benzaldehyde as oxidation product was determined as follows. At a given pH 10ml of a solution 0.5M in substrate and 20mM in 3-(3-sulphopropyl)-lumiflavin was irradiated at 25°C under anaerobic conditions with a 300W tungsten lamp until the absorption of flavoquinone at 445nm reached half the initial value. Flavohydroquinone was then reoxidized by molecular O_2. The pH was adjusted to pH10.9 by addition of solid Na_2CO_3. Benzaldehyde was extracted three times with 5ml of chloroform. The combined chloroform extracts were twice washed with 3ml of aq. $NaHCO_3$ and dried over anhydrous Na_2SO_4. The chloroform was evaporated *in vacuo* and the residue was dissolved in 0.5ml of carbon disulphide. From integrated ^1H n.m.r. spectra the ratios of aromatic protons ($\delta \sim 7.5$p.p.m. relative to tetramethylsilane) to the aldehyde $C_{(\alpha)}$ proton ($\delta = 10$p.p.m.) were determined to be: 5.0:0.98 for the reaction with C_6H_5–$CH(OH)$–CO_2^- in ^2H_2O at p^{2}H4.2 and 8.0 (20mM-sodium phosphate buffer), 5.0:0.01 for the reaction with C_6H_5–$C^2H(OH)$–CO_2^- in H_2O at pH3.8, 8.0 (20mM-sodium phosphate buffer) and pH10.9, 5.0:0.94 for the reaction with C_6H_5–$CH(OCH_3)$–CO_2^- in ^2H_2O at p^{2}H7.8, and 5.0:0.91 for the reaction with C_6H_5–$CH(NH_2)$–CO_2^- in ^2H_2O at p^{2}H10.4.

Isotope retention of the α-hydrogen of phenylacetate in the benzylated dihydroflavin product of flavin photoreduction was determined as follows. A 1ml volume of an anaerobic ^2H_2O solution, p^{2}H9.8, containing 0.14mmol of 3-carboxymethyl-lumiflavin and 1.4mmol of ammonium [α-^{2}H]phenylacetate was sealed in an n.m.r. tube and irradiated in a thermostatically controlled water bath at 15°C with a 300W tungsten lamp for 24h. To rearrange 8- and 5-benzylated dihydroflavin to the 4a-benzyl isomer the solution was heated under anaerobic conditions at 50°C for 24h. From the integrated ^1H n.m.r. spectrum of the resulting 4a-benzyl-1,5-dihydroflavin the ratio of N^{10}-methyl hydrogen at $\delta = 3.3$p.p.m. to α-hydrogen of the 4a-benzyl group at $\delta = 2.8$p.p.m. was determined as 3.0:1.9.

Results and Discussion

Product determination and proton balance in flavin-sensitized photo-oxidation of carboxylic acid anions

Under alkaline (pH10) anaerobic conditions phenylacetate is known to undergo decarboxylation and benzyl group transfer to photoexcited flavoquinone, yielding approximately equal amounts of three benzylated dihydroflavins (Walker *et al.*, 1970; Hemmerich *et al.*, 1973), while CO_2 is liberated, according to eqns. (1*a*), (1*b*) and (1*c*) (cf. also Scheme 1):

$$\mathrm{Fl}^*_{ox.} + \mathrm{RCH_2CO_2^-} + \mathrm{H_2O} \xrightarrow{a} 5\text{-}\mathrm{RCH_2}\text{-}\mathrm{Fl}_{red.} + \mathrm{CO_3^{2-}} + 2\mathrm{H^+} \quad (1a)$$

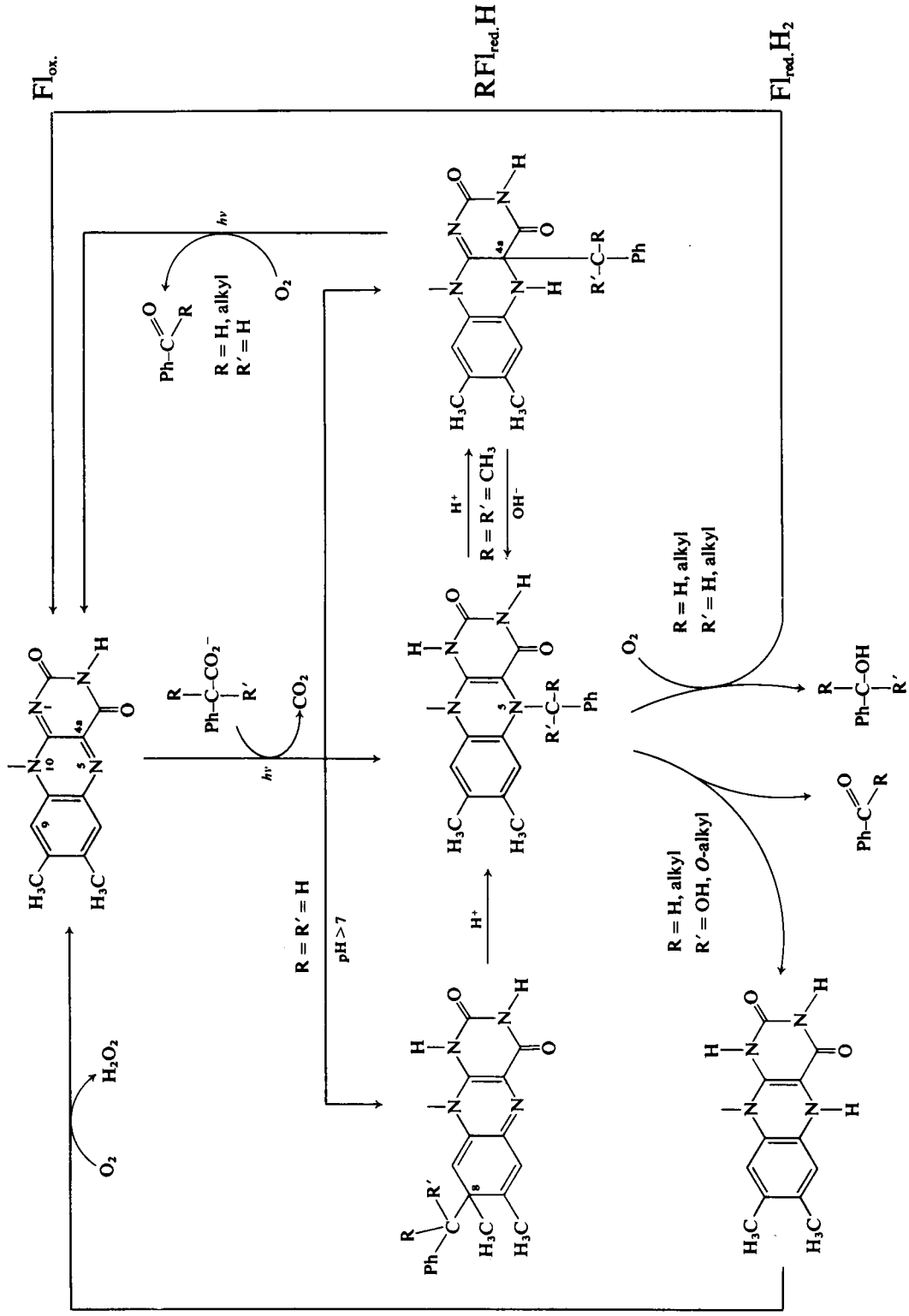
$$\mathrm{Fl}^*_{ox.} + \mathrm{RCH_2CO_2^-} + \mathrm{H_2O} \xrightarrow{b} 4a\text{-}\mathrm{RCH_2}\text{-}\mathrm{Fl}_{red.}\text{-}5\text{-}\mathrm{H} + \mathrm{CO_3^{2-}} + \mathrm{H^+} \quad (1b)$$

$$\mathrm{Fl}^*_{ox.} + \mathrm{RCH_2CO_2^-} + \mathrm{H_2O} \xrightarrow{c} 8\text{-}\mathrm{RCH_2}\text{-}\mathrm{Fl}_{red.} + \mathrm{CO_3^{2-}} + 2\mathrm{H^+} \quad (1c)$$

($R = C_6H_5$)

Although in the cited previous studies the physical properties, quantity and structure of the 'flavin benzyl adducts' were all elucidated, we now wished to quantify CO_2 liberation and proton balance. For this purpose 3-(3-sulphopropyl)-lumiflavin was photoreduced in the presence of phenylacetate down to 50% absorption decrease at $\lambda = 445$nm, while pH10.9 was constantly maintained. 3-(3-Sulphopropyl)-lumiflavin was used as $Fl_{ox.}$ because of its photostability (see below) and its high water-solubility. Two of the products, 5-benzyl-$Fl_{red.}^-$ and 4a-benzyl-$Fl_{red.}$-5-H, are transparent at $\lambda = 445$nm (eqns. 1*a* and 1*b*), but correction had to be made for absorption of the 33% of 8-benzyl adduct [eqn. 1*c*, $\varepsilon_{445}^{pH\,11} = 6500\,M^{-1}\cdot cm^{-1}$ (Hemmerich *et al.*, 1973)] formed (cf. eqn. 1*c*), yielding the amount of bleached reduced flavin as 60.5%. The actual amount of protons to be neutralized was 1.58 ± 0.04 (2)mol/mol of flavin reduced. This amount is compatible with the average requirement of 1.67mol of protons to be liberated/mol of flavin reduced when the reactions (1*a*), (1*b*) and (1*c*) are equally probable, and confirms that two out of the three adduct isomers exist in the anionic state at pH11.

Carbonate in the photoreaction mixture was then determined in two different ways. (*a*) The alkaline solution was cautiously titrated under anaerobic conditions with standard $HClO_4$ and the amount of bicarbonate formed was derived from the titration curve. 4a-Benzyl-$Fl_{red.}$-5-H has no measurable pK_a in aqueous solution, whereas the pK_a of 5-benzyl-$Fl_{red.}$-1-H is 7.2 (Walker *et al.*, 1967) and that of 8-benzyl-$Fl_{red.}$-1-H is even lower (Haas, 1973). This acidimetric carbonate determination (end point found at pH8.7) thus needs no correction for product pK_a. The yield was 0.94 ± 0.06 (3)mol of (bi)carbonate/



Scheme 1. *Reaction pathways of flavin photoreduction by carboxylic acid anions*

mol of flavin reduced. As required, no significant change of absorption was found to be associated with this neutralization. (*b*) At pH7.1 bicarbonate was directly assayed with the aid of a CO_2 electrode, yielding 0.98 ± 0.06 (3) mol of bicarbonate/mol of flavin reduced, in good agreement with the acidimetric data.

These results call for quantitative liberation of CO_2 during the photodecarboxylation at alkaline pH also. This requires revision of a preliminary interpretation given by Hemmerich (1970) with respect to the observed selective retention of the 8-benzyl adduct in the alkaline phase whereas 4a- and 5-benzyl adducts are extracted into chloroform. This distribution pattern was tentatively assigned to the formation of a dihydroflavincarboxylate according to eqn. (2):

$$Fl^{*}_{ox.} + C_6H_5CH_2CO_2^- \rightarrow 8\text{-}C_6H_5CH_2\text{-}Fl_{red.}\text{-}CO_2^- \quad (2)$$

In fact, however, it is due, as we know now, to the use of ammonia to maintain the pH at 11. This requires a large excess of ammonia compared with flavin. Consequently the ammonia content of the chloroform phase causes the deprotonation at N-1 of the otherwise extractable (e.g. from carbonate buffer, pH11) acidic isomer $8\text{-}C_6H_5CH_2\text{-}Fl_{red.}H$ and forces the anion back into aqueous phase.

If phenylacetate, as a flavin photosubstrate, is replaced by mandelate, no adducts can be trapped and the only flavin product to be found under anaerobic conditions at any pH is 1,5-dihydroflavin (Walker *et al.*, 1967) (cf. Scheme 1 and eqn. 3*a*). On admission of air, the latter is reconverted quantitatively within seconds into starting flavoquinone. The mandelate substrate is, as we find, quantitatively degraded to benzaldehyde (eqn. 3*a*), determined as dinitrophenylhydrazone. No trace of the alternative product phenylglyoxylate (eqn. 3*b*) could be detected, which confirms 100% decarboxylation (eqn. 3*a*) in favour of dehydrogenation (eqn. 3*b*). Phenylglyoxylate, however, is known to be itself a flavin photosubstrate (Brüstlein & Hemmerich, 1968). This secondary reaction (eqn. 3*c*) could have been faster than the primary one, irrespective of the large excess of mandelate applied as primary donor. This possibility was excluded by the absence of significant amounts of benzoate from the reoxidized reaction mixture, as checked by ^1H n.m.r. after chloroform extraction of the aldehyde at pH8.

$$Fl^{*}_{ox.} + C_6H_5CH(OH)CO_2^- + H_2O \rightarrow Fl_{red.}H_2 + C_6H_5CHO + HCO_3^- \quad (3a)$$

$$Fl^{*}_{ox.} + C_6H_5CH(OH)CO_2^- \rightarrow Fl_{red.}H_2 + C_6H_5COCO_2^- \quad (3b)$$

$$Fl^{*}_{ox.} + C_6H_5COCO_2^- + H_2O + OH^- \rightarrow Fl_{red.}H_2 + C_6H_5CO_2^- + HCO_3^- \quad (3c)$$

Table 1. *Initial rates and products of anaerobic flavin photoreduction by carboxylic acid anions*

Anaerobic solutions of 70 μM-3-methyl-lumiflavin and 0.1 M-substrate in water/methanol (1:1, v/v), 0.1 M in $NaClO_4$ and 10 mM in sodium phosphate buffer, pH8, were irradiated in 1 cm cuvettes with a light-intensity of 120 klx. For amino acids the pH was 10.5 and the light-intensity was 26 klx.

Substrate	Initial rate of anaerobic bleaching (ΔA_{445}/min)	% of A_{445} restored after half-bleaching and 1 min aeration	Products of anaerobic flavin photoreduction
Phenylacetate	2.21	8	8-, 5- and 4a-Alkyl-$Fl_{red.}H$
2-Phenylpropionate	2.38	10	5- and 4a-Alkyl-$Fl_{red.}H$
2-Methyl-2-phenylpropionate	2.61	25 (100 after 3 h)	5-Alkyl-$Fl_{red.}H$
2,2-Dimethylpropionate (pivalate)	0.25	24	5- and 4a-Alkyl-$Fl_{red.}H$
α-Hydroxyphenylacetate (mandelate)	3.04	100	$Fl_{red.}H_2$
α-Methoxyphenylacetate	>4	99	
2-Hydroxy-2-phenylpropionate	3.56	100	
2-Methoxy-2-phenylpropionate	>4	97	
2-Methoxy-2-methylpropionate	0.39	97	
Aminoacetate (glycine)	1.56	98	
2-Aminopropionate (alanine)	1.58	99	
2-Amino-2-methylpropionate	1.58	97	

Similarly phenylglycinate yields 1,5-dihydroflavin, benzaldehyde and ammonia in quantitative yield, and phenylglyoxylate is not detectable, in agreement with the g.l.c. investigation by Penzer & Radda (1968) as well as Frisell *et al.* (1959). From this, the conclusion can be drawn that substrate photodecarboxylation quite generally prevails over photodehydrogenation, provided that the substrate acid is present in its anionic state. It must be kept in mind, however, that electron-donating α-substitution is a prerequisite for efficient decarboxylation by $Fl^{*}_{ox.}$. Hence neutral α-amino acids are non-photoreactive in their dipolar form, and only the tautomer N_2N–CHR–CO_2H, present to small extent in excited flavin–substrate complexes, reacts by dehydrogenation in favour of decarboxylation, as shown below.

Apart from this exception, attack by light-excited flavin of carboxylic acids always involves rupture of the $C_{(\alpha)}$–C bond. The most efficient reactions of this type are indeed found with carboxylates devoid of α-hydrogen (Table 1), with the reaction course and product types remaining the same. Thus the 'simplest' carboxylic acid anion, which is efficiently photodecarboxylated by $Fl_{ox.}$, is pivalate $(CH_3)_3CCO_2^-$ (Haas & Hemmerich, 1972). αα-Dimethylphenylacetate, for example, yields a normal pattern of 4a- and 5- adducts, but these, however, undergo pH-dependent 4a–5-migrations (Scheme 1) at room temperature owing to increased substituent lability (Hemmerich & Haas, 1975). If indeed a $C_6H_5C(CH_3)_2$- group is able to migrate on the flavin surface at ambient temperature, it is not surprising that the $C_6H_5CH(OH)$- residue from mandelate cannot be stabilized at all in an adduct (eqn. 4*a*), not even when flavin N-5 is modified to CH, as in 5-deazaflavins. There, however, the intermediate formation of mandelate adducts (eqn. 4*b*) can be traced indirectly (Duchstein, *et al.*, 1979)

$$Fl_{ox.}+RCH(OH)CO_2^- \xrightarrow[\text{slow}]{h\nu,\,-CO_2} RCH(OH)\text{–}Fl_{red.}H \xrightarrow[\text{fast}]{} Fl_{red.}H_2+RCHO \qquad (4a)$$

$$5\text{-}dFl_{ox.}+RCH(OH)CO_2^- \xrightarrow[\text{slow}]{h\nu,\,CO_2} RCH(OH)\text{-}5\text{-}dFl_{red.}H \xrightarrow[\text{fast}]{h\nu} \tfrac{1}{2}[H\text{-}dFl]_2+RCHO \qquad (4b)$$

Table 2. *Kinetic isotope effect of flavin photoreduction by mandelate and [α-²H]mandelate*

Initial rates were determined by irradiating anaerobic solutions in 1 cm cuvettes at 25°C with a light-intensity of 120 klx. Solutions at pH 8 contained 20 μM-sodium phosphate buffer. Acetonitrile was freshly distilled over CaH_2. Mean values of at least three determinations are given. Standard deviation did not exceed ±5% of the values given.

		k_H/k_{2H} from initial rate				
		Present study				Penzer *et al.* (1970)
	Excess [Substrate]/[Fl]	3-(3-Sulphopropyl)-lumiflavin (70 μM)	3-Methyl-lumiflavin (70 μM)	Lumiflavin (60 μM)	Taylor (1970) Lumiflavin (30 μM)	Lumiflavin (30 μM)
pH 8	0 (extrapolated)					3.5
	≃2			1.04		
	14	1.04				
	80	0.89			1.44	
	330	1.09			0.86	
	1070	0.97				
	1430	1.00				
	∞ (extrapolated)					0.62
pH 4	0 (extrapolated)					1.9
	11	1.05			1.43	
	33				1.25	
	143	1.04				
	1430	0.97				
	∞ (extrapolated)					1.0
CH_3CN	7		1.07			
	70		1.00			
	700		1.01			

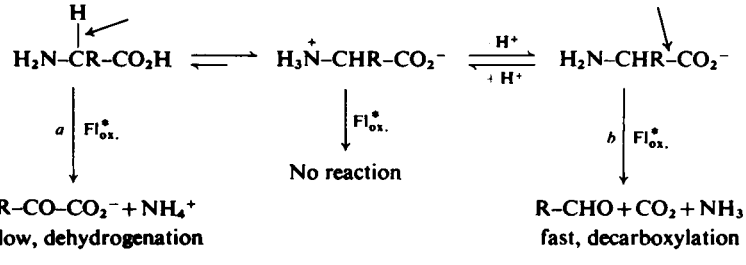
Flavin-dependent substrate photoreduction versus flavin autophotolysis and claimed deuterium isotope effects

The question of 'C–C versus C–H rupture' in these reactions has been discussed extensively in the preceding section, because there is still the unrefuted claim by Penzer *et al.* (1970) of an extrapolated kinetic isotope effect up to $k_H/k_{2H} = 3.5$ observed in the flavin-dependent photo-oxidation of α-deuterated mandelate. From the very beginning it was unclear why mandelate should give such an effect in contrast with phenylacetate. Despite meticulous efforts, we were unable to reproduce this effect. In our case, benzaldehyde obtained by flavin-sensitized photo-oxidation of mandelate in ^2H_2O on the one hand, and of α-deuteromandelate in H_2O on the other hand, shows more than 95% isotope retention on careful relative integration of aldehyde and phenyl protons by ^1H n.m.r. Any kinetic isotope effect must therefore be secondary and could hardly exceed a value of 2 (Krumbiegel, 1970). As documented in Table 2, we have not been able to demonstrate any effect that would exceed the error limit of 1.0 ± 0.1 at any pH.

Taylor (1970) and Penzer *et al.* (1970) worked with unsubstituted lumiflavin, which even at the low concentration of 30 μM is in supersaturated solution and will form a precipitate slowly, which increases error limits. We have therefore conducted most experiments with the more soluble 3-methyl-lumiflavin and the highly water-soluble sulphonate. $k_H/k_{2H} = 1.0 \pm 0.1$ was found for phenylacetate, in agreement with Taylor (1970) and Penzer *et al.* (1970). With mandelate the measured $k_H/k_{2H} \sim 1.5$ quoted by these authors could, however, not be reproduced, nor could a significant dependence of this effect on substrate concentration be verified. The same degree of isotope retention in benzaldehyde is observed with *O*-methylmandelate and phenylglycinate as photosubstrates. Correspondingly no trace of label can be introduced into the 'adduct' $C_6H_5CH_2$–Fl_{red}.H in the active CH_2 group, if phenylacetate is decarboxylated in ^2H_2O at any p^2H, as was confirmed by integrated ^1H n.m.r. spectra.

Thus it is emphasized that amino acid anions react with $Fl^*_{ox.}$ by decarboxylation (eqn. 5*b*), in agreement with previous reports (Frisell *et al.*, 1959; Enns & Burgess, 1965; Penzer & Radda, 1968). Approaching the isoelectric point, however, a strong decrease in rate (Fig. 1) is accompanied by a change in product pattern. This was first shown by Byrom & Turnbull (1967) for phenylalanine and by Enns & Burgess (1965) for EDTA as substrates. In the latter case glyoxylate replaces formaldehyde as product, a phenomenon for which the authors had no explanation.

Betaine $(CH_3)_3N^+CH_2CO_2^-$ is, in our experience, exceedingly stable to attack by $Fl^*_{ox.}$. Thus if an amino acid does react at the isoelectric point, even though very slowly, the activity must be attributed to the non-zwitterionic tautomer $H_2NCH(R)CO_2H$, which is generated within the reaction cage. This may already exist to some extent in a ground state complex with $Fl_{ox.}$ or at least in an excited complex formed with $Fl^*_{ox.}$ (see below). Hence it is apparent that a C–H bond, though inferior to C–CO_2^-, is superior to C–CO_2H as site of $Fl^*_{ox.}$-induced cleavage if adjacent to a strongly activating functional group such as NH_2 (eqn. 5*a*):



(5)

The kinetic isotope effect to be expected is indeed found in this case, as shown in Table 3 for the prototype case of the more rapidly reacting benzylamine, where it reaches values up to $k_H/k_{2H} = 3$. The photoactivity is, as expected, entirely blocked on protonation of the amino group (Fig. 1).

Flavin autophotolysis

If we switch over from the strongly activating H_2N donor to alcoholic substrates, we frequently reach a range of lower activity, where substrate oxidation is competing with flavin autophotolysis, i.e. with self-dismutation of $Fl_{ox.}$. Recently it has been shown by P. Hemmerich, W.-R. Knappe, H. E. A. Kramer & R. Traber (unpublished work) that the main path in the chemical self-decay of the triplet is as in eqn. (6):

$$[Fl_{ox.}, Fl_{ox.}]^* \rightarrow \dot{F}l^- + \dot{F}l^+ \rightarrow 2Fl_{ox.} \qquad (6)$$

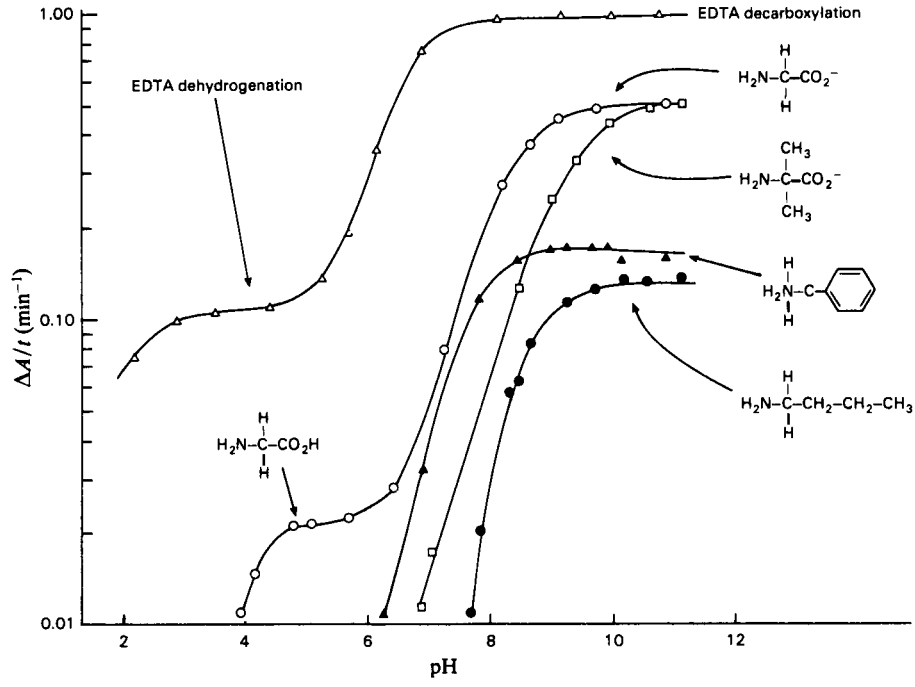


Fig. 1. *pH-dependence of flavin-sensitized photo-oxidation: variation of substrate*
Relative anaerobic reaction rates measured by the decrease of the long-wavelength flavin absorption at 445nm are plotted against pH. In water/methanol (1:1, v/v) substrate was of 0.1 M, 3-methyl-lumiflavin at 70 μM, $NaClO_4$ at 0.1 M and buffer at 10mM (pH 1–3.5, sulphate; pH 3.5–6, acetate; pH 6–11, phosphate). Light-intensity was 26klx and the temperature 25°C. Note that substrate pK gives rise to an increase of reaction rate with increasing pH, whereas flavin photo-pK leads to a decrease. In the present Figure the flavin photo-pK (cf. Fig. 2) is over compensated by the much greater effect of substrate pK. Bonds to be cleaved are printed **bold**.

Table 3. *Kinetic isotope effect of flavin photoreduction by benzylamine and [αα-$^2H_2$]benzylamine and by benzyl alchohol and [αα-$^2H_2$]benzyl alcohol*
The concentration of 3-methyl-lumiflavin was 70 μM, that of $NaClO_4$ 0.1 M, and that of buffer if necessary 10mM (pH 4, sodium acetate; pH 8, sodium phosphate; pH 10, sodium carbonate). To avoid precipitation of reduced flavin, aqueous solutions contained 20% (v/v) acetonitrile, freshly distilled over CaH_2. Other experimental conditions were as indicated in Table 1. Mean values of two experiments are given.

Substrate	Concn. (M)	pH	k_H/k_{2H} from initial rate
$C_6H_5CH_2NH_2$ and	0.1	CH_3CN	3.1 ± 0.3
$C_6H_5C^2H_2NH_2$	0.1	8	2.3 ± 0.3
	0.001	8	1.5 ± 0.1
	0.1	10	1.5 ± 0.2
	0.001	10	1.4 ± 0.1
$C_6H_5CH_2OH$ and	0.1	4	2.2 ± 0.1
$C_6H_5C^2H_2OH$	0.1	8	2.1 ± 0.1
	0.1	10	1.5 ± 0.2
	0.001	10	1.4 ± 0.2

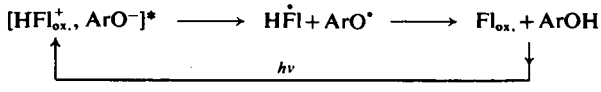
This reaction starts from a dimeric excited flavin complex yielding a pair of radicals, which rapidly returns to the ground state by back donation of electrons, and irreversible photolysis is only a minor side reaction. This side reaction could either start directly from the excited flavoquinone or from the 'oxidized radical' $Fl^{\bullet+}$. Various types of autophotolysis must be considered, as follows.

(1) *Dehydrogenation of hydroxylic side chain.* This involves dehydrogenation of a hydroxylic side chain that can fold back towards the excited chromophore, as in riboflavin (Yang & McCormick, 1965; Moore *et al.*, 1963).

(2) *Attack at N- or C-methyl groups of the flavin nucleus.* We found a decreasing sensitivity in the sequence N^3-$CH_3 > C^7$-$CH_3 > C^8$-$CH_3 > N^{10}$-CH_3. For example, oxidation of a 3-methyl group competes with methanol photodehydrogenation in aqueous 50% solution at pH 3.5 (Fig. 2) and results in the formation of lumiflavin without alteration of the flavin chromophore. If an N^{10}-methyl group is lost

under alkaline conditions, it is a dark reaction. Thus 10-methylisoalloxazine is found to be the flavin derivative most stable towards autophotolysis.

(3) *Light-induced solvolysis.* Light-induced solvolysis, as observed with alloxazine (Dekker *et al.*, 1973), is obtained with 10-methylisoalloxazine only under conditions that stabilize the cation $HFl^{+}_{ox.}$. (Schölln-



(8)

hammer & Hemmerich, 1974). In principle, however, this reaction channel remains open, as shown by the addition of ammonia to $Fl^{*}_{ox.}$ (S. Kasai & P. Hemmerich, unpublished work).

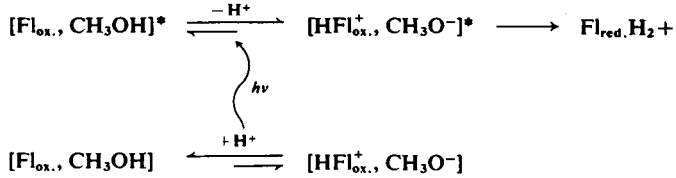
(4) *Photo-dealkylation at N-10.* The long-known photo-dealkylation at N-10 has been shown to be a fragmentative process encountered only with N^{10}-alkyl residues bigger than ethyl (Gladys & Knappe, 1974). This is the only photolytic reaction that would not yield bleaching, i.e. formation of dihydroflavin, under anaerobic conditions. Flavin bleaching is by definition reversible with O_2 and must be distinguished from unspecific photodestruction of flavin.

(5) *Unspecific photodestruction of flavin.* Unspecific photodestruction of flavin was observed in long-time irradiation experiments by Vaish & Tollin (1970) as well as in the present work (cf. Fig. 2). Such unspecific destruction of the chromophore is not a 10-dealkylation and thus would not yield significant amounts of alloxazine. It arises presumably from solvolytic ring cleavage in the oxidized radical $\dot{F}l^{+}$ (eqn. 6). Flavin bleaching and its reversibility in a system where, depending on pH, autophotolysis competes with substrate (i.e. solvent) dehydrogenation is illustrated in Fig. 2. Anaerobic lumiflavin autophotolysis is prevalent up to pH 7, as shown by the low reversibility of the bleaching by O_2, The increased photoreaction rate at alkaline pH is accompanied by increased reversibility by O_2 and must be assigned to flavin reduction by methanol dehydrogenation due to enhanced methanol dissociation within an excited flavin–solvent complex (eqns. 7*a* and 7*b*):

This is even more pronounced with phenol (Vaish & Tollin, 1970). The phenolic substrate represents the special case of a substrate, which contains no σ-bond to be activated and thus can donate single π-electrons only. Hence no net reaction results from electron transfer between substrate and flavin, induced by the excited flavin species (eqn. 8):

This ready reversible electron transfer even prevents flavin autophotolysis (Table 4). The radical pair of eqn. (8) conceivably returns much more slowly to the starting ground state than the more reactive pair of eqn. (6).

Green & Tollin (1968) have interpreted a comparable quantum yield for flavin photobleaching with propan-2-ol and 2-methylpropan-2-ol as successful competition between O–H and $C_{(\alpha)}$–H bond fission. To check this claim we compared in aqueous solution benzyl alcohol with its methylated derivatives, to obtain an elevated photoactivity as compared with that of aliphatic alcohols. In this series we find no significant decrease of flavin bleaching on *O*-methylation (Table 4), whereas $\alpha\alpha$-dimethylation results in a drastic loss of photoactivity down to that of lumiflavin autophotolysis. In agreement with Song & Metzler (1967), who demonstrated that there is no kinetic isotope effect in lumiflavin photoreduction with EtOH and $EtO^{2}H$, this casts even more doubts on the O–H bond rupture claimed to occur by Green & Tollin (1968). Furthermore flavin photoreduction with [$\alpha\alpha$-$^{2}H_2$]benzyl alcohol shows an appreciable kinetic isotope effect (Table 3), indicating successful $C_{(\alpha)}$–H bond fission. Hence phenylcarbinols react with $Fl^{*}_{ox.}$ sufficiently rapidly in aqueous solution to yield quantitative amounts of dihydroflavin and the corresponding carbonyl compound with kinetic isotope effects in the range 1.4–2.2 (Table 3). Thus Green & Tollin (1968) failed to consider autophotolysis (partially O_2-irreversible) as a competitive event with solvent dehydrogenation (100% O_2-reversible).



(7*a*)

(7*b*)

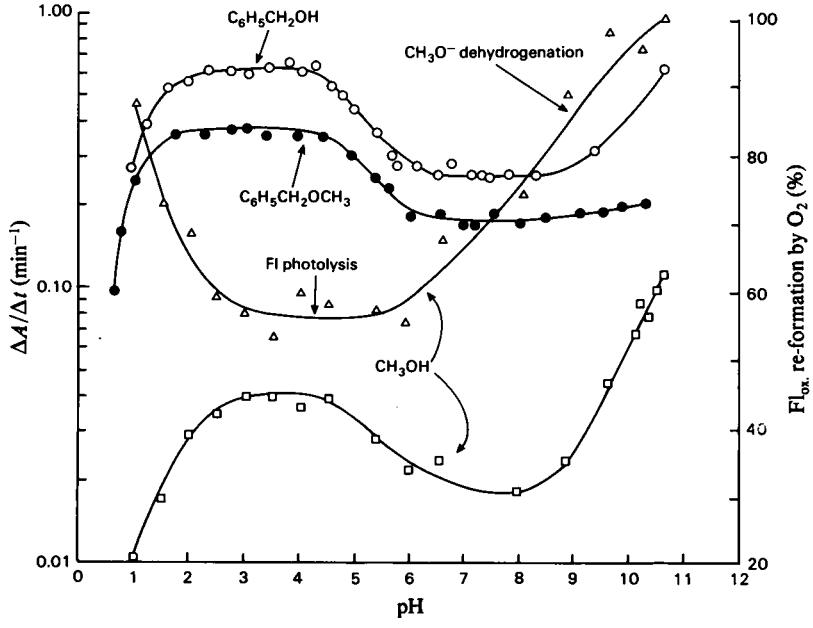


Fig. 2. *pH-dependence and reversibility of flavin autophotolysis and photoreduction*
Relative anaerobic reaction rates measured by the decrease of $Fl_{ox.}$ absorption at 445 nm are plotted against pH. Solvent, flavin, buffers and temperature were as given in Fig. 1. Light-intensity was 120 klx. □, No substrate (△, percentage restoration of A_{445} by O_2 after 50% reduction); ○, 0.1 M-benzyl alcohol; ●, 0.23 M-benzyl methyl ether; both are 100% O_2-reversible at any pH. Note that rate decrease at pH < 2 is due to increased dynamic quenching of $^1Fl^*_{ox.}$ by protons whereas rate decrease at pH 4→6 ('flavin photo-pK'') is due to deprotonation of $H^3Fl^{+\bullet}_{ox.}$. Autophotolysis of 3-methyl-lumiflavin (□) is a mixture of unspecific photolysis, which destroys the flavin chromophore, and specific photolysis (intermolecular N^3-methyl dehydrogenation), which leaves the chromophore intact. At pH > 9 solvent dehydrogenation (100% O_2-reversible reduction of $Fl_{ox.}$) prevails over autophotolysis.

Table 4. *Rates of anaerobic flavin photoreduction by alcohols*
Experimental conditions were as indicated in Table 1, except for dry methanol, which serves as both substrate and solvent.

Substrate	Initial rate of anaerobic bleaching (ΔA_{445}/min)	$t_{\frac{1}{2}}$(min)	% of A_{445} restored after half-bleaching and 5 min aeration
Benzyl alcohol	0.352	3.0	100
Benzyl methyl ether	0.244	7.0	94
2-Phenylpropan-2-ol	0.072	Within the error limit of photolysis	
2-Phenylpropan-2-ol, but at 1.0 M	0.076		
Phenol	0.004	Partial fluorescence quenching, no permanent bleaching, but suppression of autophotolysis	
None (photolysis; cf. Fig. 2)	0.064	>90	80
Methanol (dry)	0.112	68	98

Radical fission of a C–H bond shows higher k_H/k_{2H} values compared with ionic dehydrogenation; e.g. attack of organic peroxy radical at [α-^{2}H]cumene shows an isotope effect of about 12 and autoxidation of [αα-$^2H_2$]benzyl t-butyl ether yields an overall value $k_H/k_{2H} \approx 20$, whereas ionic perchromic acid oxidation of [2-^{2}H]propan-2-ol shows a value $k_H/k_{2H} = 7$. Even smaller values are to be expected for more easily oxidizable alcohols, e.g. benzyl alcohol (Krumbiegel, 1970). Kinetic isotope effects measured in enzymic dehydrogenation reactions are usually not higher than 7 and frequently as low as $k_H/k_{2H} = 2.3$ in NAD^+-dependent enzymic alcohol dehydrogenation (Mahler *et al.*, 1962), which is

clearly a hydride transfer (Blankenhorn, 1977), 2.5 for the succinate dehydrogenase reaction (Thorn, 1951) and 2.3 for the oxidation of tyramine by monoamine oxidase (Belleau *et al.*, 1960). Though there is no exact correlation between the value of a kinetic isotope effect and the kind of hydrogen transfer as H^+, $H^\bullet$ or H^-, the last-mentioned results are further argument against a compulsory radical course of flavin-dependent photo-oxidation.

References

Belleau, B., Fang, M., Burba, J. & Moran, J. (1960) *J. Am. Chem. Soc.* **82**, 5752–5754

Blankenhorn, G. (1977) in *2nd Symposium on Pyridine Nucleotide-Dependent Dehydrogenases* (Sund, H., ed.), pp. 185–205, H. W. de Gruyter Verlag, Berlin

Brodhag, A. & Hauser, C. R. (1955) *J. Am. Chem. Soc.* **77**, 3024–3031

Bruice, T. C. & Topping, R. M. (1963) *J. Am. Chem. Soc.* **85**, 1480–1488

Brüstlein, M. & Hemmerich, P. (1968) *FEBS Lett.* **1**, 335–338

Byrom, P. & Turnbull, J. H. (1967) *Photochem. Photobiol.* **6**, 125–131

Clifford, A. A. & Waters, W. A. (1965) *J. Chem. Soc.* 2796–2804

Dekker, R. H., Srinivasan, B. N., Huber, J. R. & Weiss, K. (1973) *Photochem. Photobiol.* **18**, 457–466

Duchstein, H. J., Fenner, H., Hemmerich, P. & Knappe, W. R. (1979) *Eur. J. Biochem.* in the press

Enns, K. & Burgess, W. H. (1965) *J. Am. Chem. Soc.* **87**, 5766–5770

Frisell, W. R., Chung, C. W. & Mackanzie, C. G. (1959) *J. Biol. Chem.* **234**, 1297–1302

Gladys, M. & Knappe, W.-R. (1974) *Chem. Ber.* **107**, 3658–3673

Green, M. & Tollin, G. (1968) *Photochem. Photobiol.* **7**, 129–143

Haas, W. (1973) Thesis, University of Konstanz

Haas, W. & Hemmerich, P. (1972) *Z. Naturf. Teil B* **27**, 1035–1037

Hemmerich, P. (1964) *Helv. Chim. Acta* **47**, 464–475

Hemmerich, P. (1970) *Vitam. Horm. (N.Y.)* **28**, 467–487

Hemmerich, P. (1976) *Fortschr. Chem. Org. Naturst.* **33**, 431–527

Hemmerich, P. & Haas, W. (1975) in *Reactivity of Flavins* (Yagi, K., ed.), 1–13, University Park Press, Baltimore

Hemmerich, P., Bhaduri, A. P., Blankenhorn, G., Brüstlein, M., Haas, W. & Knappe, W.-R. (1973) in *Oxidases and Related Redox Systems* (King, T. F., Mason, H. S. & Morrison, M., eds), pp. 3–24, University Park Press, Baltimore

Kemp, T. J. & Waters, W. A. (1964) *J. Chem. Soc.* 1192–1194

Krumbiegel, P. (1970) *Isotopieeffekte*, Akademie Verlag, Berlin

Lambooy, J. P. (1967) *Heterocycl. Compd.* **9**, 118–223

Mahler, H. R., Baker, R. H. & Shiner, V. J. (1962) *Biochemistry* **1**, 47–52

Moore, W. M., Spence, J. T., Raymond, F. A. & Colson, S. D. (1963) *J. Am. Chem. Soc.* **85**, 3367–3371

Penzer, G. R. & Radda, G. K. (1968) *Biochem. J.* **109**, 259–268

Penzer, G. R., Radda, G. K., Taylor, J. A. & Taylor, M. B. (1970) *Vitam. Horm. (N.Y.)* **28**, 441–466

Schöllnhammer, G. & Hemmerich, P. (1974) *Eur. J. Biochem.* **44**, 561–577

Song, P.-S. & Metzler, D. E. (1967) *Photochem. Photobiol.* **6**, 691–709

Taylor, J. A. (1970) Thesis, University of Oxford

Thorn, M. B. (1951) *Biochem. J.* **49**, 602–609

Vaish, S. P. & Tollin, G. (1970) *Bioenergetics* **1**, 181–192

Walker, W. H., Hemmerich, P. & Massey, V. (1967) *Helv. Chim. Acta* **50**, 2269–2279

Walker, W. H., Hemmerich, P. & Massey, V. (1970) *Eur. J. Biochem.* **13**, 258–266

Weizmann, C., Sulzbacher, M. & Bergmann, E. (1948) *J. Am. Chem. Soc.* **70**, 1153–1158

Yang, C. S. & McCormick, D. B. (1965) *J. Am. Chem. Soc.* **87**, 5736–5765