

Oxidation of 2-Thioflavins by Peroxides

FORMATION OF FLAVIN 2-S-OXIDES*

(Received for publication, November 15, 1982)

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The reaction of 2-thioriboflavin (sulfur replacing the oxygen substituent at position 2 α) with hydrogen peroxide at pH ~10 leads to a blue flavin ($\lambda_{\max} = 565$ nm) which was purified in stable, homogeneous form. Titrations of 2-thioflavins with *m*-chloroperoxybenzoic acid also yield the same blue flavins with consumption of 1 eq of peracid. Anaerobic reduction of the blue flavin by sodium dithionite requires 4e⁻ eq, and leads to formation of 1,5-dihydro-2-thioflavin. Oxidation of the latter with O₂ restores the original 2-thioflavin. pH titration of the blue flavin shows two pK_a values of 2.4 and 6.6, with no apparent ionization in the pH range 8-11. These results suggest that the blue flavin is a flavin 2-S-oxide. The visible absorption spectra of flavin 2-S-oxides show a pronounced dependence on solvent polarity. This property suggests that these flavin analogs may be useful hydrophilic/hydrophobic probes of flavoprotein active sites. Flavin 2-S-oxides can be oxidized further to the 2-sulfinate and 2-sulfonate analogs, some properties of which are described.

2-Thio-FAD, an analog of FAD which carries a sulfur atom instead of oxygen at the isoalloxazine position 2 α , has been used successfully in the reconstitution of a series of artificial flavin enzymes (1). It has proven useful in studies of the accessibility of the coenzyme at the active center (1, 2), and, in general, it has turned out to be a versatile tool for mechanistic and labeling studies. In this respect, it has been shown (3) that 2-thio-FAD-reconstituted *p*-hydroxybenzoate hydroxylase, an orange-reddish enzyme, can be oxidized by H₂O₂ to a blue species ($\lambda_{\max} = 620$ nm). This product decays slowly in a peroxide-dependent reaction to yield a different chromophore, which is remarkable in that it is now covalently linked to the apoprotein (3).

In earlier studies, Dudley *et al.* and Müller *et al.* (4, 5) reported the formation of a transient blue species during the reaction of 2-thiolumiflavin with excess peroxide in methanolic solution. This reaction was described to yield flavins functionally substituted at the 2-position as final products. The original assumption that the blue species is a radical has, however, turned out to be erroneous, as the species is diamagnetic (3). This species is, on the other hand, a probable key intermediate in the reaction leading to formation of the covalent flavin-protein linkage in the enzyme *p*-hydroxyben-

zoate hydroxylase. Thus, this class of modified flavin coenzymes appears to possess considerable potential as a regio-specific labeling agent for flavoproteins and as a spectroscopic probe for catalytic centers. These considerations prompted efforts aimed at the elucidation of the structure of the blue chromophore, at an understanding of its properties, and at studies of its mechanism of formation and reaction. It will be shown that the structure of the blue compound is that of a flavin 2-S-oxide; for ease of description, the term flavin 2-S-oxide will be used interchangeably with that of the blue intermediate.

MATERIALS AND METHODS

2-Thioriboflavin, 2-thiolumiflavin, and *N*-3-methyl-2-thiolumiflavin were synthesized by the method of Müller and Hemmerich (6); all other flavins were obtained according to the references given in Table I. MCPB¹ was from EGA Chemie or from Aldrich, and was standardized by iodometric titration before use (7). Benzene (AR grade) was from Mallinckrodt. Acetonitrile, spectral grade, from J. T. Baker was either used as purchased or was purified by refluxing over phosphorus pentoxide, and subsequent distillation over CaH₂ (8). Dimethylformamide (from Merck) was purified by fractional distillation, and was stored over molecular sieve beads. Chloroform, methanol, ethyl acetate, and acetic acid, reagent grade, were from Merck. Silica Gel 60 for column chromatography and precoated silica gel thin layer plates were from Merck. Quaternary (QAE)-Sephadex A-25 was from Pharmacia, and bovine catalase was from Calbiochem or Böhlinger.

Anaerobic dithionite titrations were performed in an all glass apparatus similar to that described elsewhere (9). Solutions of sodium dithionite (Baker or Merck) were standardized against lumiflavin-3-acetate (9). All anaerobic samples were prepared by alternate evacuation and flushing with oxygen-free nitrogen.

Reactions of the flavin 2-S-oxides with substoichiometric amounts of MCPB were slow, thus requiring kinetic monitoring at the appropriate wavelength as each MCPB addition was made before recording of the final spectra (~10-15 min).

The following proteins and apoproteins were prepared as previously described: flavodoxin from *Megasphaera elsdenii* (10, 11), Old Yellow enzyme from brewers bottom yeast (12, 13), riboflavin-binding protein from hen egg white (14, 15), D-amino acid oxidase from hog kidney (16, 17), and *p*-hydroxybenzoate hydroxylase from *Pseudomonas fluorescens* (18, 19). Apoglucose oxidase (*Aspergillus niger*) was a gift from Miles Laboratories, Elkhart, IN.

Riboflavin 2-S-Oxide Preparation—50 ml of 2-thioriboflavin (~20 μ M) in 10 mM bicarbonate, pH 9.8 at 25 °C, were mixed with 6.2 mM H₂O₂, and the reaction was monitored spectrophotometrically at 620 nm. At 6-7 min after peroxide addition, when the maximal A₆₂₀ was reached, catalase was added to a final concentration ~10 nM; the reaction mixture was cooled in ice and then applied to a column (16 \times 2 cm) of QAE-Sephadex A-25, equilibrated with 10 mM bicarbonate, pH 9.9 at 4 °C, and run in the dark. A concentrated blue band adsorbed to the column, while contaminating fluorescent yellow riboflavin passed through during elution with 250 ml of the starting buffer.

* This work was supported in part by Grant GM 11106 from the United States Public Health Service. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ Recipient of Grant Gh 2 4/4 from the Deutsche Forschungsgemeinschaft.

|| Died October 3, 1981.

¹ The abbreviations used are: MCPB, *m*-chloroperoxybenzoic acid; tlc, thin layer chromatography; HPLC, high pressure liquid chromatography.

TABLE I
R_F values of 2- and 8-substituted flavins on thin layer chromatography

Plates were precoated silica gel plates from Merck. Literature references are given in parentheses.

	System		
	A	B	C
Lumiflavin	0.44	0.75	0.52
Riboflavin	0.49	0.55	0.48
<i>N</i> -3-Me-lumiflavin	0.56	0.71	0.56
2-Thiolumiflavin	0.68	0.87	0.73
2-Thioriboflavin	0.69	0.72	0.60
<i>N</i> -3-Me-2-thiolumiflavin	0.79	0.80	0.75
Lumiflavin 2-S-oxide	0.37	0.22	0.40
Riboflavin 2-S-oxide		0.25	0.28
<i>N</i> -3-Me-lumiflavin 2-S-oxide	0.35	0.39	0.28
2-Morpholino-2-deoxyriboflavin (6)			0.42
2-Methoxylumiflavin (5)	0.50	0.81	.053
2-S-Methylriboflavin (6)	0.36	0.53	0.44
Lumiflavin 2-SS-2-dimer	0.15	0.41	0.17
Lumiflavin 8-SS-8-dimer (20)	0.00	0.00	0.00
8-Mercaptolumiflavin (20)	0.32		

^a Systems: A, butanol/ethyl acetate/water (4:3:1); B, methanol/chloroform/ethyl acetate (4:3:3); C, butanol/methanol (1:1) + 1% water.

A linear NaCl gradient (0–0.2 M NaCl; 300-ml total) in the same bicarbonate buffer was then applied, and fractions of the riboflavin 2-S-oxide were collected, eluting in the range of 0.09–0.12 M NaCl. This preparation of the nonfluorescent riboflavin 2-S-oxide could be stored in the dark at 4 °C for up to 2 weeks without detectable decay to riboflavin.

The 2-S-oxide of FAD was prepared by titration of a solution of ~30 μM 2-thio-FAD in ~10 ml of 10 mM bicarbonate buffer, pH 10, at 4 °C in the dark with MCPB (60 mM in methanol) to maximal *A*₅₈₀. This material was then loaded on a column (13 × 0.9 cm) of QAE-Sephadex A-25 equilibrated at 4 °C with 10 mM bicarbonate buffer. Both the blue 2-S-oxide and the fluorescent contaminating FAD stuck to the column. The FAD contaminant was eluted in 10 mM bicarbonate, pH 10, containing 0.1 M NaCl. The remaining 2-S-oxide was then eluted in the same buffer containing 0.2 M NaCl. Spectral analysis allowed the pooling of homogeneous FAD-2-S-oxide fractions, which were stored in the dark at 4 °C. The FMN-2-S-oxide was prepared by treatment at neutral pH and 25 °C of the FAD derivative with a few grains of *Naja naja* venom (phosphodiesterase) from Sigma.

Preparation and Purification of *N*-3-Methylumiflavin 2-S-Oxide by HPLC—25 ml of a 5 × 10⁻⁵ M solution of pure *N*-3-methyl-2-thiolumiflavin in chloroform/ethyl acetate (1:1) was oxidized with 1 eq of MCPB (1.35 × 10⁻³ M in acetonitrile) until the absorption at 630 nm had reached a maximum. When the fast first decay phase of the *N*-3-methylumiflavin 2-S-oxide came to an end after 3 h, a total of 4 ml of the S-oxide solution was separated in several batches on a Silica Gel Si 100 Knauer (Bad Hamburg, W. Germany) HPLC column (250 × 16 mm) equilibrated with ethanol/chloroform/ethyl acetate (1:1:1). Elution was carried out with the same solvent mixture with a flow rate of 4.0 ml/min. Product elution was detected spectrophotometrically at 270 nm. MCPB, *N*-3-methyl-2-thiolumiflavin, *N*-3-methylumiflavin, and *N*-3-methylumiflavin 2-S-oxide eluted within 20 min in that order. After adding 30 ml of water to the samples of the S-oxide (~60 ml), the organic solvent was flash evaporated at room temperature and the resulting blue aqueous solution of the S-oxide was found to be stable without any detectable decay (in the dark at 4 °C) for at least 48 h. This solution was freeze dried and the residue consisted of virtually pure (by tlc and absorption spectrum) *N*-3-methylumiflavin 2-S-oxide in 86% yield.

RESULTS

The 2-electron Oxidation of 2-Thioflavins—In contrast to the reaction observed with 2-thio-FAD-reconstituted *p*-hydroxybenzoate hydroxylase (3), when 2-thioflavins (*i.e.* 2-thio derivatives of FAD, FMN, riboflavin, lumiflavin, 3-methylumiflavin, etc.) are reacted in aqueous solution with peroxide under similar conditions (pH 7, 25 °C), direct formation of normal flavin (*i.e.* with a carbonyl function at position 2) with

very little appearance of a blue intermediate is observed. Reaction with stoichiometric amounts of MCPB increases the yield of the blue product up to 40% but does not lead to its stoichiometric formation. At high pH values, however, 2-thioflavins are converted essentially quantitatively, and isospectically, and with the consumption of 1 eq of *m*-chloroperbenzoic acid to this new blue species which is characterized by an absorption maximum at 565 nm. Fig. 1 shows the results with 2-thiolumiflavin. This is the first evidence that the blue flavin is a 2-S-oxide. The further reaction with ~1 additional eq of MCPB leads to quantitative conversion to lumiflavin, as evidenced by the isosbestic course of the reaction (Fig. 1), and by tlc analysis of the product (Table I). Qualitatively and quantitatively, the same reaction course is observed on oxidation of 2-thioriboflavin. The final product, riboflavin, was identified by its absorption and fluorescence spectra, tlc, and its stoichiometry of binding to riboflavin-binding protein (3). 2-Thio-FMN and 2-thio-FAD behave similarly. The difference in reactivity at the pH values 7 and 10 is noteworthy. At pH 7, the rate of oxidation of 2-thioflavins by peroxide is rapid, and the yield of the blue species is maximally 40%, due to competing further oxidation. In the region of, and above, pH 10 (the p*K* of *N*-3-H dissociation of 2-thioflavins), reactivity with peroxides and in particular the further oxidation of the S-oxides is considerably slower, and essentially 100% yield of S-oxide is observed (Fig. 1, *inset*). Further, the conversion of the blue species to the final products requires close to 1 eq of MCPB at pH 10, while the stoichiometry at pH 7 varied between 1.5 and 2 in several experiments.

Reduction of the Blue Flavin S-Oxides—Anaerobic illumination of the blue flavin in the presence of EDTA leads to complete disappearance of the absorption at 560 nm, and to formation of a chromophore closely similar to that of 1,5-dihydro-2-thioflavin (*cf.* Fig. 2, *curve* 5). Addition of oxygen at this stage restores approximately 70% of the spectrum of the parent 2-thioflavin (not shown). This result demonstrates that the oxidation to the blue compound is substantially reversible by reduction. A quantitative study of these reduc-

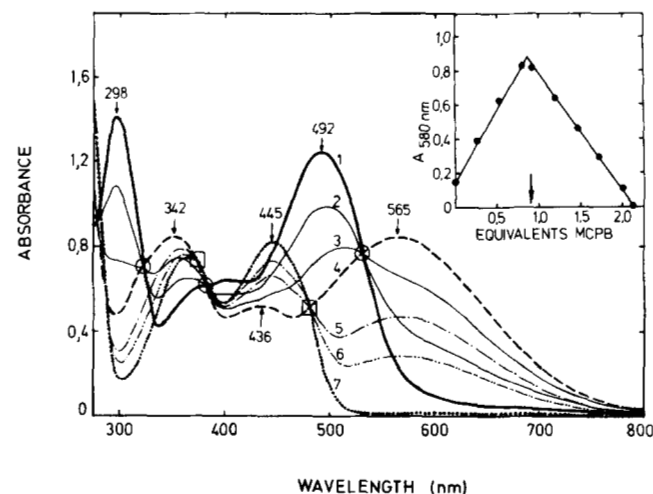


FIG. 1. Spectral course of the oxidation of 2-thioriboflavin. Sequential formation of riboflavin 2-sulfoxide and of riboflavin. 2-Thioriboflavin, 6.5 × 10⁻⁵ M in 0.01 M carbonate buffer, pH 9.9 (*curve* 1, —), was titrated with aliquots of MCPB (6.35 mM in acetonitrile). *Curve* 4 (---) was obtained after addition of 0.9 eq of MCPB, and represents the spectrum of the riboflavin 2-sulfoxide. Further titration leads to formation of riboflavin, which is complete after ~2.1 eq. *Curves* 2, 3, 5, and 6 represent selected intermediate spectra which were obtained after addition of 0.27, 0.53, 1.47, and 1.73 eq of MCPB. → ○ symbols indicate the isosbestic points obtained during the formation of the sulfoxide, and → □ are those observed during its conversion to riboflavin.

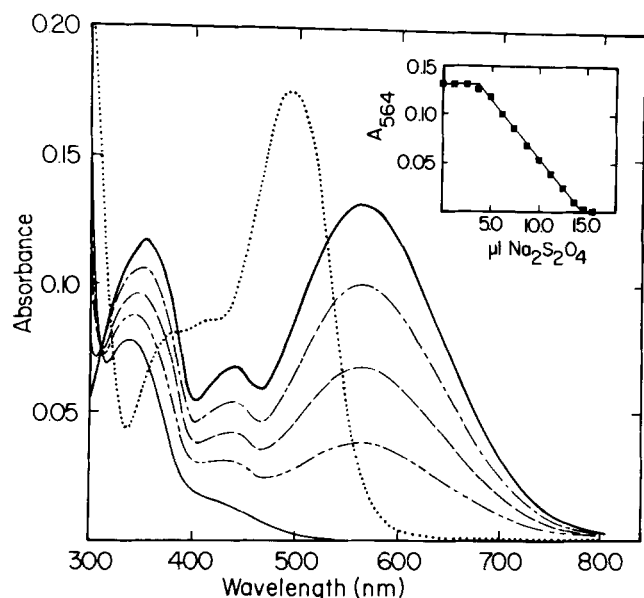


FIG. 2. Dithionite titration of the blue flavin. Purified blue flavin (—; $9.3 \mu\text{M}$ in 10 mM NaHCO_3 , pH 10.5, plus 0.1 M NaCl) was titrated with a 2.0 mM solution of standardized $\text{Na}_2\text{S}_2\text{O}_4$ at 4°C . Intermediate spectra were taken after addition of 0.55 (· · · ·), 1.09 (— — —), 1.62 (· · · · ·), and 2.5 (—) molar eq of dithionite, respectively. The inset shows the change in absorbance at 564 nm as a function of dithionite addition. The spectrum of the flavin appearing on readmission of air (· · · · ·) is also shown.

tion/oxidation processes was done by titration with dithionite, as shown in Fig. 2. The spectra shown are isosbestic throughout the titration at 311 and 260 nm . Even after thorough scrubbing of contaminating oxygen (the lag phase in the titration; inset to Fig. 2), it was observed that rapid flavin reduction (decrease in A_{560}) was followed by a gradual equilibration process (increase in A_{560}), which was complete after $\sim 5 \text{ min}$. This internal equilibration process was seen with every dithionite addition up to full flavin reduction.

The spectrum of the reduced flavin (Fig. 2, curve 5; $\lambda_{\text{max}} = 338, 288 \text{ nm}$) is very similar to that of the reduced anionic form of 2-thioflavin. Readmission of air to the reduced flavin results in the appearance of oxidized 2-thioriboflavin (Fig. 2, curve 6). From the known (1) extinction coefficient of 2-thioflavins at pH 10.5 ($\epsilon_{491} = 19,000 \text{ M}^{-1} \text{ cm}^{-1}$), an extinction coefficient of $14,300 \text{ M}^{-1} \text{ cm}^{-1}$ can be determined for the blue flavin at 560 nm under these conditions. This value is in reasonable agreement with that determined from titration of the blue compound with aporiboflavin-binding protein ($\epsilon_{560} = 13,300 \text{ M}^{-1} \text{ cm}^{-1}$, results not shown).

The dithionite titration data of Fig. 2 show that 4 electrons/mol blue flavin were required for its full reduction, suggesting a 2-electron reduction of the flavin isoalloxazine ring system and a 2-electron reduction of the oxidized sulfur substituent. This result conclusively demonstrates that the sulfur substituent at position 2 in the initial 2-thioflavin sample is not lost in the formation of the blue flavin, and that the latter has the structure of a 2-S-oxide.

Purification of Flavin 2-S-Oxides—Due to the relative instability encountered with this class of compounds, a few comments appear appropriate in the context of the purification procedures outlined in the experimental section. As described below, the rate of decay of the flavin 2-S-oxides is highly dependent on light and on the concentration of the S-oxide. Therefore, most purification procedures must be carried out in the dark using relatively dilute samples. In particular, eluates from chromatographic separation procedures cannot be concentrated by flash evaporation without some break-

down to normal flavin. The only practical method which we have found is lyophilization and subsequent storage as a dry powder in the dark.

Stability and Decay Modes of Flavin 2-S-Oxides—In our hands, the flavin 2-S-oxides have proved to be of sufficient stability to be readily used in studies where they are incorporated into apoproteins (see next section). However, as reported in the original literature describing the properties of 2-thioflavins and their derivatives (4–6), they are susceptible to decay to a variety of products, including loss of the sulfur substituent and reformation of native flavin. The mechanisms of these decay reactions are still unclear; some possibilities will be considered under “Discussion.” The most serious decay problem which is encountered is photochemical in nature. For example, exposure of an aerobic sample in chloroform/ethyl acetate (1:1) to light from a 250-watt projector (light intensity, $\sim 3 \times 10^6 \text{ ergs cm}^{-2} \text{ s}^{-1}$) results in total conversion of 3-methylumiflavin 2-S-oxide to 3-methylumiflavin in a few seconds. The same effect is given by normal room light in approximately 1 h. This photolability severely complicates the accurate determination of the dark decay kinetics. Nevertheless, it may be stated that the dark decay processes of the flavin 2-S-oxides are quite dependent on pH, the nature of the solvent, the concentration of S-oxide, and the presence of O_2 . The pH range of maximal stability is pH 8–10. For example, an aerobic $40 \mu\text{M}$ sample of riboflavin S-oxide at pH 8, 25°C , has a $t_{1/2}$ for decay of $\sim 100 \text{ h}$, while at pH 7 the $t_{1/2}$ is 70 h. At 50°C , pH 10, the $t_{1/2}$ for decay under aerobic conditions of $40 \mu\text{M}$ lumiflavin 2-S-oxide was 6 h, while a 4-fold lower concentration under the same conditions had a $t_{1/2}$ of 11 h. The product of aerobic decay was lumiflavin, *i.e.* a desulfuration and replacement with oxygen had occurred, as described in the original literature (4–6). Oxygen appears to play a role in this process, since under anaerobic conditions the decay is approximately four times slower. Moreover, the products of anaerobic decay are different from those obtained aerobically; while they have not been characterized, the anaerobic breakdown products appear to involve rupture of the flavin ring system.

The process of aerobic decay is also influenced by the nature of the solvent and the nature of the substituent at the flavin position N-3. In general, *N*-3-methylumiflavin 2-S-oxide is less stable than lumiflavin 2-S-oxide; in both cases, but particularly with the *N*-3-methyl derivative, the presence of even 5% H_2O has a pronounced stabilizing effect, increasing the half-life by approximately 1 order of magnitude. Depending on the starting concentration of the S-oxide, either complete oxidation to 3-methylumiflavin (at concentrations $\sim 10^{-5} \text{ M}$) or disproportionation to an almost equimolar mixture of 3-methyl-2-thiolumiflavin and 3-methylumiflavin (at concentrations $\sim 10^{-4} \text{ M}$) was found. The latter effect is illustrated in Fig. 3 for a starting concentration of $7.8 \times 10^{-5} \text{ M}$ 3-methylumiflavin 2-S-oxide in CHCl_3 /ethyl acetate (1:1). The disappearance of the S-oxide, monitored by its absorbance at 630 nm , is accompanied by the appearance of 3-methylumiflavin (450 nm) and 3-methyl-2-thiolumiflavin (510 and 318 nm). The identity of the products was confirmed by tlc. The inset shows the markedly biphasic time course of the reaction. This appears to be due to the presence of two distinct isomers, which might be envisaged as being *cis* or *trans* to the *N*-3-methyl substituent (*cf.* Scheme 2 below). Evidence for this hypothesis comes from experiments in which the S-oxide was isolated by HPLC (described under “Materials and Methods”) after the faster decay phase had taken place. Thus, when an aliquot of the solution was subjected to re-isolation by HPLC after 3 h of decay, the pure S-oxide obtained ($3.6 \times 10^{-5} \text{ M}$) now decayed monophasically, with a $t_{1/2} \sim 9 \text{ days}$.

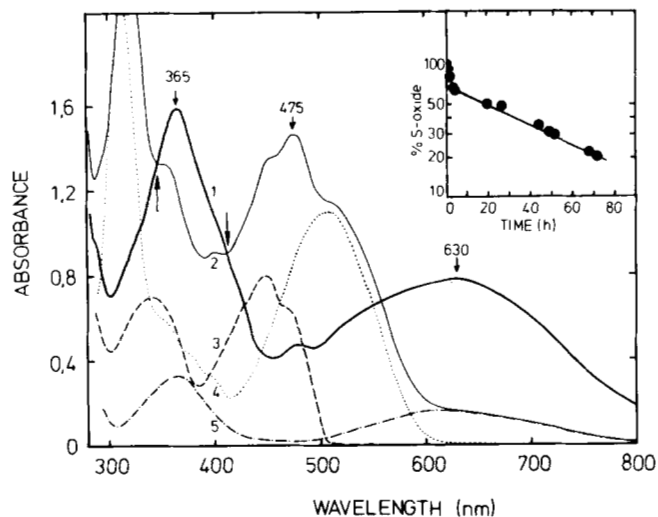


FIG. 3. Spectral course of the biphasic disproportionation of *N*-3-methyllumiflavin 2-S-oxide in chloroform/ethyl acetate (1:1). Curve 1 represents the spectrum of *N*-3-methyllumiflavin 2-S-oxide, 7.8×10^{-5} M in chloroform/ethyl acetate (1:1) with a small contamination of *N*-3-methyllumiflavin and *N*-3-methyl-2-thiolumiflavin; curve 2 shows the end spectrum of the disproportionation of the blue flavin after 73 h at room temperature in the dark. This spectrum represents a mixture of approximately 46% *N*-3-methylumiflavin, 43% *N*-3-methyl-2-thiolumiflavin, and 10% residual blue flavin; these spectra are shown by curves 3, 4, and 5, respectively (calculated curves). The arrows denote the isosbestic points observed during the disproportionation. The inset shows the biphasic decay of the 630-nm absorption of the *N*-3-methylumiflavin 2-S-oxide.

Further Oxidation of Flavin 2-S-Oxides—As to be expected for S-oxides, flavin 2-S-oxides can be oxidized further with peroxides. In aqueous solvents, normal “2-oxo” flavins are obtained directly upon addition of 2 or more eq of MCPB to 2-thioflavins, probably because of fast hydrolysis of any intermediate species. In aprotic solvents, however, addition of 1 eq of MCPB to lumiflavin 2-S-oxide leads to a new species, the spectrum of which (Fig. 4) is similar to that of normal flavin (cf. Fig. 1, curve 7). This species is, however, not identical with lumiflavin, as upon addition of small amounts (0.5%) of water a further minor, but significant, spectral change occurs. This goes along with a shift of the UV band of species B from 357 (Fig. 4) to 340 nm, to yield a spectrum which is virtually identical with that of lumiflavin under the same conditions. Lumiflavin is also the only product which can be identified by analytical methods (thin layer chromatography, HPLC). All attempts to isolate species B, Fig. 4, by the same methods failed, lumiflavin being the only product which could be detected. Addition of further amounts of MCPB to species B, Fig. 4, leads slowly to a complex spectrum, which in turn decays to that of lumiflavin.

However, when an excess of solid MCPB is added directly to the 2-S-oxide (Fig. 4, species A), the color changes from blue to yellow within seconds, and a species is formed which has the spectrum shown by curve C. In the absence of moisture, this species very slowly decays to the lumiflavin spectrum (not shown). Species C, as shown on Fig. 4, is probably contaminated by species B and/or lumiflavin, as denoted by the shoulders in the 350- and 470-nm regions. Again, all attempts to isolate this compound by HPLC or standard chromatographic methods failed, lumiflavin being the only compound which could be obtained. Addition of water to C immediately leads to formation of the spectrum of normal lumiflavin (not shown).

When a small excess of solid MCPB is added in a similar experiment to *N*-3-methyl-2-thiolumiflavin (or the corre-

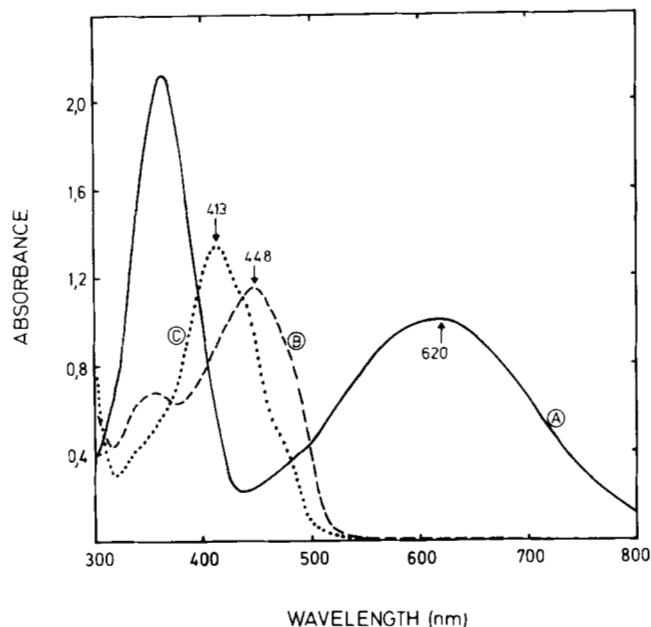


FIG. 4. Course of oxidation of lumiflavin 2-S-oxide with MCPB in acetonitrile. 2-Thiolumiflavin, 1.06×10^{-4} M in acetonitrile, was titrated with MCPB (9×10^{-3} M in the same solvent) to a maximal yield of lumiflavin 2-S-oxide (curve A). Upon addition of 1 further eq of MCPB, the absorption at 550 nm disappears almost completely within seconds, and spectrum B was obtained. The spectrum of the third species (C) was obtained immediately after addition of an excess of solid MCPB to the sample of spectrum B.

sponding 2-S-oxide), a species is formed that again is spectrally closely similar to, but not identical with, 3-methylumiflavin. It is converted to the latter either slowly upon standing, or rapidly upon addition of 1 volume % of water. Addition of a large excess of MCPB to 3-methyl-2-thiolumiflavin leads to a species the spectrum of which (not shown) is composed of the two main flavin transitions in the 350- and 450-nm regions, and also of a species absorbing around 420 nm. This mixture decomposes slowly to 3-methylumiflavin.

Spectral Properties of the Blue Flavin 2-S-Oxides—The two basic ionizations characteristic for the isoalloxazine system are retained in the blue flavin derivatives as demonstrated by the pH-dependent spectra shown in Fig. 5. From these reversible spectral changes, ionization constants of 6.6 and of 2.4 can be estimated by pH titration. They compare with the values of ~ 10 and 9.8 for the position N-3—H of normal FAD, and 2-thio-FAD (1, 4), and to the value of ~ 0 for the protonation of flavins and 2-thioflavin (4) in acidic medium. The significance of these differences will be considered under “Discussion.” The blue flavins also show pronounced solvatochromy (Fig. 6). This effect is reversible, and is observed on dilution of aliquots of blue flavin in acetonitrile into solvents of various polarity, as well as by direct oxidation of 2-thioflavins with MCPB in these solvents.

Reconstitution of Artificial Flavoproteins from Apoflavoproteins and Flavin 2-S-oxides—The large spectral changes shown in Fig. 6 as a function of solvent polarity suggest that blue flavins might be sensitive probes for the flavin-binding site, and in particular for the apoprotein environment near the pyrimidine moiety of the bound flavin.

Fig. 7 shows the absorption spectra of the blue FAD derivative free and reconstituted with apo-*p*-hydroxybenzoate hydroxylase and apo-*D*-amino acid oxidase, respectively. The dramatic shifts observed are primarily in the λ_{\max} positions for the long wavelength band and are similar to the effects of solvent polarity upon the free blue flavin spectra.

As described in the previous paper (3), the blue 620-nm

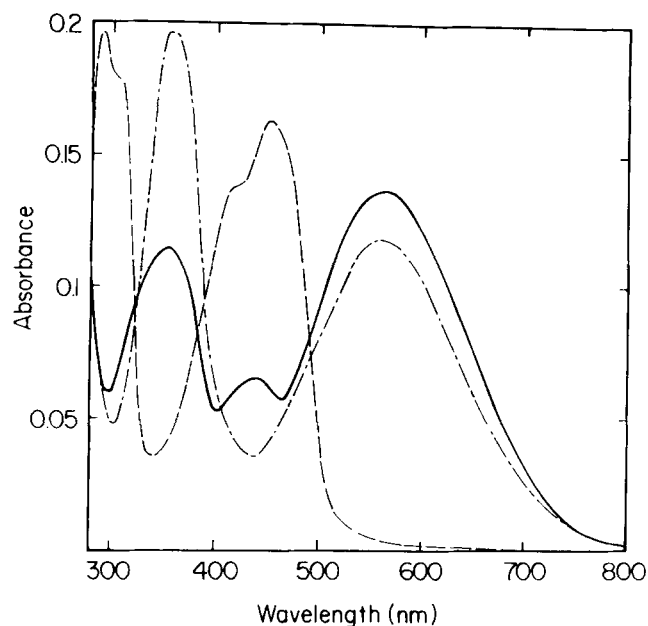


FIG. 5. pH-dependent spectral properties of riboflavin 2-S-oxide. Purified blue flavin (—; 9.6 μM , in 10 mM NaHCO_3 , pH 10.8, plus 0.1 M NaCl) was titrated to pH 5.6 (---) and pH 0.8 (- - -) with H_2SO_4 at 4 $^\circ\text{C}$.

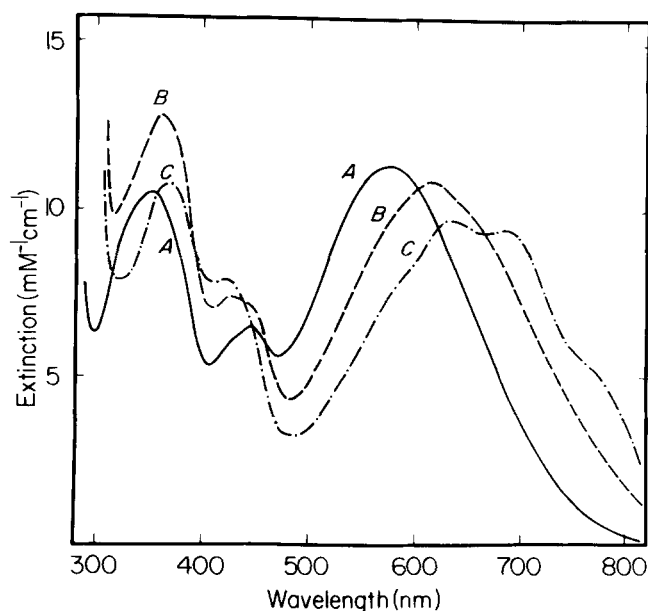


FIG. 7. Spectra of FAD-2-S-oxide, free (A) and bound to apo-*p*-hydroxybenzoate hydroxylase (B), and apo-D-amino acid oxidase (C). All spectra were recorded at 4 $^\circ\text{C}$ in either Tris- SO_4 or sodium pyrophosphate buffers, pH 8.5. Apoproteins were reconstituted as outlined under "Materials and Methods."

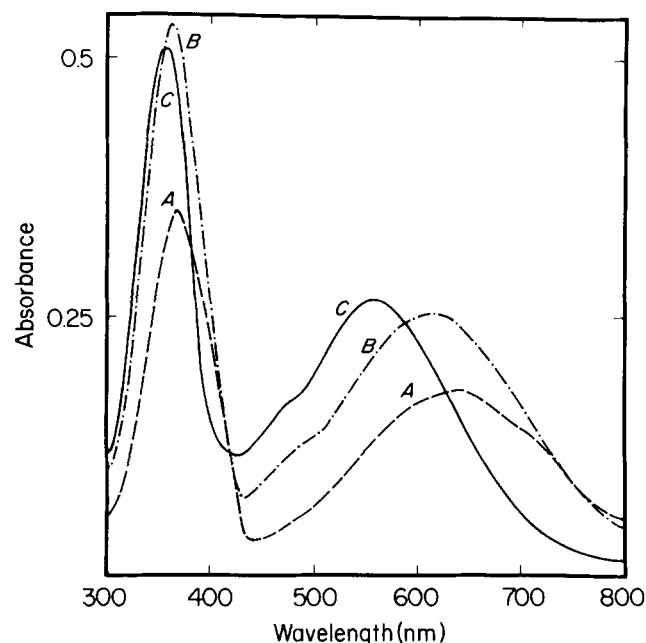


FIG. 6. Solvent perturbations of the absorption spectrum of the blue lumiflavin derivative. 2-Thiolumiflavin (25 μM) was titrated at 4 $^\circ\text{C}$ with MCPB (1.0 ± 0.1 eq) to yield maximal 2-S-oxide in benzene (A), spectral grade acetonitrile (B), and 10 mM NaHCO_3 , pH 9.9. Spectrum C represents the aqueous sample after acidification to pH 3.8 (i.e. N-3 position fully protonated in C). Titrations were carried out in stoppered 1-ml cuvettes, with the appropriate solvent reference.

intermediate in the reaction of 2-thio-FAD-*p*-hydroxybenzoate hydroxylase with H_2O_2 can be stabilized by removal of excess peroxide. When treated with 5% trichloroacetic acid, the flavin is released quantitatively into the supernatant, but with an accompanying shift in long wavelength maximum from 620 to 570 nm. Thus, it appears likely that when bound to the apoenzyme of *p*-hydroxybenzoate hydroxylase the blue flavin is in a more hydrophobic environment than in free

solution. D-Amino acid oxidase and L-lactate oxidase (not shown) exhibit by far the most pronounced spectral changes with the blue FAD and FMN derivatives, indicating hydrophobic flavin-binding sites. These results are in accord with those of previous studies (1) in which the 2-thio substituent in 2-thio-FAD-reconstituted D-amino acid oxidase and 2-thio-FMN-L-lactate oxidase were found not to react with the thiol reagent methyl methanethiolsulfonate while protein bound; similarly, they also failed to react with H_2O_2 or MCPB (this work, results not shown). Table II summarizes the spectral data for different flavin 2-S-oxides free in solution and bound to various apoflavoproteins.

The $1e^-$ Oxidation of 2-Thioflavins—As was detailed in previous sections, peroxide or peracid oxidation of 2-thioflavins in aqueous media leads directly to the formation of a blue chromophore, the flavin 2-S-oxide. The similar formation of the 2-S-oxide was also obtained in spectral grade acetonitrile by the addition of 1 eq of MCPB (cf. Fig. 6). By contrast, when the oxidation was conducted in acetonitrile purified by refluxing over phosphorus pentoxide, and subsequent distillation over CaH_2 (cf. "Materials and Methods"), a product is formed reproducibly (Fig. 8) the spectrum of which is markedly shifted to shorter wavelengths as compared to that of the starting 2-thioflavin. Complete disappearance of the starting 2-thioflavin is obtained with 0.5 eq of MCPB, i.e. with $1e^-$ /flavin. This oxidation is readily and completely reversed by 0.5 eq of dithiothreitol (cf. inset of Fig. 8). The oxidation product is stable to air in acetonitrile, and also in aqueous solvent. On thin layer chromatography (Table I), it shows an R_F value which is markedly lower than that of the starting material, and of other 2-substituted flavins. Such an R_F value is, however, characteristic for flavin dimers. The spectrum of the oxidation product is closely similar to the spectra of e.g. 2-SS- CH_3 flavins, a class of compounds which is obtained from reaction of 2-thioflavins with methyl methanethiolsulfonate (1). This oxidation product is not obtained when position N-3 of the flavin is blocked, e.g. when the oxidation is attempted with 3-methyl-2-thiolumiflavin. The product has no dissociable protons, as evidenced by the lack of immediate spectral changes at pH >11. Under such conditions, it, how-

TABLE II
Spectral properties of various apoflavoproteins reconstituted with flavin 2-S-oxides

Flavin	Enzyme	Spectral properties ($\lambda_{\text{max}}^{\text{nm}}$, ϵ_{max} · cm)
Lumiflavin 2-S-oxide (pH 9.9)		565, 345; 13.5, 12.9
N-3-methylumiflavin 2-S-oxide (pH ~6)		545, 351; 12.1, 18.3
Riboflavin 2-S-oxide (pH 10.7)		560, 356; 13.8, 12.3
FMN-2-S-oxide (pH 7.1)	Riboflavin-binding protein	560, 328; 12.6, 11.8
	Old Yellow enzyme	564, 356; 10.7, 12.5
	Old Yellow enzyme plus pentafluorophenol	564, 354; 10.1, 14.0
	Flavodoxin	580, 363; 9.8, 13.9
	Lactate oxidase	592, 365; 8.6, 13.5
FAD-2-S-oxide (pH 8.4)		630, 670, 760 (sh ^a); 9.1, 9.1, 360 15.0
	<i>p</i> -Hydroxybenzoate hydroxylase	574, 352; 11.3, 10.5
	<i>p</i> -Hydroxybenzoate hydroxylase plus <i>p</i> -hydroxybenzoate	616, 427, 360; 11.3, 7.7, 13.7
	Glucose oxidase	588, 445, 353; 11.0, 6.8, 12.9
	D-Amino acid oxidase	624, 328; 11.6, 21.7
		633, 688, 780 (sh); 9.7, 9.4
	D-Amino acid oxidase plus benzoate	421, 368 7.9, 10.7
	Same	

^a sh, shoulder.

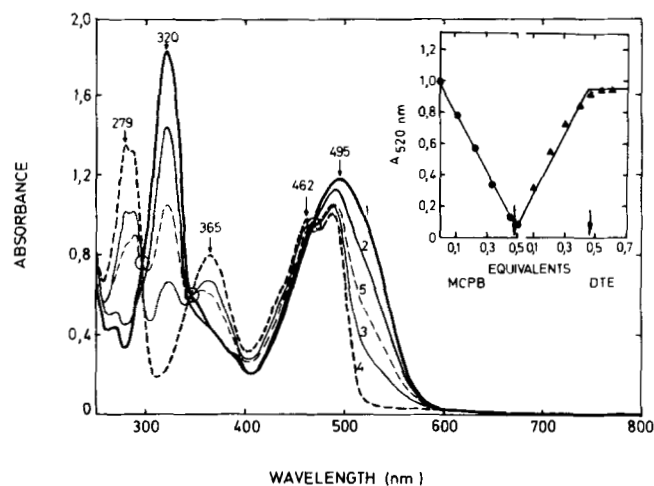


FIG. 8. Course of oxidation of 2-thioflavin with MCPB and of reduction of the product, the flavin dimer, with dithioerythritol. 2-Thiolumiflavin (7.1×10^{-5} M in acetonitrile; curve 1, —) was titrated with aliquots of MCPB (8.06 mM in the same solvent). Curve 4 (---) shows the spectrum obtained after addition of 0.5 eq of MCPB (see inset). Subsequent to titration with MCPB, the chromophore of curve 4 was titrated with 5.4 mM dithioerythritol (DTE) (in acetonitrile) to yield a final spectrum identical with that of 2-thiolumiflavin (curve 1). Curves 2, 3, and 5 represent selected intermediate spectra which were obtained after addition of 0.11 and 0.3 eq of MCPB, and 0.2 eq of dithioerythritol, respectively. The arrows denote the isosbestic points observed during the reduction and oxidation. The inset shows the dependence of the absorbance at 520 nm on the equivalents of MCPB and dithioerythritol added. It must be noted that such results were obtained only in acetonitrile which had been refluxed over P_2O_5 with subsequent distillation over CaH_2 (8). In spectral grade acetonitrile to which small amounts of isocyanates had been added, formation of dimer can also be observed, although not quantitatively. See text for more details.

ever, decays slowly and forms lumiflavin and 2-thiolumiflavin as final products, which were identified by tlc. In addition, small quantities (~20%) of lumiflavin 2-S-oxide are formed, as evidenced by the transient formation of a blue species absorbing in the 600-nm region. In acidic medium (at pH < 0; 6 N HCl or glacial formic acid), a chromophore (λ_{max} , 422 and 284 nm; $\epsilon_{422} = 17,500/\text{flavin chromophore}$; not shown) is obtained, which is similar to the protonated form of 2-S- CH_3 -lumiflavin (4).

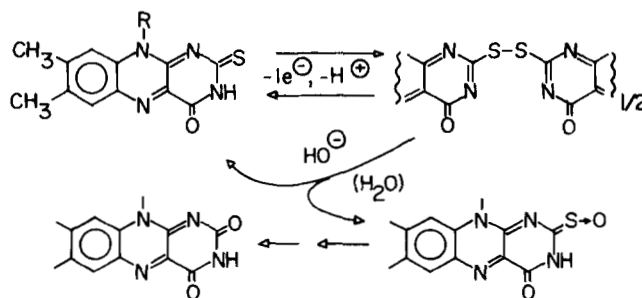
Initially, it was thought that the different pathway of oxidation shown with the redistilled acetonitrile as compared with spectral grade solvent was due to some trace contaminant

in the latter. However, this was made unlikely by the finding that the redistilled solvent had more UV absorbance than the original (a component absorbing maximally at ~250 and ~320 nm) which could be removed by passage over a column of alumina. In solvent purified by this procedure, titration with MCPB resulted in the stoichiometric formation of the blue flavin S-oxide, in the same way as with the initial unpurified solvent. Analysis of the distilled acetonitrile by gas chromatography-mass spectroscopy (kindly performed by Dr. T. Kuster in the laboratory of Professor H-C. Curtius, Kinderspital, Zurich) suggested that the contaminant introduced in the distillation procedure was an isocyanate. This was confirmed by the finding that addition of 0.1–0.5% isocyanate (as trimethylsilylisocyanate) to spectral grade acetonitrile resulted in the formation of a mixture of dimer and 2-S-oxide (results not shown).

DISCUSSION

Structure of the $1e^-$ Oxidation Product of 2-Thioflavins—The stoichiometry of the oxidation and of the reduction reactions under the conditions of Fig. 8, the analogy of the optical spectra to those of flavins carrying a 2-SS-R substituent (1), and the chromatographic properties listed in Table I are all consistent with formation of a flavin-2-SS-2-flavin (dimer) as shown on Scheme 1.

This dimeric structure requires also the removal of the N-3 proton, as in this molecule an exocyclic double bond is not possible (Scheme 1); in agreement with this, no dimer formation could be observed under similar conditions when the starting flavin was N-3-methyl-2-thiolumiflavin. The decay reactions of the dimer observed at high pH are accounted for



SCHEME 1. Reversible conversion of 2-thioflavins to the corresponding dimers via $1e^-$ oxidoreduction, and possible pathways for the decay, to form normal flavin and starting 2-thioflavin.

by the reaction sequences shown in Scheme 1; nucleophilic attack by OH^- will lead in a formal disproportionation to 1 eq of 2-thioflavin, and 2-S-oxide, as observed qualitatively. The latter, however, will desulfurize under alkaline conditions to yield the unsubstituted isoalloxazine (Scheme 1). The solvolysis of disulfides to yield "sulfide" + sulfenic acid/S-oxide is a well known process, and has been described particularly for the structurally similar pyrimidine disulfides (21).

Structure and Properties of the Blue Flavin 2-S-Oxides—In aqueous media, and in particular at high pH values, 2 electrons/flavin are involved in the reversible oxidation of 2-thioflavins to the blue species (cf. Scheme 3). The fact that these blue flavins bind stoichiometrically to apoenzymes (Table II), and their behavior on tlc exclude that they have a dimeric structure such as that shown in Scheme 1. They thus logically should have the structure of a flavin 2-S-oxide as shown in Scheme 2, *structures B*. Such an oxidation product of an asymmetric thioamide function (i.e. the N-1—C-2=SN-3 region of thioflavins) can exist in several isomeric, tautomeric, and mesomeric forms, the three most important ones being represented by *structures B1*, *B2*, and *B3* (Scheme 2). The fact that blocking of position N-3 with a methyl substituent does not preclude formation of the blue species, is consistent with the sulfoxide forms *B1* or *B3*, in contrast to the sulfenic acid (or sulfenate) *B2* being the correct tautomer.

The superior stability to be expected for a bridged structure such as that of *B1* (21, 22) as compared to *B3* for the N-3-unsubstituted flavins suggests that *B1* might be the predominant form, at least for the neutral species. That two spectrally similar species having different stability might indeed be formed is suggested by the strongly biphasic decay kinetics of the primary product(s), which is absent upon purification of the S-oxide after the first decay phase has gone to completion. In the case of 3-methylflavins, however, the *trans* form *B3* might be energetically favored, this difference being possibly the origin of the different stability of 3-unsubstituted *versus* 3-substituted derivatives.

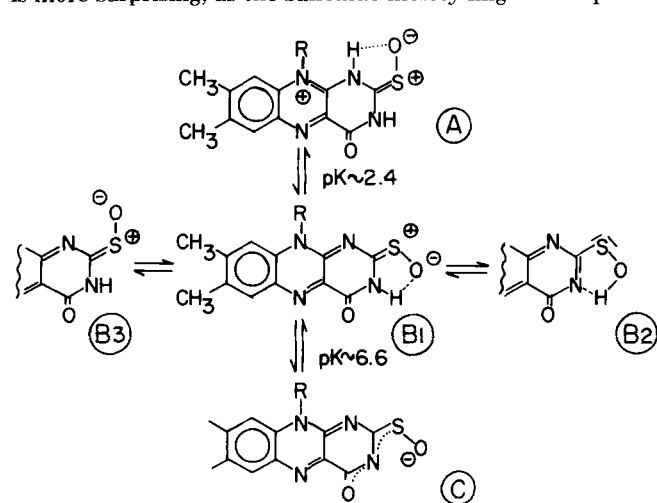
The pK value of 2.4 undoubtedly corresponds to a protonation of the nucleus; the observed blue shift of the lowest energy-visible absorption band coincides qualitatively with that known to accompany the protonation of isoalloxazine (4), i.e. a blue shift of the lowest energy band, and a red shift of the transition in the 350-nm region to yield a single absorption band around 400 nm (4). The increase of the pK value to 2.4 as compared to ~ 0 for both normal flavin and 2-thioflavin (4) is more surprising, as the sulfoxide moiety might be expected

to render the nucleus more electron deficient, i.e. to lower its pK, as compared to 2-thioflavin. This effect could result from anchimeric stabilization of the protonated species, as shown by *structure A*, Scheme 2. On the other hand 2 α -substituted flavins such as 2-SR, 2-OR, and 2-NR₂ flavins have pK values in the range 3.7–4.4 (6) for the protonation at N-1. That the sulfoxide substituent indeed does lower the electron density of the pyrimidine moiety is demonstrated by the lowering of the pK for the deprotonation of N-3—H to 6.6 from around 10 for normal and for 2-thioflavins (1, 4). The direction of this shift is as expected; its magnitude is surprising, however, as an anchimeric effect in a structure such as *B1* should counterbalance the expected decrease. The structure of the anionic species can again be represented by several mesomeric structures, and by two isomeric structures, the more likely of which is shown by *structure C*, Scheme 2.

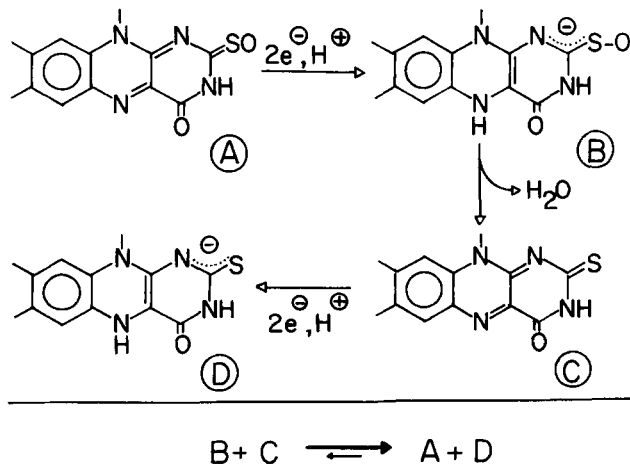
The large negative solvatochromy observed with the flavin 2-S-oxides is clearly in agreement with the ground state existing in a rather dipolar structure, a case encountered also with the neutral flavin radical (23, 24) and with other organic dyes (25). This effect is in agreement with a dipolar structure of the sulfoxide group (cf. *structures B1* and *B3*, Scheme 2).

Mechanisms of Formation and Decay of the Flavin 2-S-Oxides—From the experiments of Fig. 2, no spectral contribution from the oxidized 2-thioflavin is apparent during the dithionite titration. The partial reformation of flavin 2-S-oxide absorption after each addition of reducing agent suggests that the overall 4e⁻ reduction proceeds by the steps shown in Scheme 3. Thus, first a rapid reduction of the flavin 2-S-oxide (*A*) occurs, to yield the 1,5-dihydro form (*B*). The latter can eliminate water slowly to yield oxidized 2-thioflavin (*C*). This undergoes a fast (thermodynamic) equilibration with unreacted dihydroflavin 2-S-oxide (*B*), which leads to the formation of 1,5-dihydro-2-thioflavin (*D*) and the partial reappearance of the 560-nm absorbance of the oxidized flavin 2-S-oxide (*A*) according to the equation: $C + B \rightleftharpoons A + D$.

The decay mechanisms and routes of the flavin 2-S-oxides are extremely complex. Their detailed elucidation was beyond the scope of the present work. However, from the results described the following interpretations appear to be reasonable. (a) The flavin S-oxides appear to be stable for practical purposes in the absence of oxidizing agents and at pH values around neutrality. This indicates that the major decay routes proceed through oxidation to the corresponding sulfenic acid, and that direct hydrolysis to lumiflavin does not play an



SCHEME 2. Ionization of flavin 2-S-oxides, and possible isomeric structures. The isomers *B1*, *B2*, *C*, and *A* are referred to as *cis* (with respect to the N-3 substituent). Isomer *B3* is *trans*.



SCHEME 3. Schematic course of (dithionite) reduction of flavin 2-S-oxides (*A*) to yield the 1,5-dihydro form (*B*). This is formally reoxidized by elimination of H_2O to form 2-thioflavin (*C*), which in turn undergoes reduction to 1,5-dihydro-2-thioflavin (*D*). Cf. text for further explanations.

important role (Scheme 4). (b) Disproportionation to 2-thioflavin and sulfenic acid appears to be the major decay route upon (anaerobic) light irradiation. (c) The flavin 2-sulfenic acid formed upon $2e^-$ oxidation (or disproportionation) of the flavin 2-S-oxide appears to hydrolyze immediately in aqueous solvents and to form the normal 2-oxo flavins. (d) That the conversion of the flavin 2-S-oxide to normal flavin can require up to 4 electron eq (at pH values ~ 7) might result from (partial) further oxidation to the flavin 2-sulfonate, or by oxidation to sulfite of the sulfenic acid resulting from hydrolysis, or from a combination of both oxidation processes (see also Scheme 4).

Evidence of Formation of Flavin 2-Sulfonates and Flavin 2-Sulfonates upon Oxidation of Flavin 2-S-Oxides: Properties and Reactivity—The observation of two distinct species upon further oxidation of the flavin 2-S-oxide (cf. Fig. 4 and Scheme 4) and the requirement of ~ 2 eq for formation of the first one can be rationalized by the sequential formation of the flavin sulfinate and sulfonate (Scheme 4, structures B and C, respectively). That the spectrum of the sulfinate (Fig. 4, species B) is closely similar to that of normal 2-oxo flavins (cf. Fig. 1, curve 7) is unexpected in view of the large spectral differences existing between the 2-oxo, 2-thio, and 2-S-oxide flavins (cf. Fig. 1). This similarity means that these two species must have similar electronic structures, suggesting that of the two possible tautomeric structures, B2 in Scheme 4 better describes the flavin sulfinate. A species with a similar absorption can be observed transiently also on oxidation with excess MCPB of N-3-blocked flavin 2-S-oxides, e.g. of 3-methylflavin 2-S-oxide. This species is, however, very labile, pre-

cluding its characterization (3).

In contrast to this, the absorption spectrum of the putative flavin 2-sulfonate is closely related to the spectra of N-1-protonated flavin cations (4). This is readily explained by the strong inductive effect of the sulfonate group, and by its inability to form an exocyclic double bond with the pyrimidine ring. Thus, the sulfonate group will induce a mesomeric structure (cf. C2, Scheme 4) closely similar to that encountered with flavin cations (4). These observations suggest that, in all the three cases of the flavin 2-sulfoxides, sulfonates, and sulfonates, the N-3—H amide orbital does not contribute to a major extent to formation of a C-2—N-3 double bond, implying that structures A, B2, and C2, Scheme 4, reflect the predominant electronic configurations of these species. This would appear to be in contrast to what has been reported for the pteridine system (26, 27). The very high reactivity of the flavin sulfinate and sulfonates toward nucleophilic displacement is documented by the fact that these species could be observed only in aprotic solvents, by the impossibility, in our hands, of isolating either of them, and by the observation of immediate hydrolysis on addition of small amounts of nucleophilic solvent, e.g. water.

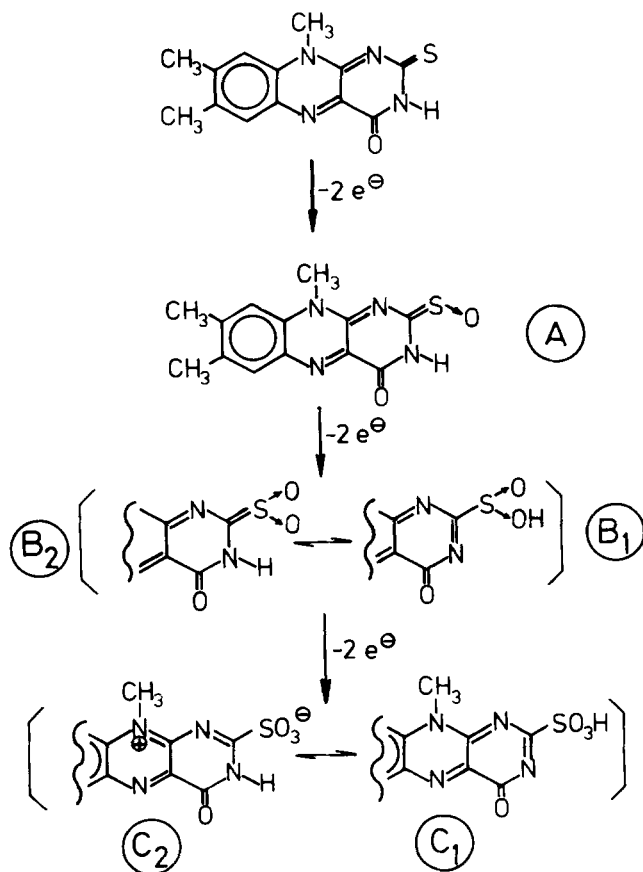
The observed high lability toward hydrolytic reagents explains the observation of formation of a covalent bond with an apoprotein residue on oxidation of the flavin 2-S-oxide in *p*-hydroxybenzoate hydroxylase (3). Thus, nucleophilic displacement with a variety of protein residues (e.g. imidazole, thiolate, phenolate, or carboxylate) might occur at the level of the sulfinate or, less likely, at that of the sulfonate (cf. Scheme 4).

Flavin 2-S-Oxides as Active Site Probes of Flavoproteins—The results summarized in Table II indicate that flavin 2-S-oxides may prove to be useful probes of flavoprotein active sites, particularly with respect to hydrophobicity of the protein around the C-2 position of the bound flavin. The finding that binding of riboflavin 2-S-oxide to riboflavin-binding protein does not result in any pronounced spectral shift indicates that the flavin is in a hydrophilic environment. This conclusion is consistent with that from previous studies of Choi and McCormick (28) and from our studies with methyl methanethiolsulfonate and protein-bound 2-thioriboflavin (1), which show that the pyrimidine ring of the bound flavin is exposed to solvent. The reaction of H_2O_2 with bound 2-thioflavin can also be used as a probe of the solvent accessibility of the flavin 2-position. In keeping with the data of Table II, H_2O_2 reacts readily with 2-thioriboflavin bound to egg white riboflavin-binding protein.

With flavodoxin, the absorption maximum of the FMN-2-S-oxide is shifted to 592 nm, indicating a somewhat hydrophobic environment. This result is consistent with the known three-dimensional structures of flavodoxins (29), which show the pyrimidine ring of the flavin to be fairly buried in the protein matrix.

The results with *p*-hydroxybenzoate hydroxylase are very informative. As shown in the preceding paper (3), the 2-thio-FAD enzyme reacts readily with H_2O_2 to form the S-oxide, showing that solvent-borne H_2O_2 is able to reach the flavin 2-position. In the presence of *p*-hydroxybenzoate, the formation of the S-oxide is slowed by a factor of 6, consistent with a conformational change of the protein accompanying substrate binding, which makes the flavin 2-position less accessible.

These results are consistent with those obtained previously on reaction of the 2-thio-FAD enzyme with methyl methanethiolsulfonate (1) and with the known structure of the enzyme-substrate complex, as determined by x-ray crystallographic analysis (30). The positions of the wavelength maxima of the enzyme-bound FAD-2-S-oxide indicate that in the absence of



SCHEME 4. General scheme for the oxidation of 2-thioflavins. The primarily formed flavin 2-S-oxide (A) yields the sulfinate (B), which can exist in the tautomeric forms (B1) or (B2), the latter being the most probable. Further oxidation leads to the sulfonate (C), which is proposed to be best described by the structure C2.

substrate the flavin is in a more hydrophobic environment than it is in the enzyme-substrate complex. These results are only compatible if the flavin 2-position is accessible to solvent via a protein channel in the case of the substrate-free enzyme, and that this channel is substantially closed, at the same time as the immediate environment of the flavin 2-position becomes more hydrophilic as a result of the conformational change accompanying substrate binding. Polarization of the flavin by the positively charged NH₂ terminus of helix H5, as suggested by Wierenga *et al.* (31), could account for the increased hydrophilic environment of the flavin in the presence of substrate.

Finally, the protein environment surrounding the flavin pyrimidine ring appears to be fairly hydrophobic in the case of the three flavoprotein oxidases studied. These results are consistent with the previously found lack of reactivity of these 2-thio-FAD and FMN proteins with methyl methanethiolsulfonate (1) and the similar lack of reaction with H₂O₂ found in the present work. From the limited results so far presented, it is clear that flavin 2-S-oxides offer another useful probe of the active site environment in flavoproteins.

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