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Dye-sensitized photo-oxidation of enzymes

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Heart lipoamide dehydrogenase, liver alcohol dehydrogenase and egg-white lysozyme are photo-oxidized in the presence of various dye sensitizers. The photodynamic process is preceded by the binding between the enzyme and the sensitizers. Among the commonly used dyes, halogenated xanthines and thiazine are effective sensitizers for the photo-inactivation of these three enzymes. Histidine residues are the primary target for the sensitized photo-oxidation that inactivates lipoamide dehydrogenase and alcohol dehydrogenase. However, the destruction of tryptophan residues is responsible for the photo-inactivation of lysozyme. The deuterium medium effect and the quenching effect by various scavengers of the potential photo-oxidative intermediates implicate the participation of the mixed type I–type II mechanism, with the involvement of singlet oxygen being of greater importance, in the photo-inactivation of the enzymes.

In spite of some obvious limitations, chemical modification remains as one of the easiest and most direct approaches in studying the chemical basis of enzyme function. Dye-sensitized photo-oxidation, which is carried out under mild conditions, allows the modification of a restricted number and types of amino acid residues. Histidine was the only amino acid residue affected in the sensitized photo-oxidation of ribonuclease (Weil & Seibles, 1955), yeast enolase (Westhead, 1965), *Escherichia coli* tryptophanase (Nihira *et al.*, 1979) and spinach nitrate reductase (Vargas *et al.*, 1982). The photosensitized inactivation of lysozyme oxidized tryptophan residues (Kepka & Grossweiner, 1973). Disulphide and peptide bonds are not cleaved by this process (Spikes & MacKnight, 1970). Thus dye-sensitized photo-oxidation is potentially applicable to the investigation of the functional importance of these amino acid residues in enzymic catalyses. However, the interpretation of experimental results is hindered by the lack of knowledge concerning the primary events of sensitization and of a rationale for the choice of dyes with respect to their selectivity and effectiveness as photosensitizers.

The photo-inactivation of lipoamide dehydrogenase in the presence of Rose Bengal (Tsai *et al.*, 1982) displays some interesting features that prompted us to investigate, in greater detail, the photo-oxidation of several enzymes sensitized by various dyes.

Materials and methods

Materials

Alcohol dehydrogenase (NAD^+:alcohol oxidoreductase, EC 1.1.1.1) from horse liver, lipoamide dehydrogenase (NADH:lipoamide oxidoreductase, EC 1.6.4.3) from pig heart, NAD^+, NADH, pyridoxal 5′-phosphate, haematoporphyrin, DL-lipoamide and dry cells of *Micrococcus lysodeikticus* were supplied by Sigma Chemical Co. Lysozyme (*N*-acetylmuramide glycanohydrolase, EC 3.2.1.17) was obtained from Miles Laboratories. Acridine Orange, Erythrosin B, Eosin Y, fluorescein, Methylene Blue and Rose Bengal were provided by Fisher Scientific Co. Riboflavin was purchased from Eastman Kodak Co. Bio-Gel P6, Cellex CM and Cellex T were products of Bio-Rad Laboratories. Absolute ethanol came from Consolidated Alcohol. Toronto, Ont., Canada. Dihydrolipoamide was prepared from lipoamide by $NaBH_4$ reduction (Reed *et al.*, 1958).

Sensitized photo-oxidation

Enzyme solution (1.0 μM–15 mM in 0.20 M-potassium phosphate buffer) in the presence of a dye sensitizer (0–100 μM) was placed in a water-jacketed cell and illuminated from a distance of 25 cm with a 250 W tungsten lamp. Samples were withdrawn at time intervals for enzymic assays. Pseudo-first-order rate constants of inactivation ($k_{obs.}$) were evaluated from the slopes of plots of

$\log A_t$ versus t in accordance with $\log(A_0/A_t) = -k_{obs.}t$, where A_0 and A_t are initial activity and residual activity at time t respectively. To purify the photo-oxidized enzyme, cationic dyes were removed by treatment with Cellex CM exchanger and anionic dyes with Cellex T exchanger.

Enzyme assays and kinetic studies

Alcohol dehydrogenase and lipoamide dehydrogenase activities were assayed spectrophotometrically by a change in absorbance at 340nm of reaction mixtures containing 1.0mM-NAD^+ and 100mM-ethanol in 0.10M-sodium pyrophosphate buffer, pH9.0, or 50μM-NADH and 250μM-lipoamide in 50mM-sodium phosphate buffer, pH7.0, respectively. Kinetic studies of alcohol dehydrogenase (Tsai, 1978) and lipoamide dehydrogenase (Tsai, 1980) in the presence of dye sensitizers were carried out and analysed for inhibition constants (Cleland, 1963) for intercept effect (K_{ii}) as well as slope effect (K_{is}). Lysozyme activity was monitored by a decrease in turbidity at 540nm of *M. lysodeikticus* cell suspensions ($A_{540} = 0.50 \pm 0.005$) in 0.10M-sodium citrate buffer, pH5.0. All spectrophotometric measurements were made with a Beckman model 25 spectrophotometer.

Other methods

For amino acid analyses, salt-free freeze-dried enzymes (0.5–2.0mg) were hydrolysed in 1.0–1.5ml of 6.0M-HCl in Pierce Reacti-Therm vacuum hydrolysis tubes, which were heated in a heating block (Lab Line Instruments) at 100°C for 22–24h. The hydrolysates were evaporated to dryness under reduced pressure and taken up in a minimum volume of water. Amino acid analyses were performed on a Beckman 119BL analyser. The pD values of deuterated buffers were obtained by addition of 0.44 to pH-meter (Radiometer PHM612) readings (Mikkelson & Nielson, 1960).

Results and discussion

Heart lipoamide dehydrogenase is a dimeric flavoenzyme containing one FAD molecule per active site, where the redox-active disulphide and an essential histidine are located (Tsai *et al.*, 1982). Illumination of lipoamide dehydrogenase in the presence of Rose Bengal inactivates its reductase activity owing to destruction of the active-site histidine residue (Tsai *et al.*, 1982). Fig. 1 shows that a total of five histidine residues per subunit were photo-oxidized, resulting in 90% inactivation. Rates of the sensitized photo-inactivation increased hyperbolically with Rose Bengal concentrations (Fig. 2), suggesting an initial binding of the dye preceding the photo-inactivation. The reciprocal rate plot was linear (Fig. 2 inset) and gave an estimate of the association constant of 48.0mM. Similar results with Methylene Blue and Eosin Y gave the association constants of 22.0 and 23.0mM respectively.

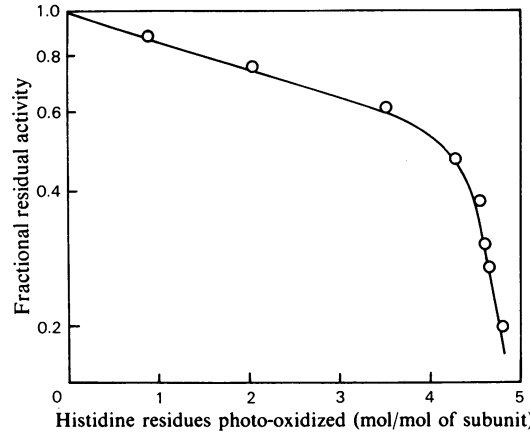

Fig. 1. *Oxidative destruction of histidine residues during Rose Bengal-sensitized photo-inactivation of lipoamide dehydrogenase*

Lipoamide dehydrogenase (0.50μM) was photo-oxidized in the presence of 50μM-Rose Bengal at pH6.5. Samples were withdrawn at time intervals for enzymic assays and amino acid analyses.

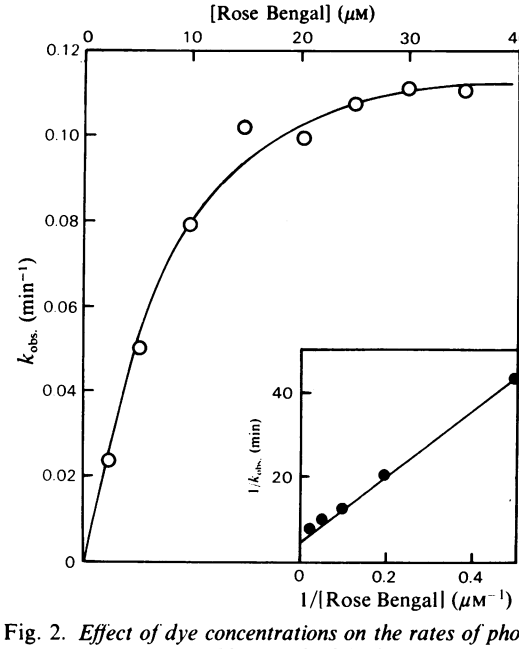

Fig. 2. *Effect of dye concentrations on the rates of photo-inactivation of lipoamide dehydrogenase*

Lipoamide dehydrogenase (0.50μM) was photo-oxidized in the presence of various concentrations of Rose Bengal at pH6.0.

The photo-inactivation of lipoamide dehydrogenase followed first-order kinetics for the given dye concentration. The pseudo-first-order rate constants ($k_{obs.}$) for the photo-inactivation sensitized by various dyes are given in Table 1. These organic dyes also act as inhibitors of enzymes by virtue of their ability to interact with protein molecules (Brand *et al.*, 1967; Steinhardt & Reynolds, 1969). Table 2 lists inhibition constants of representative dyes for lipoamide dehydrogenase. Rose Bengal inhibits the heart enzyme competitively with respect to lipoamide but non-competitively with respect to NAD^+ (Tsai *et al.*, 1982). All the other dyes studied are non-competitive inhibitors with respect to both substrates.

Fig. 3 illustrates three typical pH–rate profiles for sensitized photo-inactivation of lipoamide dehydrogenase. The rates of riboflavin sensitization are pH-independent. The sigmoidal pH–rate profile with increasing rates toward high pH characterizes the Rose Bengal effect, whereas Methylene Blue sensitization displays the opposite trend. The pH effects on the rates of sensitized photo-inactivation reflect presumably the charge character of sensitizers and enzyme residues that are involved in the binding.

Two histidine residues, His-51 and His-67, are strategically situated within the catalytic domain of liver alcohol dehydrogenase (Eklund *et al.*, 1976). Table 1 lists the pseudo-first-order rate constants for the photo-inactivation of alcohol dehydrogenase sensitized by various dyes that also act as inhibitors (Table 3). However, a lack of correlation between the sensitizing efficiency and the inhibition constants of sensitizers implicates that factor(s) other than the affinity between the sensitizers and the enzymes are of primary importance in the photo-inactivation. This is to be expected if the sensitized photo-oxidation proceeds via singlet oxygen (see below), which can diffuse and react with a distant target acceptor (Shnuriger & Bourdon, 1968; Lindig & Rodgers, 1981). An earlier observation for the sensitized photo-oxidation of amino acids reported that histidine was converted into aspartate (Tomita *et al.*, 1969). This is confirmed by the present study on alcohol dehydrogenase, though the conversion was not quantitative (Table 3).

Egg-white lysozyme contains a unique histidine residue, His-15 (Canfield, 1963), and the active site of lysozyme lies a cleft (Blake *et al.*, 1967) where three catalytically essential tryptophan residues,

Table 1. *Rates of photo-inactivation of enzymes sensitized by various dyes*

Lipoamide dehydrogenase ($0.90 \pm 0.10\,\mu$M), alcohol dehydrogenase (3.7 ± 0.3 mM) and lysozyme (15 ± 2 mM) were photo-oxidized in the presence of $50 \pm 5\,\mu$M dye at pH 6.0, 7.0 or 8.1, as indicated. The pseudo-first-order rate constants ($k_{obs.}$) of photo-inactivation are the average values ($\pm 50\%$) for three separate determinations.

	$10^3 \times k_{obs.}$ (min^{-1})		
Sensitizing dye	Lipoamide dehydrogenase	Alcohol dehydrogenase	Lysozyme
Thiazine: Methylene Blue (pH 7.0)	65.6	45.2	25.2
Porphyrin: Haematoporphyrin (pH 8.1)	50.8	46.0	21.3
Xanthine: Rose Bengal (pH 6.0)	62.1	45.9	26.8
Erythrosin B (pH 6.0)	54.6	42.9	16.5
Eosin Y (pH 7.0)	33.9	34.7	13.5
Fluorescein (pH 7.0)	1.56	2.74	1.52
Acridine: Acridine Orange (pH 7.0)	0.98	1.68	1.21
Alloxazine: riboflavin (pH 7.0)	1.43	0.86	1.26
Pyridoxine: pyridoxal 5′-phosphate (pH 7.0)	0.70	1.29	1.48

Table 2. *Inhibition constants of dyes for lipoamide dehydrogenase*

The lipoamide dehydrogenase (20 nM)-catalysed reaction was carried out at [NADH] = $25\,\mu$M with varied concentrations of lipoamide or at [lipoamide] = $250\,\mu$M with varied concentrations of NADH in the presence of 0, 100, 200, 300, 400 and $600\,\mu$M of dye. Data are the averages ($\pm 20\%$) for duplicate determinations.

		Inhibition constants of dyes (μM)		
Varied substrate		Haematoporphyrin	Eosin Y	Acridine Orange
NADH	K_{ii}	412	57.4	241
	K_{is}	1587	254	1090
Lipoamide	K_{ii}	1053	121	453
	K_{is}	549	29.3	1772

Trp-62, Trp-63 and Trp-108, are located (Hartdegen & Rupley, 1967; Shechter *et al.*, 1972; Imoto *et al.*, 1974). Both histidine and tryptophan residues were photo-oxidized in the presence of sensitizers. However, the destruction of tryptophan residues is considered to be responsible for the photo-inactivation (Asquith & Rivett, 1971; Kepka & Grossweiner, 1973). The pH-independence of the rate of photo-inactivation by all the sensitizers examined (results not shown) requires that a non-titratable group be involved and is consistent with the assignment of tryptophan as the relevant residue.

All dye sensitizers tested, depending on their sensitizing efficiency, fall into two groups. In three enzymes studied, rates of photo-inactivation are at least an order of magnitude faster with efficient sensitizers (Methylene Blue, haematoporphyrin, Rose Bengal, Erythrosin B and Eosin Y) than with inefficient ones (fluorescein, Acridine Orange, riboflavin and pyridoxal 5′-phosphate), Similar observations were made for various chemical (Kearns *et al.*, 1967; Gardin *et al.*, 1983) and biological (Ito, 1978; Houba-Herin *et al.*, 1982) systems. No apparent correlation exists between the sensitizing efficiency and dye structures. Although the physicochemical parameters characterizing the sensitizing efficiency are yet to be defined, halogenated xanthines and thiazine with low triplet-state energies are shown to be effective photosensitizers.

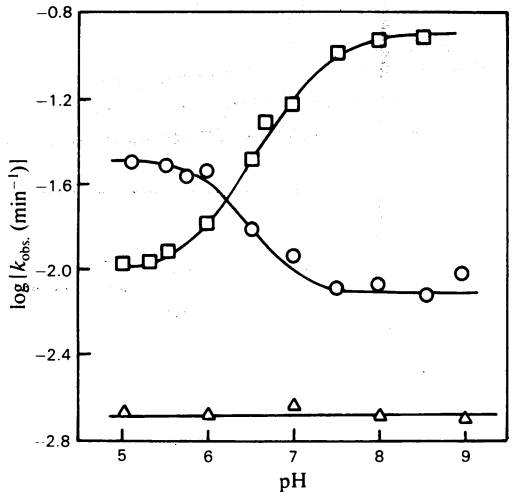

Fig. 3. *pH–rate profiles for the inactivation of lipoamide dehydrogenase*

Lipoamide dehydrogenase (0.50–0.90 μM) was photo-oxidized in the presence of 50–60 μM-Rose Bengal (○), -Methylene Blue (□) or -riboflavin (△) at various pH values.

Two mechanisms have been proposed to explain the dye-sensitized photo-oxidation (Foote, 1968; Grossweiner, 1969; Kramer & Maute, 1972). In the type I mechanism, the acceptor reacts initially with excited triplet sensitizer, and thereafter with oxygen via a radical intermediate. In the type II mechanism, energy is transferred from the excited-triplet sensitizer to molecular oxygen to form singlet oxygen ($^1O_2$), which, in turn, reacts with the acceptor. The operation of the type II mechanism is evidenced by the participation of $^1O_2$. Of the two singlet oxygens, $^1\Delta_g$ and $^1\Sigma_g^+$ (Kasha & Brabham, 1979), only $^1\Delta_g$ has a long enough half-life to be effective in aqueous systems (Lindig & Rodgers, 1979; Rodgers & Snowden, 1982; Ogilby & Foote, 1983). Therefore the participation of the $^1\Delta_g$ singlet oxygen provides a reliable avenue for differentiating between the two photo-oxidation mechanisms (Ito, 1978).

Useful diagnostic tests for singlet oxygen are based on the large deuterium solvent effect on the half-life of $^1O_2$ (Merkel *et al.*, 1972) and the specific quenching effect of N_3^- on $^1O_2$ (Hasty *et al.*, 1972). The half-life of $^1O_2$ is 53–68 μs in ^2H_2O (Lindig & Rodgers, 1979; Ogilby & Foote, 1983) as compared with the half-life of approx. 4 μs in H_2O (Rodgers & Snowden, 1982). Thus the reaction rate, neglecting solvent isotope effects for photo-oxidation, should be increased by a factor of 15-fold in going from H_2O to ^2H_2O. The rate constant for quenching of $^1O_2$ by N_3^- is 2.2×10^8 M^{-1}·s^{-1} (Hasty *et al.*, 1972), so that the half-life of $^1O_2$ in 10 mM-N_3^- solution would be approx. 0.5 μs,

Table 3. *Inhibition of alcohol dehydrogenase by dyes and changes in amino acid residues of photo-inactivated enzymes*

Liver alcohol dehydrogenase is a dimer containing 14 aspartate, 7 histidine and 4 tyrosine residues per subunit. Data are the averages (±20%) for duplicate experiments.

	Inhibition constants (μM)				Amino acid residues affected per subunit	
	Versus NAD$^+$		Versus ethanol			
Dye	K_{ii}	K_{is}	K_{ii}	K_{is}	Decrease	Increase
Methylene Blue	21	13	20	60	7 His, 1 Tyr	4 Asp
Rose Bengal	–	46	44	81	7 His, 1 Tyr	4 Asp
Acridine Orange	36	42	62	51	1 His, 1 Tyr	1 Asp

Table 4. *Effect of scavengers on dye-sensitized photo-oxidation of enzymes*

Dye (25–50 μM)-sensitized photo-oxidations of lipoamide dehydrogenase, alcohol dehydrogenase and lysozyme were carried as indicated in Table 1 legend in the presence of scavenger [N_3^- or $Fe(CN)_6^{3-}$].

Enzyme	Sensitizing dye	pH	Scavenger ...	$10^3 \times k_{obs.}$ (min^{-1}) None	N_3^- (10 mM)	$Fe(CN)_6^{3-}$ (0.50 mM)
Lipoamide dehydrogenase	Methylene Blue	7.0		57.1	7.81	20.3
	Haematoporphyrin	8.1		40.6	4.5	5.4
	Eosin Y	7.0		25.1	2.6	6.3
	Acridine Orange	7.0		1.07	0.28	0.31
Alcohol dehydrogenase	Erythrosin B	6.0		40.4	13.0	20.9
Lysozyme	Eosin Y	7.0		16.5	2.17	7.38

Table 5. *Deuterium effect of dye-sensitized photo-oxidiation of enzymes*

Dye-sensitized photo-oxidation of enzymes were carried out as indicated in Table 4 legend in a proton (H_2O) buffer and a deuterium (^2H_2O) buffer.

Enzyme	Sensitizing dye	pH/pD	$10^3 \times k_{obs.}$ (min^{-1}) H_2O	^2H_2O (95%)
Lipoamide dehydrogenase	Methylene Blue	7.0	57.1	151
	Rose Bengal	6.0	45.8	236
	Fluorescein	7.0	2.03	10.4
Alcohol dehydrogenase	Erythrosin B	6.0	40.4	127
	Acridine Orange	7.0	2.50	12.0
Lysozyme	Eosin Y	7.0	16.5	55.6

corresponding to a decrease in the rate of inactivation by a factor of 8-fold. The suppression factor of 4–9-fold in the presence of 10 mM-N_3^- for three enzymic systems investigated (Table 4) is within the range expected for the $^1O_2$ participation. However, the deuterium enhancement of 3–5-fold (Table 5) suggests that the type II is not the only mechanism involved in the sensitized photo-inactivation of enzymes. The type I mechanism is implicated by the quenching effect of the electron acceptor $Fe(CN)_6^{3-}$ (Dewey & Stein, 1970; Rossi *et al.*, 1981). The mixed type I–type II mechanism that has been observed in a number of chemical systems (Kramer & Maute, 1973; Sconfienza *et al.*, 1981; Grossweiner *et al.*, 1982) is implicated in the present study for the dye-sensitized photo-inactivation of enzymes.

Among the commonly used sensitizing dyes, halogenated xanthines and thiazine with low triplet-state energies are the most effective for the photo-inactivation of enzymes. Histidine residues are the primary target of oxidation via the mixed type I–type II mechanism, with the involvement of singlet oxygen (type II) being of greater importance.

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References

Asquith, R. S. & Rivett, D. E. (1971) *Biochim. Biophys. Acta* **252**, 111–116

Blake, C. C. F., Johnson, L. N., Mair, G. A., North, A. C. T., Phillips, D. C. & Sarma, V. R. (1967) *Proc. R. Soc. London Ser. B* **167**, 378–388

Brand, L., Gohlke, J. R. & Rao, D. S. (1967) *Biochemistry* **6**, 3510–3518

Canfield, R. E. (1963) *J. Biol. Chem.* **238**, 2698–2707

Cleland, W. W. (1963) *Biochim. Biophys. Acta* **67**, 173–187

Dewey, D. L. & Stein, G. (1970) *Radiat. Res.* **44**, 345–358

Eklund, H., Nordström, B., Zeppezauer, E., Soderlund, G., Ohlsson, I., Boiwe, T., Soderberg, B. O., Tapia, O., Branden, C.-I. & Akeson, A. (1976) *J. Mol. Biol.* **102**, 27–59

Foote, C. S. (1968) *Acc. Chem. Res.* **1**, 104–110

Gandin, E., Lion, Y. & Van de Vorst, A. (1983) *Photochem. Photobiol.* **37**, 271–278

Grossweiner, L. I. (1969) *Photochem. Photobiol.* **10**, 183–191

Grossweiner, L. I., Patel, A. S. & Grossweiner, J. B. (1982) *Photochem. Photobiol.* **36**, 159–167

Hartdegen, F. J. & Rupley, J. A. (1967) *J. Am. Chem. Soc.* **89**, 1743–1745
Hasty, N., Merkel, P. B., Radlick, P. & Kearns, D. R. (1972) *Tetrahedron Lett.* **1**, 49–52
Houba-Herin, N., Calberg-Bacq, C. M., Piette, J. & Van de Vorst, A. (1982) *Photochem. Photobiol.* **36**, 297–306
Imoto, T., Fujimoto, M. & Yagishita, K. (1974) *J. Biochem. (Tokyo)* **76**, 745–753
Ito, T. (1978) *Photochem. Photobiol.* **28**, 493–508
Kasha, M. & Brabham, D. E. (1979) in *Singlet Oxygen* (Wasserman, H. H. & Murray, R. W., eds.), pp. 1–33, Academic Press, New York
Kearns, D. R., Hollins, R. A., Khan, A. U., Chambers, R. W. & Radlick, P. (1967) *J. Am. Chem. Soc.* **89**, 5455–5456
Kepka, A. G. & Grossweiner, L. I. (1973) *Photochem. Photobiol.* **18**, 49–61
Kramer, H. E. A. & Maute, A. (1972) *Photochem. Photobiol.* **15**, 7–23
Kramer, H. E. A. & Maute, A. (1973) *Photochem. Photobiol.* **17**, 413–423
Lindig, B. A. & Rodgers, M. A. J. (1979) *J. Phys. Chem.* **83**, 1683–1688
Lindig, B. A. & Rodgers, M. A. J. (1981) *Photochem. Photobiol.* **33**, 627–634
Merkel, P. B., Nilsson, R. & Kearns, D. R. (1972) *J. Am. Chem. Soc.* **94**, 1030–1031
Mikkelson, K. & Nielson, S. O. (1960) *J. Phys. Chem.* **64**, 632–637
Nihira, T., Torays, T. & Fukui, S. (1979) *Eur. J. Biochem.* **101**, 341–347
Ogilby, P. R. & Foote, C. S. (1983) *J. Am. Chem. Soc.* **105**, 3423–3430
Reed, L. J., Koike, M., Levitch, M. E. & Leach, F. R. (1958) *J. Biol. Chem.* **232**, 143–158
Rodgers, M. A. J. & Snowden, P. T. (1982) *J. Am. Chem. Soc.* **104**, 5541–5543
Rossi, E., Van de Vorst, A. & Jori, G. (1981) *Photochem. Photobiol.* **34**, 447–454
Sconfienza, C., Van de Vorst, A. & Jori, G. (1981) *Photochem. Photobiol.* **31**, 351–357
Shechter, Y., Burstein, Y. & Patchornik, A. (1972) *Biochemistry* **11**, 653–660
Shnuringer, B. & Bourdon, J. (1968) *Photochem. Photobiol.* **8**, 361–368
Spikes, J. D. & MacKnight, M. L. (1970) *Ann. N.Y. Acad. Sci.* **171**, 149–162
Steinhardt, J. & Reynolds, J. A. (1969) *Multiple Equilibria in Proteins*, pp. 302–307, Academic Press, New York
Tomita, M., Irie, M. & Ukita, T. (1969) *Biochemistry* **8**, 5149–5160
Tsai, C. S. (1978) *Biochem. J.* **173**, 483–496
Tsai, C. S. (1980) *Int. J. Biochem.* **11**, 407–413
Tsai, C. S., Wand, A. J., Godin, J. R. P. & Buchanan, G. W. (1982) *Arch. Biochem. Biophys.* **217**, 721–729
Vargas, M. A., Maurino, S. G., Maldonado, J. M. & Aparicio, P. J. (1982) *Photochem. Photobiol.* **36**, 223–228
Weil, L. & Seibles, T. S. (1955) *Arch. Biochem. Biophys.* **54**, 368–377
Westhead, E. W. (1965) *Biochemistry* **4**, 2139–2144