The reaction of diphenyliodonium chloride with free reduced flavins has been studied by stopped flow spectrophotometry under anaerobic conditions, and second order rate constants were determined as a function of pH. The reactive flavin species was identified as the reduced anion, based on an observed reaction pK of 6.7. The product mixture was independent of the initial concentration of reactant and contained ~20% oxidized flavin. The results can be modeled quantitatively on a modification of the mechanism proposed by Tew (Tew, D. G. (1993) Biochemistry 32, 10209–10215). The composition of the complex reaction mixture has been analyzed, and four flavin-phenyl adducts with distinctive absorbance and fluorescence characteristics have been identified, involving substitution at the flavin C4a, N5, and C8 positions. Inactivation of flavoprotein enzymes by diphenyliodonium has also been studied, and several examples were found where inactivation occurs readily, despite noninvolvement of radical intermediates in their reaction mechanisms. It can be concluded that inactivation by phenyliodonium species is not a valid indicator of catalytic mechanism involving radical intermediates. One of the several factors determining inactivation is maintenance of the enzyme flavin in the reduced form in the steady state of catalysis, the other factors being redox potential and accessibility of the inhibitor to the flavin active site.

Recent studies have provided many examples of inhibition of flavoprotein enzymes by diphenyliodonium salts and similar iodonium compounds. The known capability of iodonium compounds to undergo radical reaction (1–4), the fact that most of the known enzymes to be severely inhibited, such as neutrophil NADH oxidase (5, 6), cytochrome P-450 reductase (7), xanthine oxidase (8), nitric-oxide synthase (9), and sulfite reductase (10), involve radical chemistry in their mechanisms has led to the concept that inhibition by iodonium compounds is a marker of flavoprotein enzymes functional by radical mechanisms, particularly because other flavoproteins, such as D- and L-amino acid oxidases, glucose oxidase, and glutathione reductase, which do not involve radicals in their reaction, are not inhibited (8). Previous work has demonstrated that the enzyme flavin needs to be reduced for inhibition to occur, and that the reaction of the reduced flavin with iodonium compounds results in phenyl adducts of the flavin (8, 10). Tew has proposed the mechanism shown in Scheme 1 for the formation of the flavin N5-phenyl adduct, involving the one electron oxidation of the reduced flavin to form the semiquinone, at the expense of reduction of diphenyliodonium to the corresponding diphenyliodanyl radical. The latter is known to fragment rapidly to give iodobenzene and a phenyl radical, and the flavin semiquinone and phenyl radical are proposed to undergo a radical recombination to yield a flavin-phenyl adduct (7).

\[
\begin{align*}
F_{\text{red}} + \text{Ph}_2I^+ & \rightarrow F+ + \text{Ph}I \\
\text{Ph}_2I^+ & \rightarrow Ph + + \text{PhI} \\
F+ + \text{Ph} & \rightarrow \text{adduct}
\end{align*}
\]

**Scheme 1**

We have reinvestigated the reaction of a number of flavoproteins with Ph₂I⁺, as well as the reaction of reduced FAD and FMN with Ph₂I⁻. Ph₂I⁻ has been demonstrated to inhibit catalysis of several enzymes that do not operate by radical mechanisms. The main criteria for inhibition appear to be maintenance of the enzyme flavin in the reduced form during the steady state of catalysis and a system redox potential sufficiently negative to act as a reductant for Ph₂I⁺. Most flavoproteins studied formed phenyl adducts when they were reduced prior to reaction with Ph₂I⁻ under anaerobic conditions. Our results with reaction of free reduced flavin with Ph₂I⁻ indicate that, in addition to the simple radical pathway of Tew (7), a substantial portion of flavin adducts formation proceeds either by direct attack of Ph₂I⁻ on the reduced flavin or via radical recombination from a primary caged radical pair. A series of flavin-phenyl adducts have been isolated and characterized, involving the flavin C4a, N5, and C8 positions.

**MATERIALS AND METHODS**

Diphenyliodonium chloride and FAD were obtained from Sigma. The enzymes used were prepared and assayed as described in the literature: Old Yellow Enzyme (OYE; Ref. 11), glucose oxidase (12), pig kidney D- and L-amino acid oxidase (13), bovine milk xanthine oxidase (14), pig heart lipopamide dehydrogenase (15), methylamine dehydrogenase (16), anthranilate hydroxylase (17), MHPC oxygenase (18), phenol hydroxylase (19), and p-hydroxybenzoate hydroxylase (20). Human red blood cell glutathione reductase was a gift from Dr. George Schulz (University of Freiburg, Freiburg, Germany) and E. coli thioredoxin reductase and thioredoxin reductase were gifts from Dr. Charles Williams (University of Michigan, Ann Arbor, MI). Flavodoxin from Clostridium beijenkeri was a gift from Dr. Richard Swenson (Ohio State University, Columbus, OH) and protocatechuate dioxygenase was a gift from Dr. David P. Ballou (University of Michigan, Ann Arbor, MI).

† Deceased August 26, 2002.

‡ To whom correspondence should be addressed: Dept. of Biological Chemistry, University of Michigan Medical School, 1301 Catherine St., Ann Arbor, MI 48109-0606. Fax: 734-763-4581; E-mail: sumita@umich.edu.

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The abbreviations used are: Ph₂I⁺, diphenyliodonium chloride; Ph₂I⁻, diphenyliodanyl radical; Ph⁺, phenyl radical; PhI, iodobenzene; Fl₆, reduced flavin; Flg, oxidized flavin; Fl₆, semiquinone flavin; F⁺, neutral flavin radical; MHPC, 2-methyl-3-hydroxy pyridine-5-carboxylate; OYE, Old Yellow Enzyme; HPLC, high performance liquid chromatography; Fldox, Fldq, the oxidized, semiquinone, and reduced forms of flavodoxin.
For the isolation of reaction products, for practical reasons we chose to use FMN for the isolation of reduced flavin with Ph$_2$I$^-$, because it is much more soluble than riboflavin and simpler to deal with in subsequent analytical procedures than FAD. Commercial FMN is only 85% pure, whereas commercial FAD is >95% pure, with the impurity being authentic FMN. Hence we used FAD as starting material and converted it to FMN using the phosphodiesterase of snake venom. In a typical experiment, 400 mg of FAD was dissolved in ~50–60 ml of 0.01 M potassium P$_2$, pH 7.0, and converted to FMN by incubating overnight at 25 °C with ~50 mg of Naja naja venom, a potent source of phosphodiesterase. The course of the conversion was monitored by the spectral change associated with the conversion ($e_{380}$ FAD = 11,300 M$^{-1}$ cm$^{-1}$, $e_{450}$ FMN = 12,500 M$^{-1}$ cm$^{-1}$), using suitably diluted aliquots for the spectrophotometric measurements. On complete conversion, the reaction mixture was boiled for 1–2 min to precipitate the venom and the solution was centrifuged to get rid of the precipitate. A sample was tested by HPLC to make sure that no unconverted FAD remained. The FMN solution was further purified by Sep-Pak C-18 reversed phase cartridges, eluting with 10% CH$_3$CN in water, and concentrated to ~250 ml using a rotary evaporator at 38–40 °C.

The pure FMN solution thus obtained was brought up to 0.1 M potassium P$_2$, pH 7.0; placed in a 500-ml three-necked round-bottomed flask; 20 ml of 0.3 M EDTA was added in the dark and made anaerobic by bubbling argon, at 0–5 °C for 45 min. The argon- equilibrated solution was then irradiated with a sun-gun for 10 min until all of the green fluorescence had disappeared, indicating full reduction. Diphenyliodonium chloride, 50 ml of 0.025 M, previously made anaerobic by bubbling with argon for 30 min was added to the reduced FMN solution with argon gas still flowing. The solution immediately turned orange with some precipitate, which, however, disappeared on incubating at 25 °C for ~1 h. The reaction mixture was opened to air and left overnight on ice.

The solution was then loaded on four 35-ml Sep-Pak prepacked columns, prewashed with methanol and equilibrated with water. After washing with water to remove salts, the flavin reaction products were eluted with acetonitrile-water mixtures. The columns were protected from light throughout. The solutions were concentrated as quickly as possible using a rotary evaporator at 38–40 °C.

The solutions were concentrated to ~250 ml using a rotary evaporator at 38–40 °C.

Spectroscopic Studies—UV-visible spectra were recorded with a Hewlett-Packard diode array spectrophotometer, or a Cary (Varian) model 219 double-beam spectrophotometer. NMR spectra were recorded in D$_2$O with a Bruker 500-MHz instrument, and chemical shift values are reported in ppm relative to tetramethylsilane.

HPLC Analysis—Reversed phase HPLC analyses were carried out with a Beckman System Gold instrument (model 126), using a UV detector and a C-18 250 × 10-mm column. The column was equilibrated and washed with 20% acetonitrile in 20 mM ammonium acetate, pH 5.7, for 5 min, and then the compounds were eluted by applying a gradient to 50% acetonitrile in 20 mM ammonium acetate over a period of 30 min. The eluate was monitored at 350 nm.

Stopped Flow Kinetics—Stopped flow experiments were carried out using a Hi-Tech SF-61 instrument in absorbance mode. For anaerobic reactions, the system was flushed and incubated overnight with a solution containing 3,4-dihydroxybenzoate (protocatechuic acid) and a catalytic amount of protocatechuate dioxygenase was placed in the side arm of the tonometer. The contents of the tonometer were made anaerobic by repeated cycles of evacuation and subsequent equilibration with high quality argon, and finally left under positive pressure of argon for transfer to the stopped flow apparatus. The flavin was reduced by shining visible light from a commercial sun-gun, mixed with the protocatechuate dioxygenase, and allowed to equilibrate for 30 min (to remove any residual oxygen) before loading into the stopped flow spectrophotometer at 25 °C and mixed with various concentrations of Ph$_2$I$^-$ or other solutions, as described in the text.

For experiments with flavoproteins, the same protocol was followed.

The results described are not fully consistent with the model of Tew and require a substantial portion of the reaction to occur through what appears to be direct reaction of reduced flavin with air-equilibrated buffer in absence of Ph$_2$I$^-$; spectrum 6 shows the corrected spectrum for the flavin-phenyl adduct mixture, obtained by subtracting 20% of spectrum 5 from the average of spectra 2–4 except that reduction of the enzyme was accomplished by introducing the reductant from a side arm.

RESULTS AND DISCUSSION

Stopped Flow Studies

The reaction of Ph$_2$I$^-$ with reduced FAD at pH 7.0 results in a relatively rapid spectral change, as shown in Fig. 1. The extent of spectral change was independent of the concentration of Ph$_2$I$^-$, a finding of considerable mechanistic importance, as will be developed later. The progress curves at all concentrations of Ph$_2$I$^-$ and at all wavelengths from 320 to 500 nm were fit satisfactorily to a single exponential, yielding $k_{obs}$ values that were linearly dependent on Ph$_2$I$^-$ concentration, and that in each case did not show any systematic variation with the wavelength of observation. Such experiments were repeated over the pH range of 6.0–8.5, with similar results in terms of the extent of spectral changes involved and the linear dependence of reaction rate constant on Ph$_2$I$^-$ concentration. The observed second order rate constants were, however, dependent on pH (Fig. 2), indicating a $pK_a$ value (23).

In separate experiments carried out under identical conditions, but in anaerobic cuvettes, similar spectral changes to those shown in Fig. 1 were observed. In those experiments it could be further shown that exposure to air there was no further spectral change, indicating that there was no remaining reduced flavin, and that most of the reduced flavin had been consumed in formation of adducts. Some, however, had been converted to oxidized flavin in the anaerobic reaction. As will be described below, this was estimated to be ~20% of the total flavin. Fig. 1 shows the calculated spectrum of the reaction mixture with this percentage of oxidized flavin subtracted from the observed final spectrum. The spectrum of the adduct mixture indicates species with remaining absorbance in the 400–500-nm region, as well as absorbance maxima at 370 and 330 nm. The nature of this mixture will be considered below.
with Ph₂I⁺, without any radical intermediates. This conclusion is based on comparison with simulations to Scheme 2.

\[
\begin{align*}
1) & \quad \text{Fl}^{\text{red}} + \text{Ph}_2\text{I}^- & \rightarrow & \quad \text{FI}^- + \text{Ph}^+ + \text{PhI}^0 \\
2) & \quad \text{FI}^- + \text{Ph}^+ & \rightarrow & \quad \text{adduct 1} \\
3) & \quad \text{Fl}^{\text{red}} + \text{Ph}_2\text{I}^- & \rightarrow & \quad \text{adduct 2} + \text{PhI}^0 \\
4) & \quad 2\text{FI}^- & \rightarrow & \quad \text{Fl}^{\text{red}} + \text{Fl}^{\text{red}} \\
5) & \quad 2\text{Ph}^+ & \rightarrow & \quad \text{products (e.g. phenyl dimer)}
\end{align*}
\]

**Scheme 2**

In this scheme, reaction steps 1 and 2 represent the mechanism proposed by Tew (7), simplified to take into account that the primary iododiphenyl radical fragments on a picosecond time scale to the more stable phenyl radical and iodobenzene (24).

However, this mechanism must be expanded to include reaction 4, the well known rapid dismutation of flavin radical to a mixture of oxidized and reduced flavin (25). The rate constants \(k_3\) and \(k_5\) are known, at pH 7.0, being \(6 \times 10^8\) and \(2 \times 10^6\) M⁻¹ s⁻¹, respectively (25, 26). Thus, for an appreciable yield of adducts and finite formation of oxidized flavin, \(k_3\) in the above scheme has to be very large, \(\sim 10^7\) M⁻¹ s⁻¹ or greater. However, in the absence of direct reaction between reduced flavin and Ph₂I⁺ (reaction 3 in Scheme 2, with rate constant \(k_3\)), simulations show that the percentage of oxidized flavin increases markedly with the concentration of Ph₂I⁺, the reaction time profile is frequently not fit with a single exponential, and the observed rate constants are not linear with Ph₂I⁺ concentration, appearing to reach a saturation value at high concentrations. It is also necessary to include a step involving the non-productive removal of phenyl radicals (exemplified by reaction 5 and rate constant \(k_6\) in Scheme 2). In the absence of such a step, simulations show frequently the transitory accumulation of oxidized flavin in excess of the final, a situation that we have never encountered experimentally. The only situation that approximates to the observed results is inclusion of \(k_3\) that can compete effectively with the radical formation step, \(k_1\). The best fits to the pH 7.0 data are in fact determined with a ratio of \(k_1/k_3\) - 0.5, with numerical values of -250 and -500 M⁻¹ s⁻¹, respectively, and with negligible contribution from the radical recombination step \(k_2\). The extent of total reaction proceeding through step 2 is of course quite dependent on the initial concentration of reduced flavin. Within experimental error we have not observed dependence of the composition of the final products on the initial reduced flavin concentration, provided that the latter is no greater than one-tenth of the lowest Ph₂I⁺ concentration used, i.e. that the observed reaction is pseudo first order.

**Adduct Characterization**

Addition of a phenyl residue to the reduced flavin ring could give rise to different products, with the phenyl substitution at the C4a, N5, or C8a positions, as shown in Structures 1–3. Reaction of reduced FMN with Ph₂I⁺, described under “Materials and Methods,” resulted in a mixture of products, as shown by HPLC (Fig. 3). A similar result was reported by Covès et al. (10) for reaction of reduced FAD with Ph₂I⁺.

Unfortunately, some of the products were not very stable, making identification difficult. To obtain enough material for characterization, the bulk reaction mixture obtained from the reaction of reduced FMN and Ph₂I⁺ was chromatographed on C-18-based Sep-Pak columns. After loading on the column, buffer salts and excess Ph₂I⁺ were removed by thorough washing with water before fractionation with acetonitrile-water mixtures. The first compound, which was eluted with water and 1% CH₃CN, was undervatized FMN (~20% of the starting material).

The next compound isolated from the reaction mixture was eluted with 2% CH₃CN. Evaporation of the solvent yielded a compound with a blue fluorescence, characterized by an excitation maximum at 380 nm and an emission maximum at 430 nm. This compound was found to be unstable; hence, we could not obtain good NMR and mass spectral data. However, its visible spectrum showed an absorption band at 364 nm with tailing into the 400 nm region, and the absorption maximum was shifted to 342 nm in 6 N HCl. This hypochromic shift in acid is reported to be a general characteristic of flavin N5 adducts (27). Based on this property, it is concluded that this adduct probably has the structure shown as Structure 2. This adduct is presumably responsible for the peak at 18 min in the HPLC separation (Fig. 3). The spectroscopic properties of this and the other isolated adducts are summarized in Table I.

Elution of the Sep-Pak column with 2–2.5% CH₃CN gave a fraction whose UV-visible spectrum changed slowly over the course of days, corresponding to the peak at 15 min in the HPLC profile of Fig. 3. Immediate evaporation of the solvent by Speed-Vac resulted in a yellow solid with absorption maxima at 320, 332 (sh), and a broad band centered around 400 nm. The fluorescence spectrum showed excitation peaks at 320 and 460 nm with emission maximal at 530 nm. The nonagreement of the absorbance and fluorescence excitation spectra indicated that this fraction was a mixture of at least two compounds. An apoflavodoxin column (28) was employed for further purification. Adducts were bound to the column as a pink band. After washing with 10 mM potassium Pi, 0.3 mM EDTA, pH 7.0, what proved to be still a mixture of adducts was eluted with 5% TCA in 0.3 mM EDTA. After removal of the TCA by extraction with ether, the solution was rechromatographed on a small Sep-Pak column with H₂O:CH₃CN mixtures. Several minor fractions
were eluted with water that had 320- and 332-nm maxima, but with variable secondary bands between 360 and 500 nm. The main fraction, eluted with 5% CH₃CN, had absorbance maxima at 320, 332, and 432 nm (Fig. 4). This fraction was evaporated to dryness at room temperature to prevent any further decomposition and dissolved only when ready to use.

Fig. 4 shows the titration of this adduct with apoflavodoxin in 0.05 M potassium Pi, pH 7.0, resulting in a dramatic shift in the absorption spectrum. From this titration, it can be estimated that the $K_d$ for binding to apoflavodoxin is <1 μM, and the extinction coefficients of the free flavin can be calculated to be $ε_{318} = 24,000 \, M^{-1} \cdot cm^{-1}$, $ε_{332} = 23,000 \, M^{-1} \cdot cm^{-1}$, and $ε_{430} = 8,500 \, M^{-1} \cdot cm^{-1}$.

Despite the impressive isosbestic points of the spectra of Fig. 4, the $^1$H NMR spectrum of the new flavin was quite complex and appears to be the result of a mixture of two compounds. The addition of a phenyl ring to the flavin was evidenced by the appearance of new complex signals in the aromatic region between 7.2 and 7.4 ppm, and there were three methyl signals that were shifted upfield compared with normal FMN (observed at δ 1.57, 1.61, and 1.63 ppm). An additional two-proton signal was observed at δ 4.37 ppm (Table 1). From the UV-visible spectra, it was predicted that C8 of flavin is involved in the formation of this adduct and detailed analysis of the $^1$H NMR spectrum is consistent with its being a mixture of two C8-phenyl adducts having Structures 3 and 4 in the ratio of 65:35, Structure 3 comprising CH₃ signals at 1.57 and 1.63 ppm, and the signal at δ 1.61 ppm resulting from Structure 4.

Further support for Structure 4 comes from the mass spectral data, which showed a molecular ion peak at 549 mass units; the observed increase of 94 units suggests the addition of a phenyl and a hydroxyl group to the FMN (Structure 4). The addition of one phenyl group to FMN accounts for an additional 77 mass units, giving rise to the observed peak at m/e 533 (Structure 3).

Flavins and Diphenyliodonium chloride. The starting spectrum before addition of apoprotein had an absorbance maximum at 430 nm; successive spectra, all corrected for dilution, were obtained on addition of apoflavodoxin at the concentrations shown in the inset. Inset, plot of the absorbance difference ($A_{430} - A_{510}$) as a function of apoflavodoxin concentration. Titrations were carried out in 0.05 M potassium Pi, pH 7.0, 25°C.

**TABLE I**

| Compound | UV-Vis | NMR (δ ppm) | Mass Spec (M⁻¹) | Fluorescence |
|----------|--------|-------------|----------------|-------------|
| 1        | λmax=364 nm and shifts to 388 nm in 6N HCl | CH₃ protons at 2.14 and 2.17, aromatic protons at 7.0-7.8 | 533 | Non fluorescent |
| 2        | λmax=364 nm with tailing into 400 nm region | ND | ND | λex=360 nm, λem=30 nm |
| 3        | ND | CH₃ protons at 1.57,1.63; aromatic protons at 7.2-7.4 | 533 | ND |
| 4        | λmax=320, 332 (sh), broad band at 400 nm | CH₃ protons at 1.61, CH₂ protons at 4.37; aromatic protons at 7.2-7.4 | 549 | λex = 320, 460 nm, λem = 530 nm |

were eluted with water that had 320- and 332-nm maxima, but with variable secondary bands between 360 and 500 nm. The main fraction, eluted with 5% CH₃CN, had absorbance maxima at 320, 332, and 432 nm (Fig. 4). This fraction was evaporated to dryness at room temperature to prevent any further decomposition and dissolved only when ready to use.

Fig. 4 shows the titration of this adduct with apoflavodoxin in 0.05 M potassium Pi, pH 7.0, resulting in a dramatic shift in the absorption spectrum. From this titration, it can be estimated that the $K_d$ for binding to apoflavodoxin is <1 μM, and the extinction coefficients of the free flavin can be calculated to
the double peak around 19 min in the HPLC of Fig. 3. The negative ion FAB Mass spectroscopic data showed an M⁺−1 at 533 mass units, consistent with the addition of a single phenyl group to FMN. The ¹H NMR data showed two clear singlets for the two aromatic methyls and the complex multiplets observed in the aromatic region account for the extra phenyl protons. The possible sites for the adduct formation are either N5 or C4a. Distinction between these two structures was made by making use of ¹³C NMR, as well as absorption spectral changes in 6 N HCl. An N5 adduct corresponds structurally to reduced flavin adducts, and making use of ¹³C NMR, as well as absorption spectral changes in 6 N HCl, confirming them to be isomeric compounds.

 Addition of a phenyl group to the C4a could occur from either side of the tetrahedral carbon to give rise to two diastereomers, evidenced by the double peak at 7.19 min in Fig. 3. These two isomers could be resolved by HPLC using an isocratic run with 20% CH₃CN in 20 mM NH₄OAc, pH 5.7, over a period of 20 min. The two isolated peaks were identical in their UV-visible and mass spectra and showed the same bathochromic shift in 6 N HCl, confirming them to be isomeric compounds. The NMR spectra were almost identical with the exception of one aromatic proton. In one isomer the peak was at 7.37 ppm, and in the other one it was at 7.32 ppm. The diastereomeric nature of the adducts was also confirmed by their specific binding to different apoproteins. For example, the first eluting isomer binds tightly to apoflavodoxin and apoOYE, but there is no binding of these apoproteins with the second isomer. These results and those with other proteins will be published separately.

Accurate quantification of the yields of the various adducts was difficult, owing to the instability of C8 and N5 adducts, and because of incomplete recovery of UV-visible absorbing material from HPLC and preparative Sep-Pak columns. Oxidized FMN was easy to quantify, because it is stable and elutes first. With Ph₂I” in excess, the yield of FMN was routinely found to be ~20% of the initial flavin taken. Spectral deconvolution of the visible spectrum of the remaining reaction products (cf. Fig. 1) indicate ~50% formation of the C4a adduct, 10–20% C8 adducts, and <10% N5 adduct.

As detailed in a previous section, simulations require that approximately two-thirds of the total reduced flavin react directly with Ph₂I” in a fashion not involving detectable radical intermediates, and that this is the major route of flavin adduct formation. Radical reactions do indeed occur, as detected previously by EPR (8), and are required in our own experiments to account for the formation of oxidized flavin (reaction 4 in Scheme 2). However, adduct formation from free flavin radicals does not appear to be a viable pathway. Although direct nucleophilic attack of the reduced flavin anion on Ph₂I” is feasible for formation of C4a-phenyl flavin (see Scheme 3), it is not feasible for formation of N5 and C8 adducts.

In these cases radical reaction pathways seem mandatory. This seeming dilemma may be explained if the apparent direct reaction involves a caged radical pair of neutral flavin semiquinone and diphenyliodonium radical (or its breakdown product, phenyl radical). In that case, the first three steps of Scheme 2 may be replaced by steps a–c of Scheme 4.

The 2:1 ratio of the rate constants for steps b and c, derived from simulations, was confirmed by experiments in which reduced flavin was reacted anaerobically with an equimolar concentration of Ph₂I”. Under these conditions the final reaction mixture contained 16% unreacted reduced flavin, easily quantified by the spectral changes on admission of air (results not shown). This result is consistent with 32% of the total reduced flavin being converted to the free radical species (step c of Scheme 4), which, instead of reaction to form adducts, rapidly disproportionates to an equimolar mixture of oxidized and reduced flavin.

**Studies with Enzymes**

**Kinetics of Inactivation under Steady State Turnover Conditions**—With most enzymes studied, where inactivation by Ph₂I” was observed under steady state conditions, the apparent first order rate constant for inactivation, k_{app}^{inact}, was obtained by analysis of the time course of decrease in catalytic velocity. For example, in assays involving the oxidation of NADH or NADPH, a continuous trace of the absorbance at 340 nm was recorded until complete inactivation was obtained, evidenced by the trace having approximately the same slope as that of a control reaction without enzyme. Semi-log plots versus time of the observed absorbance minus that obtained from the extrapolated slope of the inhibited reaction were linear for at least 90% of the total change, yielding a value of k_{inact}^{app}. With all of the enzymes studied where inactivation occurred, within the error of measurement, the k_{inact}^{app} values were directly proportional to the Ph₂I” concentration, consistent with lack of specific binding of Ph₂I”, and yielding a second order rate constant for the inactivation. These results differ from those reported previously with xanthine oxidase and other enzymes, where apparent specific binding of the inhibitor was deduced from inhibition progress curves (6, 7, 8, 10). Table II summarizes the data obtained with the flavoprotein enzymes studied.
Steady state inactivation of selected flavoprotein enzymes

| Enzymes                      | Substrates                        | Conditions      | $k_{inact}$  |
|------------------------------|-----------------------------------|-----------------|--------------|
| Old Yellow Enzyme            | NADPH, O$_2$                      | pH 7.0, 25°C    | None         |
| Glucose oxidase              | NADPH, cyclohexenone              | pH 7.0, 25°C    | None         |
| $p$-Amino acid oxidase       | Glucose, O$_2$                    | pH 5.6, 25°C    | None         |
| Xanthine oxidase             | Xanthine, O$_2$                   | pH 8.5, 25°C    | None         |
| Lipoamide dehydrogenase      | NADH, lipote                      | pH 6.5, 25°C    | None         |
| Glutathione reductase        | NADPH, GSSG                       | pH 7.6, 25°C    | None         |
| Thioredoxin reductase        | NADPH, DTNB, thioredoxin          | pH 7.6, 25°C    | None         |

(Morganella morganii) | NADH, O$_2$, 2-hydroxybenzoic acid | pH 7.6, 25°C | None |
| Anthranilate hydroxylase    | NADPH, O$_2$, anthranilate        | pH 7.6, 25°C   | None |
| MHPC oxygenase              | NADH, O$_2$, 2-methyl-3-hydroxypyridine-5-carboxylate | pH 7.6, 25°C | None |
| Phenol hydroxylase          | NADPH, O$_2$, phenol              | pH 7.6, 25°C   | None |
| $p$-Hydroxybenzoate hydroxylase | NADPH, O$_2$, 4-hydroxybenzoate  | pH 7.6, 25°C    | None |
|                               | NADPH, O$_2$, 2,4-dihydroxybenzoate | pH 7.6, 25°C | None |
|                               | NADPH, O$_2$, tetrafluoro-4-hydroxybenzoate | pH 7.6, 25°C | None |
|                               | NADPH, O$_2$, 2-fluoro-4-hydroxybenzoate | pH 7.6, 25°C | None |
|                               | NADPH, O$_2$, 3-fluoro-4-hydroxybenzoate | pH 7.6, 25°C | None |
|                               | NADPH, O$_2$, 4-amino benzoate     | pH 7.6, 25°C   | None |
|                               | NADPH, O$_2$, (6-hydroxy nicotinate) | pH 7.6, 25°C | None |

Parentheses indicate a non-substrate effector.

$^a$ DTNB, 5,5'-dithiobis(nitrobenzoic acid).

Reaction of Reduced Flavoproteins with Diphenyliodonium Chloride

Old Yellow Enzyme—Although OYE did not show any inhibition by Ph$_2$I$^-$ in the standard enzymatic assays with oxygen or cyclohexenone as electron acceptors (Table II), the reduced enzyme did react very slowly with Ph$_2$I$^-$, as shown in Fig. 5. In this experiment the enzyme was maintained in the reduced state under anaerobic conditions by a catalytic amount of NADPH and an NADPH-regenerating system. On tipping Ph$_2$I$^-$ from the side arm of the anaerobic cuvette, very slow spectral changes were observed, with a $t_{1/2}$ of 77 min (Fig. 5, inset), corresponding to a second order rate constant of 0.6 M$^{-1}$ s$^{-1}$. Flavin adduct formation was complete, because on exposure to air there was no sign of return of the spectral characteristics of oxidized enzyme. The modified enzyme was completely inactive in the standard catalytic assays. It was strongly fluorescent, with an emission maximum at 530 nm and excitation spectrum similar to that of the absorption spectrum, with maxima at 320 and 440 nm, characteristics of a flavin-C8-phenyl adduct. On denaturation of the protein with 50% ethanol or acetone, the fluorescence of the liberated flavin increased 5-fold, with slight shifts of the excitation maxima to 325 and 445 nm. HPLC analysis of the liberated product also revealed the presence of a small amount of flavin-C4a adduct, as evidenced by its retention time and by the characteristic shift in absorbance maximum in strong acid. Inactivation of the enzyme is clearly the result of modification of the flavin, because the modified flavin could be removed by dialysis versus 2 M KBr as with native enzyme (48) and the resulting apoenzyme could be quantitatively reconstituted with FMN with full return of catalytic activity (results not shown). Although this point has not been checked with the other enzymes studied, it is reasonable to assume that it may be a general phenomenon, i.e. the inactivation of flavoproteins by Ph$_2$I$^-$ is caused by the chemical modification of the flavin prosthetic group.

$p$-Hydroxybenzoate Hydroxylase—In catalytic assays, the inactivation of $p$-hydroxybenzoate hydroxylase by Ph$_2$I$^-$ was very dependent on the substrate (hydroxylation reaction) or effector (stimulation of oxidase reaction) that was employed (Table II). Striking differences are apparent between $p$-hydroxybenzoate as substrate, where rapid inactivation was found, and 2,4-dihydroxybenzoate, where no inactivation was observed. Nevertheless, when reduced enzyme was incubated with Ph$_2$I$^-$, with either substrate, phenyl adduct formation was found, at approximately the same rate, with essentially complete inactivation of the enzyme when subsequently assayed with either substrate. Results of a typical experiment, in the presence of $p$-hydroxybenzoate, are shown in Fig. 6. At the concentration of Ph$_2$I$^-$ used (500 $\mu$M), the spectral changes shown are complete within 15 min, with a half time of $70$ s, corresponding to a second order rate constant of $20$ M$^{-1}$ s$^{-1}$. A similar experiment, carried out in the presence of 2,4-dihydroxybenzoate, gave the same spectral changes, with a second
order rate constant of 23 m⁻¹ s⁻¹. In both the cases, the product had an intense green fluorescence, emission maximum at 530 nm and with excitation maxima at 330 nm and 420 nm, characteristics similar to those assigned to a flavin-C8-phenyl adduct. The reaction was also followed in more detail by stopped flow techniques. Enzyme, reduced with a catalytic concentration of NADPH maintained by glucose 6-phosphate and glucose-6-phosphate dehydrogenase in the presence of p-hydroxybenzoate, was reacted with concentrations of Ph₂I⁺ of 0.125, 0.25, 0.5, and 1.0 mM. The reaction curves at all wavelengths studied were fit by single exponential functions, with observed pseudo first order rate constants directly dependent on the Ph₂I⁺ concentration, yielding a second order rate constant for adduct formation of 26 M⁻¹ s⁻¹ (results not shown).

Glucose Oxidase—Diphenyliodonium chloride was without effect on the catalytic activity of glucose oxidase, assayed at its pH optimum of 5.6, and employing the coupled assay for H₂O₂ with horseradish peroxidase and o-dianisidine (33). Not only was it without effect in steady state assays, but it was also without effect on incubation of the glucose-reduced enzyme for prolonged periods (24 h) with 1 mM Ph₂I⁺, at pH 5.6, 7.0, or 8.5. In these experiments, on opening to air and allowing turnover of the enzyme, complete return to the starting spectrum of oxidized enzyme was obtained. This enzyme appears, therefore, to be remarkably resistant to reoxidation of the reduced iron-sulfur chromophores in the inactivated enzyme. The Ph₂I⁺-treated enzyme had less than 0.2% the activity of the starting enzyme in conventional xanthine-oxygen reductase assays (34).

Confirmation that it is the flavin site in xanthine oxidase that reacts with Ph₂I⁺ comes from a study with the deflavo enzyme. This form, which retains the two iron sulfur centers and the molybdopterin center, and is readily reduced by xanthine (34), was reduced with dithionite under anaerobic conditions and then mixed with 0.5 mM Ph₂I⁺. No spectral changes occurred over a period of 2 h, and on admitting air the spectrum of the original deflavo enzyme was slowly regained. Subsequent addition of xanthine resulted in rapid reduction of the deflavo enzyme, as with the untreated enzyme (results not shown).

Flavodoxin—Flavodoxins are low molecular weight, FMN-containing flavoproteins of plant and microbial origin that are involved in numerous single electron transfer reactions (see Ref. 35 for a review). They are characterized by the thermodynamic stability of the flavin semiquinone oxidation state, and hence provide an ideal opportunity to test for the involvement of radical intermediates in the inactivation of flavoproteins by Ph₂I⁺. In the following experiments we have used the flavodoxin of C. beijerinckii, which is known to yield almost quantitative stabilization of the blue neutral semiquinone on photoirradiation in the presence of EDTA (35), and which can also be converted to the fully reduced state photochemically with 5-deaza flavin as photocatalyst. Fig. 8 shows the results of an experiment in which the protein was reduced to the semiquinone level under anaerobic conditions and then reacted with Ph₂I⁺. The reaction was extremely slow, extending over a period of 4 days before completion. The final spectrum clearly showed the absence of semiquinone, with its characteristic absorbance between 600 and 700 nm, and was dominated by a major return of the absorbance of the oxidized protein. There was evidence of some modified flavin, from the absorbance in the 500–600 nm region and in the 300–400 nm region.

By contrast, when fully reduced flavodoxin was mixed with Ph₂I⁺, there was a very rapid reaction, resulting in an initial spectrum with wavelength maxima at 510, 352, and 324 nm (Fig. 9). This reaction is very rapid, requiring stopped flow

**Fig. 6. Reaction of p-hydroxybenzoate hydroxylase with diphenyliodonium chloride.** Conditions were identical to those of Fig. 5, except for pH 7.6. Solid line, spectrum of oxidized enzyme (~35 μM), in the presence of 230 μM p-hydroxybenzoate. Bottom dashed line, enzyme after reduction with NADPH-regenerating system. Subsequent curves with increasing absorbance at 440 nm were obtained at 20, 90, 150, 210, 300, and 480 s after addition of 500 μM Ph₂I⁺.

**Fig. 7. Reaction of milk xanthine oxidase with diphenyliodonium chloride.** The enzyme concentration was ~7.6 μM in 0.02 M sodium pyrophosphate, pH 8.5, 25 °C. Curve 1, oxidized enzyme; curve 2, after addition of an ~2-fold excess of sodium dithionite; curve 3, immediately after addition of 250 μM Ph₂I⁺. This spectrum remained unchanged under anaerobic conditions over a period of 2 h. Curve 4, final stable spectrum 18 h after air reoxidation. Inset, difference spectra; a, curve 3 - curve 2; b, curve 1 - curve 4.
measurement. The observed rate was directly proportional to Ph$_2$I$^+$ concentration, yielding a second order rate constant at pH 7.0, 25 °C of $2.2 \times 10^4$ M$^{-1}$ s$^{-1}$, independent of wavelength (results not shown). The spectrum of the rapidly formed species appears to be the composite of an approximately equal mixture of semiquinone and adduct, with semiquinone again disappearing over a period of 4 days (loss of absorbance in the 600–700 nm region), and clearly with much less return of the absorbance of oxidized flavoprotein. That the initial absorbance in the 600–700 nm region was indeed caused by flavin semiquinone is probably the result of a slow dismutation of the semiquinone to an equimolar mixture of oxidized and reduced flavodoxin, with the reduced flavin form reacting with Ph$_2$I$^+$ as it is formed.

Thus, several cycles of reaction of Fld$_{red}$ with Ph$_2$I$^+$, dismutation, and reaction of the re-formed Fld$_{red}$ would result in a final distribution of 67% adduct and 33% Fld$_{ox}$. By contrast, when the reaction is started with Fld$_{sq}$ and Ph$_2$I$^+$, the final distribution should be 33% adduct and 67% Fld$_{sq}$, close to that actually observed (cf. Fig. 8).

With the above assumption, the spectrum of the adduct was calculated in two ways from the data of Fig. 9. First, from the final spectrum after 4 days of reaction, 33% of the initial oxidized spectrum was subtracted and then multiplied by 100/67 to give an estimate of the spectrum if 100% adduct had been formed. Dashed line, difference spectrum obtained by subtracting 50% of the spectrum of full semiquinone (data obtained during the photoreduction, but not shown) from that of spectrum 3, and multiplying by 2, to account for the presumed equimolar mixture of semiquinone and adduct formed immediately on reaction of Ph$_2$I$^+$ with fully reduced flavodoxin.

adduct, the other flavin semiquinone, in the same way as concluded with free flavins (see Schemes 2–4).

$$
\text{Fld}_{red} + \text{Ph}_2I^+ \rightarrow \text{adduct} + \text{Phi}
$$

$$
\text{Fld}_{red} + \text{Ph}_2I^+ \rightarrow \text{Fld}_{sq} + \text{Ph}^- + \text{Phi}
$$

Reactions 1 and 2

In the case of flavodoxin, the semiquinone appears to be very unreactive, so that the fate of the unstable phenyl radical product is probably dimerization rather than adduct formation. The slow formation of phenyl adduct from the semiquinone is probably the result of a slow dismutation of the semiquinone to an equimolar mixture of oxidized and reduced flavodoxin, with the reduced flavin form reacting with Ph$_2$I$^+$ as it is formed.

$$
2\text{Fld}_{sq} \rightleftharpoons \text{Fld}_{sq} + \text{Fld}_{red}
$$

Reaction 3

Fig. 8. Reaction of flavodoxin semiquinone with diphenyliodonium chloride. 40 μM enzyme in 0.05 M potassium phosphate, pH 7.0, 25 °C was used. Curve 1, system under anaerobiosis before reduction. Curve 2, after careful photoreduction to the maximum semiquinone formation of the semiquinone. These results suggest that the reaction of reduced flavodoxin with Ph$_2$I$^+$ proceeds by two parallel pathways of equal rate, one yielding directly the

Fig. 9. Reaction of fully reduced flavodoxin with diphenyliodonium chloride. Conditions are the same as those detailed in Fig. 8. Curve 1, anaerobic oxidized protein (≈50 μM). Curve 2, after full photochemical reduction. Curves 3–5, spectra 2.5, 25, and 91 h after addition of 500 μM Ph$_2$I$^+$. All spectra have been corrected for dilution.

Fig. 10. Calculated spectra of the phenyl adduct(s) obtained on reaction of reduced flavodoxin with diphenyliodonium chloride (spectra numbers are from experiment shown in Fig. 9). Solid line, difference spectrum obtained by subtracting 33% of spectrum 1 from that of spectrum 7, and multiplying by 100/67 to estimate the spectrum if 100% adduct had been formed. Dashed line, difference spectrum obtained by subtracting 50% of the spectrum of full semiquinone (data obtained during the photoreduction, but not shown) from that of spectrum 3, and multiplying by 2, to account for the presumed equimolar mixture of semiquinone and adduct formed immediately on reaction of Ph$_2$I$^+$ with fully reduced flavodoxin.
Because iodonium compounds such as Ph2I– are well established as essentially irreversible 1-electron oxidants, because of the instability of the primary diphenyliodonyl radical, which decays on a picosecond time scale to phenyl radical and iodobenzene, it is likely that formation of flavin-phenyl adducts would involve flavin radical intermediates, as proposed by earlier workers (5–10). The present study, both with free flavins and flavoproteins, shows that this concept has to be modified to admit the possibility of direct nucleophilic attack of reduced flavin on Ph2I–, or that phenyl adducts are formed only from a caged pair of flavin radical and phenyl radical, rather than from the dissociated free radicals. The results with free flavins indicate that the reactive species is the reduced flavin anion, FIH–, rather than neutral flavin, FIH+. This situation probably applies also with flavoproteins, because NMR studies have indicated that most reduced flavoproteins are in the anion form (36). There are several factors that would be expected to influence the reactivity of a flavoprotein with Ph2I–. Given the unlikelihood of specific binding of Ph2I– at the enzyme active center, a concept supported by the second order character of the interaction, accessibility of Ph2I– to the flavin is an obvious candidate for determining whether any reaction will occur, and if it does, its contribution to rate. A second obvious factor would be expected to be the redox potential of the enzyme flavin, particularly that of the FL+/FL0 couple, because ideally this should be lower than that of the Ph2I–/Ph2I+ couple. Because the flavin species that is reactive with Ph2I– is the fully reduced form, as shown previously (6, 7), and confirmed in the present study, the steady state level of reduced flavin in catalytic turnover would also be expected to be an important factor in whether an enzyme is inactivated by Ph2I–.

The concept that inhibition of catalytic activity by diphenyliodonium and similar compounds is an indicator of a catalytic mechanism involving radical intermediates is clearly not correct, because several enzymes listed in Table II that are inactivated by Ph2I– do not involve flavin radicals in their catalytic mechanism, e.g. lipoamide dehydrogenase, thioredoxin reductase, phenol hydroxylase, and p-hydroxybenzoate hydroxylase. The latter enzyme is particularly informative, because inactivation in steady state assays is very dependent on the substrate or non-substrate effector. Thus inactivation is readily observed with p-hydroxybenzoate as substrate, but no inactivation was observed with 2,4-dihydroxybenzoate as substrate. A series of fluorine-containing substrates lead to inactivation, but at quite different rates, and no inactivation is observed with p-aminobenzoate as substrate or 6-hydroxynicotinate as a non-substrate “effector,” which causes the enzyme to function as an NADPH oxidase rather than as a hydroxylase. The different results with p-hydroxybenzoate hydroxylase do not appear to be explicable in terms of redox potential, because in general the midpoint potential of the enzyme flavin is only marginally different in the presence or absence of substrate (37). It should be noted also that, in general, the enzyme does not stabilize a flavin radical form on 1-electron reduction, indicating that the potential for the EFL+/EFL0 couple must be of the order of 100 mV more positive than that of the midpoint potential, EFL+/EFL0, i.e. ~60 mV (38). With the potential of the Ph2I–/Ph2I+ couple at ~332 mV (39), this difference makes the primary 1-electron reduction of Ph2I– thermodynamically very unfavorable, perhaps accounting at least in part for the slowness of the reaction. However, clearly this is not the reason for the big differences in reactivity in the presence of different substrates. In the case of p-hydroxybenzoate hydroxylase, the chief factor influencing the reactivity with Ph2I– would appear to be level of reduced enzyme in steady state catalysis. Under the assay conditions of Table II, it can be estimated that ~80% of the enzyme should be in the reduced state in steady state turnover with 4-hydroxy benzoate present (40), whereas with 2,4-dihydroxybenzoate less than 1% should be present in the reduced state (41). The same situation is expected in the presence of the effector, 6-hydroxynicotinate, where reduction of the enzyme flavin by NADPH is much slower than its reoxidation by molecular oxygen (42).

Phenol hydroxylase is also readily inactivated during catalytic turnover (Table II). Again, it can be estimated both with phenol and resorcinol as substrates that appreciable concentrations (30–60%) of the enzyme should be in the reduced form in the steady state of catalysis (43, 44).

Although with these two enzymes the steady state level of the reduced flavin would appear to provide a good rationale for the results, this explanation is not necessarily valid in general. For example, with another flavoprotein monooxygenase, MHPC oxygenase, it can be calculated with MHPC as substrate, ~80% of the enzyme should be in the reduced state in turnover, and with 5-hydroxynicotinate, ~60% should be in the reduced form (45, 46). However, no inactivation with Ph2I– was found with either substrate. With this enzyme the flavin radical state is stabilized on 1-electron reduction, with the potential for the EFL+/EFL0 couple estimated at ~48 mV in the presence of MHPC and ~82 mV in the presence of 5-hydroxynicotinate. Although these values indicate that the reaction of the reduced enzyme with Ph2I– is thermodynamically unfavorable, the situation is no worse than with p-hydroxybenzoate hydroxylase, where the potential of the EFL+/EFL0 couple is equally high (see discussion above). The fact that the redox potential is not the only factor determining reactivity is clear from Table II, e.g. both d-aminoc acid oxidase and Old Yellow Enzyme have redox potentials for the EFL+/EFL0 couple of ~204 and ~215 mV, respectively (47, 48), values more thermodynamically favorable for the reaction, yet no inactivation with Ph2I– was found in steady state assays. In such cases, presumably lack of accessibility of Ph2I– to the flavin, or electrostatic repulsion by suitably positioned positively charged residue(s) in the vicinity of the flavin, must be dominant in preventing reaction. In the case of glucose oxidase, which is remarkably resistant to reaction with Ph2I–, the flavin is well buried in the protein structure, accessible through a funnel-shaped pocket, which can accommodate only a single substrate molecule (49). With this protein, difficulty of access would therefore appear to be responsible for the nonreactivity with Ph2I–.

The reaction of reduced flavodoxin with Ph2I– is quite remarkable, because it results in the very rapid (k = 2.2 × 1011 M–1 s–1 at pH 7, 25 °C) equimolar mixture of flavin-C8-phenyl adduct and flavodoxin semiquinone. The very slow further reaction of the semiquinone appears to be a result of the slow disproportionation of the semiquinone to an equimolar mixture of oxidized and reduced flavoprotein, with the latter reacting with Ph2I– as it is formed. With flavodoxin the only part of the flavin that is readily accessible to solvent is the benzene sub-nucleus (35), providing a good rationale for the reaction product being a flavin-C8 adduct. Similar accessibility of the flavin-C8 position in p-hydroxybenzoate hydroxylase (50) and the Old Yellow Enzyme (51) could explain why flavin-C8-phenyl adducts are also formed with these enzymes. What remains as an intriguing unanswered problem is the large difference in reaction rates, 0.6 M–1 s–1 for Old Yellow Enzyme, 26 M–1 s–1 for p-hydroxybenzoate hydroxylase, and 2.2 × 104 M–1 s–1 for flavodoxin, especially because the latter value is 50–100 times greater than for reaction with free flavin.

Acknowledgments—We thank Drs. Y. V. S. N. Murthy and Younus Meah for help with collection and interpretation of NMR data and Drs. S. Bruce Palfrey and David Ballon for many fruitful discussions.
REFERENCES

1. Banke, D. F. (1986) *Chem. Rev.*, **66**, 243–286
2. Kunze, A., Muller, U., Tittes, K., Fouassier, J. P., and Mortillet-Savary, F. (1997) *J. Photochem. Photobiol. A: Chem.* **110**, 115–122
3. Goetz, M., Eckert, G., and Muller, U. (1999) *J. Phys. Chem. A* **103**, 5714–5721
4. Henning, H., Brede, O., Billing, R., and Shinewerk, J. (2001) *Chem. Eur. J.* **7**, 2114–2121
5. Yea, C. M., Cross A. R., and Jones, O. T. G. (1990) *Biochem. J.* **265**, 95–100
6. O’Donnell, V. B., Tew, D. G., Jones, O. T. G., and England, P. J. (1993) *Biochem. J.* **290**, 41–49
7. Tew, D. G. (1993) *Biochemistry* **32**, 10209–10215
8. O’Donnell, V. B., Smith, G. C. M., and Jones, O. T. G. (1994) *Mol. Pharmacol.* **46**, 778
9. Stuehr, D. J., Fasehun, O., Kwon, N. S., Gross, S. S., Gonzalez, J. A., Levi, R., and Nathan, C. F. (1991) *FASEB J.* **5**, 103–103
10. Coves, J., Lebrun, C., Gervasi, G., Dalbon, P., and Fontecave, M. (1999) *Biochem. J.* **342**, 465–472
11. Saito, K., Thiele, D. J., Davis, M., Lockridge, O., and Massey, V. (1991) *J. Biol. Chem.* **266**, 20720–20724
12. Swoboda, B. E. P., and Massey, V. (1965) *J. Biol. Chem.* **240**, 2209–2215
13. Fitzpatrick, P. F., and Massey, V. (1982) *J. Biol. Chem.* **257**, 1166–1171
14. Massey, V. (1960) *Biochim. Biophys. Acta.* **37**, 314–322
15. Massey, V., Brumby, P. E., Komai, H., and Palmer, G. (1969) *J. Biol. Chem.* **244**, 1682–1692
16. Strickland, S., and Massey, V. (1973) *J. Biol. Chem.* **248**, 2944–2952
17. Ploewler, J. B., Dugay, S., Massey, V., and Ballou, D. P. (1987) *J. Biol. Chem.* **262**, 69–74
18. Chaiyen, P., Ballou, D. P. and Massey, V. (1997) *Proc. Natl. Acad. Sci. U. S. A.*, **94**, 7233–7238
19. Maeda-Yorita, K., and Massey, V. (1993) *J. Biol. Chem.* **268**, 4134–4144
20. Palley, B. A., Ents, B., Ballou, D. P., and Massey, V. (1994) *Biochemistry* **33**, 1545–1554
21. Patil, P. V., and Ballou, D. P. (2000) *Anal. Biochem.* **286**, 187–192
22. Press, W. H., Teukolsky, S. A., Vetterling, W. T., and Flannery, B. P. (1992) *Numerical Recipes in C, the Art of Scientific Computing*, 2nd Ed., Cambridge University Press, Cambridge, United Kingdom
23. Muller, F. (ed) (1991) *Chemistry and Biochemistry of Flavoenzymes*, Vol. I, pp. 1–71, CRC Press, Boca Raton, FL
24. Daplatte, G., and Jonah, C. D. (1984) *Radiat. Phys. Chem.* **24**, 557
25. Land, E. J., and Swallow, A. J. (1969) *Biochemistry* **8**, 2117–2125
26. Massey, V., Palmer, G., and Ballou, D. P. (1973) in *Oxidases and Related Redox Systems* (King, T. E., Masen, H. S., and Morrison, M., eds) pp. 25–49, University Park Press, Baltimore
27. Hemmerich, P., Ghisla, S., Hartmann, U., and Muller, F. (1971) in *Flavins and Flavoproteins* (Kamin, H., ed) pp. 83–105, University Park Press, Baltimore
28. Mayhew, S. G., and Strating, M. J. (1975) *Eur. J. Biochem.* **59**, 539–544
29. Mayhew, S. G., and Massey, V. (1984) in *Flavins and Flavoproteins* (Braay, R. C., Engel, P. C., and Mayhew, S. G., eds) pp. 261–267, de Gruyter, Berlin
30. Muller, F., Vervoort, J., van Mierlo, C. P. M., Mayhew, S. G., van Berkel, W. J. H., and Bacher, A. (1987) in *Flavins and Flavoproteins* (Edmonson, D. E., and McCormick, D. B., eds) pp. 261–270, de Gruyter, Berlin
31. Brustle, M., Knapp, W. R., and Hemmerich, P. (1971) *Angew. Chem. Int. Ed. Engl.* **10**, 894–896
32. Moosen, C. T. W., Vervoort, J., and Muller, F. (1984) *Biochemistry* **23**, 4859–4867
33. Lockridge, O., Massey, V., and Sullivan, P. A. (1972) *J. Biol. Chem.* **247**, 8097–8106
34. Komai, H., Massey, V., and Palmer, G. (1969) *J. Biol. Chem.* **244**, 1692–1700
35. Ludwig, M. L., and Luschinsky, C. L. (1992) in *Chemistry and Biochemistry of Flavoenzymes* (Mueller, F., ed) Vol. III, pp. 427–466, CRC Press, Boca Raton, FL
36. Muller, F. (1991) in *Chemistry and Biochemistry of Flavoenzymes* (Muller, F., ed) Vol. III, pp. 557–595, CRC Press, Boca Raton, FL
37. Moran, G. R., Entsch, B., Palley, B. A., and Ballou, D. P. (1996) *Biochemistry* **35**, 9278–9285
38. Entsch, B., Palley, B. A., Ballou, D. P., and Massey, V. (1991) *J. Biol. Chem.* **266**, 17341–17349
39. Beringer, F. M., and Messing, S. (1972) *J. Org. Chem.* **37**, 2484–2489
40. Howell, L. G., Spector, T., and Massey, V. (1972) *J. Biol. Chem.* **247**, 4340–4350
41. Spector, T., and Massey, V. (1972) *J. Biol. Chem.* **247**, 4679–4687
42. Spector, T., and Massey, V. (1972) *J. Biol. Chem.* **247**, 7123–7127
43. Detmer, K., and Massey, V. (1984) *J. Biol. Chem.* **259**, 11265–11272
44. Xu, D., Ballou, D. P., and Massey, V. (2001) *Biochemistry* **40**, 12369–12378
45. Chaiyen, P., Brissette, P., Ballou, D. P., and Massey, V. (1997) *Biochemistry* **36**, 2612–2621
46. Chaiyen, P., Brissette, P., Ballou, D. P., and Massey, V. (1997) *Biochemistry* **36**, 2600–2607
47. Van den Berghe-Snoek, S., and Stankovich, M. T. (1985) *J. Biol. Chem.* **260**, 3373–3379
48. Stewart, R. C., and Massey, V. (1985) *J. Biol. Chem.* **260**, 13639–13647
49. Hecht, H. J., Kalisz, H. M., Hendle, J., Schmid, R. D., and Schomburg, D. (1993) *J. Mol. Biol.* **229**, 153–172
50. Schreuder, H. A., Prick, P. A. J., Wieringa, B. K., Vriend, G., Wilson, K. S., Hol, W. G. J., and Drenth, J. (1989) *J. Mol. Biol.* **208**, 679–696
51. Fox, K. M., and Karplus, P. A. (1994) *Structure* **2**, 1089–1105