Riboflavin-Catalyzed Photooxidative Decarboxylation of Dihydrophthalates*

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ABSTRACT: Riboflavin or 3-methyllumiflavin catalyze the photooxidation of 1,2-dihydrophthalates to yield benzoic acid or methyl benzoate. The rate of reaction is favored by the ionization of the carboxyl group of the reductant and impeded by the ionization of the flavin. The reaction proceeds through a reactive complex as suggested by kinetic and thermodynamic parameters. Based upon the products formed and the lack of sensitivity to ionic strength, it is concluded that the reaction involves radical production. In the case of the dimethyl esters of the 1,2-dihydrophthalic acids, this radical mechanism produces an alkylated flavin rather than a benzenoid product. However, at pH 11, this adduct will rearrange to yield dimethyl phthalate. A mechanism is proposed not only for this reaction but for similar flavin-catalyzed photooxidations. Also, this mechanism explains the photoalkylation of flavins as found in this study and by others.

Flavoenzymes catalyze many types of redox reactions; however, the reaction most unique to flavins is that involving unsaturation α,β to a carbonyl group. Our studies have been directed toward developing a model system that would reflect the uniqueness of this reaction. Two systems have been developed; one, reported here, is an oxidative decarboxylation that is photocatalyzed; the other, to be reported subsequently, is a dehydrogenation that proceeds in the dark.

Many studies have been made on flavin-catalyzed photoreactions. Most of these have involved amino acids and amines (Galston, 1949; Frisell *et al.*, 1959; Enns and Burgess, 1965; McCormick *et al.*, 1967; Byrom and Turnbull, 1968; Penzer and Radda, 1968). Some have involved carboxylic acids (Hendriks and Berends, 1958; Hemmerich *et al.*, 1967; Penzer and Radda, 1968; Bruestlein and Hemmerich, 1968), but only one (Carr, 1961) has led to α,β unsaturation. The studies presented here will suggest a similar, if not a common, mechanism for all of these reactions.

Experimental Procedures

Materials. The *cis*- and *trans*-1,2-dihydrophthalic acids were prepared by the method of Baeyer (1892). Dimethyl esters of the acids were prepared by allowing the acids (5 g) to stand for several days in absolute methanol (20 ml) which had been saturated with dry HCl. The *cis*- and *trans*-diesters were isolated and characterized by their ultraviolet spectra which were similar to those of the corresponding protonated acids. Their boiling points could not be determined due to darkening and polymerization. The monomethyl ester of the *cis*-acid (mp 101–103°) was prepared by reaction of methanol with the anhydride which has been prepared as described by Baeyer (1892).

Riboflavin was obtained gratis from Sigma Chemical Co. 3-Methyllumiflavin was a gift from Dr. Peter Hemmerich which was obtained by Dr. David Metzler. All other materials were commercial products and were used as supplied.

A Cary Model 14 spectrophotometer was used in all the studies.

Methods. Anaerobic kinetic studies were conducted in modified Thunberg tubes having a 1-cm spectrophotometer cell attached at the bottom. The flavin and buffer were placed in the tube and the reductant in the side arm. After two evacuations, for 2 min each, with a water aspirator and a vacuum pump, the tube was placed at 30° (or at the appropriate temperature) for 10 min, in the dark. The solutions were mixed and the zero time absorbance was measured at 445 nm. Subsequently, the tube was irradiated for 2-min intervals by a 100-W tungsten bulb at 30 cm and serial absorbance readings were recorded. Another tube, without reductant, served as the control. In a typical experiment, the concentrations of the reductant and flavin were about 1×10^{-3} and 1×10^{-5} M, respectively, and the buffer was at 0.05 ionic strength.

Aerobic kinetic studies were done in a similar fashion, but the tubes were not evacuated.

The studies on the effect of pH, ionic strength, heat, and concentrations were conducted aerobically in a Pyrex tube of the following dimensions: inside diameter 1.92 cm, and wall thickness 0.11 cm. This tube was placed 1 cm from the inside wall of a glass water bath and irradiated with a 100-W tungsten bulb at a distance of 30 cm. Samples were drawn at intervals and placed in a 1-cm spectrophotometer cell. The spectra of these aliquots were determined from 215 to 300 nm. In all cases, a riboflavin blank was used as the reference because riboflavin has a high absorbance in this region.

In a typical experiment, the reaction solution contained 2.50 ml of riboflavin (1.4×10^{-4} M), 1.50 ml of reductant (3×10^{-3} M), 7.50 ml of buffer (0.1 ionic strength), and 3.50 ml of water. All components were mixed, in the dark, in the reaction tube which was covered by a black plastic sleeve. The covered reaction tube was placed in the water bath for 10 min prior to removing the sleeve.

Product identification studies were done aerobically in the

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TABLE I: Identification of Reaction Products by Thin-Layer Chromatography.[a]

Compound	R_F	Compounds Found from the Oxidation of		
		trans-Diacid	*cis*-Diacid	*cis*-Half-Ester
trans-1,2-Dihydrophthalic acid	0.40	X[b]		
cis-1,2-Dihydrophthalic acid	0.21		X	
Methyl hydrogen 1,2-dihydrophthalate	0.50			X
Benzoic acid	0.91	X	X	
Methyl benzoate	0.80			X
Phthalic acid	0.15			
Methyl hydrogen phthalate	0.36			

[a] Thin-layer chromatography on silica gel. Solvent systems were 80 ml of chloroform–20 ml of cyclohexane–10 ml of glacial acetic acid for the acids and only chloroform for the esters. [b] X indicates that the compound on the left was found in the reaction products from the oxidation of the compound given in the column heading.

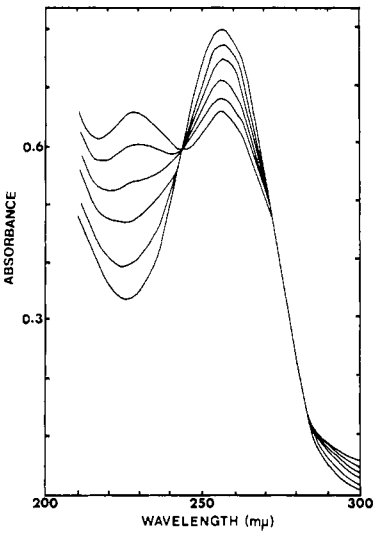

FIGURE 1: Successive spectra of the photoreaction solution of riboflavin and *trans*-1,2-dihydrophthalic acid with a solution of riboflavin as the reference. The experiment was conducted in 0.05 ionic strength phosphate buffer at pH 7.0. Curves represent 0, 4, 8, 12, 16, and 20 min, respectively, beginning with the curve lowest at 230 nm.

tube system described above at pH 7.0. After irradiation for 60 min, the reaction mixtures were taken to dryness by evaporation or lyophilization. An ethanol or ether extract of the residue was applied to a thin-layer chromatogram. The solvent systems were composed of 80 ml of chloroform, 20 ml of cyclohexane, and 10 ml of glacial acetic acid (acids) (Stahl, 1965) or chloroform only (esters). After development, spots of the appropriate R_F values were removed and the products were eluted with 0.1 M phosphate buffer (pH 7.0). The spectra of the products were obtained and compared with standards. Solutions of the dihydro acids or esters were treated in the same manner to ensure that the changes were due to the reaction and not to the isolation procedure.

Results

Reduction of the Flavins. The 1,2-dihydrophthalates (acids and esters) were investigated as to their ability to reduce riboflavin or 3-methyllumiflavin at pH 4, 7, and 11 under anaerobic conditions. In all cases, with the reductant in excess, the reduction of the flavin followed first-order kinetics. In most cases, the reduced flavin could be reoxidized completely upon the entrance of air into the reaction vessel. The exceptions were the diesters of both the *cis*- and *trans*-acids at pH 4 and 7. The explanation and importance of these two reactions will be discussed later. Also, at pH 11, the esters reduced the flavin in the dark. The studies of this system will be the subject of a subsequent paper (Weatherby and Carr, 1970).

Identification of Products. When *cis*- or *trans*-1,2-dihydrophthalic acid or methyl hydrogen *cis*-1,2-dihydrophthalate serve as reductants, one could anticipate the possibility of two reactions occurring: (1) dehydrogenation to yield phthalic acid or methyl hydrogen phthalate and (2) oxidative decarboxylation to yield benzoic acid or methyl benzoate. In order to identify the products arising from the dihydro acids or esters, the reaction was conducted under aerobic conditions at pH 4. With these conditions, the flavin remained in the oxidized form and it was possible to use the total spectra of the reaction solutions to follow the progress of the oxidations and also to identify the products. Figure 1 shows successive spectra of the photoreaction solution using riboflavin and *trans*-1,2-dihydrophthalic acid. Similar results were obtained using *cis*-1,2-dihydrophthalic acid and methyl hydrogen *cis*-1,2-dihydrophthalate as reductants. Examination of the spectral changes suggested that the products were benzoic acid or its ester; therefore, oxidative decarboxylations had occurred. Molar absorptivities were determined for all reactants and products at pH 4 and the molar concentrations were calculated for each time point. When the log of the fraction of the reductant remaining was plotted versus time, a straight line was obtained, indicating that the reaction was first order with respect to the reductant concentration.

To further verify that the products were benzoates, thin-layer chromatographic isolations were made (Table I).

When either *cis*- or *trans*-1,2-dihydrophthalic acid served as the reductant, only two migrating components could be found on the thin-layer plates. These components had R_F values which corresponded to the original reductant and benzoic acid. When methyl hydrogen *cis*-1,2-dihydrophthalate was the reductant, the only migrating components found corresponded to the *cis*-half-ester and methyl benzoate.

Elution of the thin-layer plates in the regions with R_F values corresponding to benzoic acid, resulted in solutions whose spectra were identical with authentic benzoic acid. The component which migrated with an R_F value corresponding to methyl benzoate was also eluted, and its spectrum was identical with that of authentic methyl benzoate.

Stoichiometry of the Reactions. As pointed out above, the

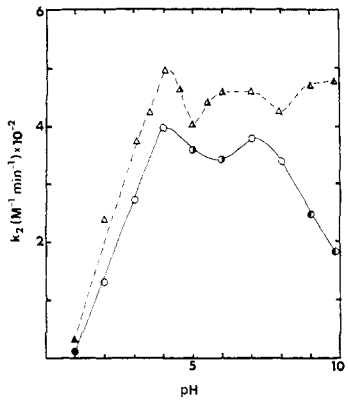

FIGURE 2: The pH dependency of the flavin-catalyzed photooxidation of *cis*-1,2-dihydrophthalic acid. All reactions were at 30° and 0.05 ionic strength. (—) Using riboflavin; (- - -) using 3-methyllumiflavin. The buffers used were (▲, ●) nitrate, (△, ○) phosphate, (◭, ◑) cacodylate, (◭, ◐) carbonate.

kinetic analyses showed that the reactions could be made to be pseudo-first order in both flavin and reductant and therefore, the reactions would have overall second-order kinetics. However, as will be shown later, the reactions probably do not occur by simple bimolecular collisions. Evidence will support the existence of a reactive complex prior to the actual electron transfer.

Consequently, the stoichiometries of the reactions were determined based upon the amount of flavin reduced and the amount of isolated product. Table II shows that the photooxidations occur by 1 mole of reductant reducing 1 mole of flavin.

Effect of pH. Since it was assured that the reactions were single product oxidations, some of the conditions were varied. The effect of pH was seen in some of the early experiments where the reductants were screened for reducing ability at pH 4, 7, and 11.

The rates of the photocatalyzed flavin oxidations of *cis*-

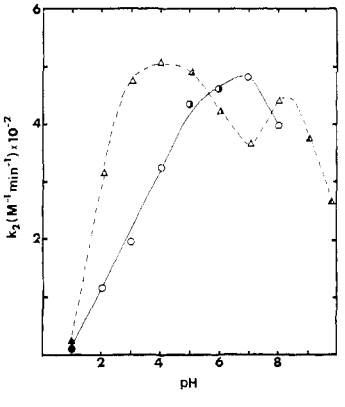

FIGURE 3: The pH dependency of the flavin-catalyzed photooxidation of *trans*-1,2-dihydrophthalic acid and methyl hydrogen *cis*-1,2-dihydrophthalate. All reactions were at 30° and 0.05 ionic strength. (- - -) Using *trans*-1,2-dihydrophthalic acid; (——) using methyl hydrogen *cis*-1,2-dihydrophthalate. The buffers used were (▲, ●) nitrate, (△, ○) phosphate, (◭, ◑) cacodylate, (◭, and ◐) carbonate.

TABLE II: Stoichiometry of the Riboflavin-Catalyzed Photooxidation of the Dihydrophthalates.

Reductant	Benzoic Acid or Methyl Benzoate Formed (μmoles)	Riboflavin Reduced (μmoles)
trans-1,2-Dihydrophthalic acid	86.1	85.8
cis-1,2-Dihydrophthalic acid	77.5	76.7
Methyl hydrogen *cis*-1,2-dihydrophthalate	38.2	38.0

and *trans*-1,2-dihydrophthalic acids and methyl hydrogen *cis*-1,2-dihydrophthalate *vs.* pH are shown in Figures 2 and 3. The rapid decrease, in all three cases, of the rate of the reaction as the pH is decreased from 4 to 1 may be a reflection of two phenomena. Proton quenching of the flavin fluorescence becomes extensive in this region (Ellinger and Holden, 1944; Kavanagh and Goodwin, 1949). At pH 1.0 about 85% of the flavin fluorescence has been quenched when compared with pH 4.0. If the triplet state is indeed the reactive species in photocatalyzed flavin oxidations, as has been supported by the work of several investigators (Radda and Calvin, 1964; Penzer and Radda, 1967; Byrom and Turnbull, 1967; Penzer and Radda, 1968; Byrom and Turnbull, 1968), then one would expect the observed marked decrease in the rate of the reaction in this pH region.

The degree of protonation of the carboxyl groups of the reductants is a second factor which may effect the reaction rate in this pH region. If the flavin triplet behaves as an electrophilic radical, as suggested by Penzer and Radda (1968), the ionized carboxyl group could serve as the center of attack. Protonation of the carboxyl group, which would occur in this pH range, would thus decrease the rate of the reaction. It should be noted that neither the *cis*- nor *trans*-dimethyl ester of the corresponding 1,2-dihydrophthalic acid would reversibly photoreduce flavins at any pH below 8.0. This suggests that the free carboxyl group is essential for the reaction, and the ionized form is probably the reactive species. However, both factors, proton fluorescence quenching and the degree of ionization of the carboxyl group, probably effect the rate of the reaction in this pH region.

The rapid decrease in the rates of the reactions at pH values greater than 8.0 probably reflects the ionization of the hydrogen of the 3 position of riboflavin. This hydrogen has been reported (Suelter and Metzler, 1960) to have a pK_a of 10. This analysis is supported by the fact that when 3-methyllumiflavin is used in place of riboflavin, one does not observe the decrease in the reaction rate at pH values greater than 8.0. Figure 3 indicates that the rate of 3-methyllumiflavin oxidation of *cis*-1,2-dihydrophthalic acid is relatively constant from pH 5.0 to 10.0. These results are consistent with the findings of Carr and Metzler (unpublished results, 1960; Carr, 1961).

No explanation is offered for the pH effects on the reaction

TABLE III: Thermodynamic Parameters for the Riboflavin-Catalyzed Photooxidation of the Dihydrophthalates.[a]

Reductant	pH 4		pH 7			
	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	pK_1	pK_2
cis-1,2-Dihydrophthalic acid	+2.2	−69	−2.8	−85	3.00	5.30
trans-1,2-Dihydrophthalic acid	+0.46	−75	−2.6	−85	3.55	5.10
Methyl hydrogen *cis*-1,2-dihydrophthalate	−3.4	−88	−2.8	−85	3.90	

[a] Experiments were conducted aerobically at pH 4 and 7, phosphate buffer at 0.05 ionic strength. $\Delta H^{\ddagger}$ values are in kcal/mole; $\Delta S^{\ddagger}$ values are in cal/deg per mole.

rates in the pH region 4 to 8. Since no sound theoretical basis is available for interpreting the data in this region, any explanation for the subtle effects which were observed would be pure speculation.

Effect of Ionic Strength. The photocatalyzed riboflavin oxidations of *cis*- and *trans*-dihydrophthalic acids and methyl hydrogen *cis*-1,2-dihydrophthalate were only slightly effected by the ionic strength in the range of 0.025 to 0.75. In general, there was a slight decrease in the rate of the reaction with increasing ionic strength. This effect would be expected if the reactions proceed by a radical mechanism. An alternative explanation would be that a complex between the riboflavin and the reductants protects any polar intermediates, if formed, from the solvent. Evidence will be presented in a later section which demonstrates the formation of a complex between the riboflavin and the reductants, but it is felt that the ionic strength effect best reflects the nonpolar, radical nature of these reactions.

Effect of Temperatures. Pseudo-first-order rate constants were determined for the oxidation of the dihydrophthalates at 10 to 60° and at pH 4 and 7. Upon plotting the data in terms of the Eyring equation (Wynne-Jones and Eyring, 1935), the thermodynamic values of Table III were obtained. When comparing the results at pH 4 and 7, it can be seen that the enthalpy of activation at pH 4 decreases as one proceeds to structures which have less stabilization of the monoionized carboxylate due to intramolecular hydrogen bonding. The monoionized *cis*-1,2-dihydrophthalic acid would be expected to form a much stronger intramolecular hydrogen bond than would the monoionized *trans*-1,2-dihydrophthalic acid and the ionized *cis*-half-ester is not capable of such bonding. The importance of a free ionized carboxyl group as the site of attack by the photoexcited flavin has already been mentioned in regard to the effect of pH.

The methyl hydrogen *cis*-1,2-dihydrophthalate, in its monoionized form, can not form intramolecular hydrogen bonds at pH 4. In this case as well as all those at pH 7, one observes a negative enthalpy of activation. This would be the expected effect if the *cis*-half-ester or dianion acids formed complexes with the photoexcited flavin prerequisite to the oxidations. Increasing the temperature could slow the rate of the reactions by decreasing the life time of the complexes so that there is insufficient time for the transfer of electrons from the reductant to the photoexcited flavin. Kinetic evidence will be presented in the next section to support the hypothesis of such complexes.

Kinetic Analyses. Although some kinetic data were obtained in the initial screening experiments, more detailed studies were made at pH 4 to clarify the role of the reactive complex suggested above. The reactions were conducted both anaerobically (reduction of the flavin) and aerobically (appearance of product). The rate constants from both methods agreed. Plots of the reciprocal of the pseudo-first-order rate constants *vs.* the reciprocal of the reductant concentrations were made. The plots resemble those made for enzyme kinetic results, which are used to determine K_m and V_{max}. Figure 4 shows the results of such a plot for methyl hydrogen *cis*-1,2-dihydrophthalate. Similar plots were obtained for both *cis*- and *trans*-1,2-dihydrophthalic acids.

From these results it is apparent that a reactive complex is formed between the riboflavin and the dihydrophthalates prerequisite to the redox reactions. If the reaction followed collision kinetics, the extrapolated line in Figure 4 would have passed through the origin. The dissociation constants (K_{diss}) for the reactive complexes were calculated from the straight-line portions of the plots. These are given in Table IV.

Similar studies were made at other concentrations of riboflavin and the dissociation constants were found to be inde-

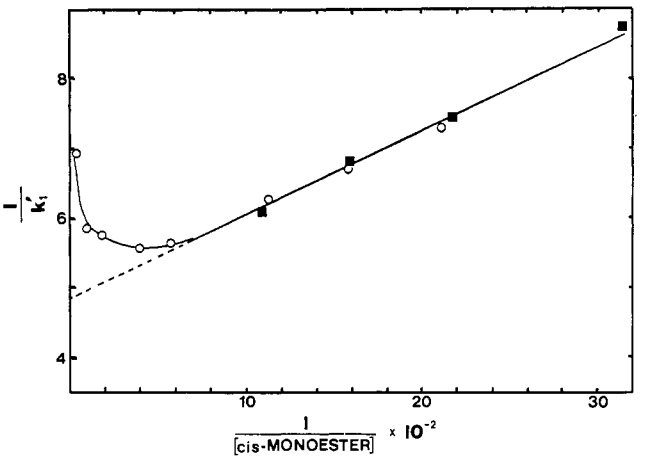

FIGURE 4: Effect of reductant concentration on the flavin-catalyzed photooxidation of methyl hydrogen *cis*-1,2-dihydrophthalate. Experiments were conducted in 0.05 ionic strength phosphate buffer at pH 4.0 and at 30°. The riboflavin concentration was 3×10^{-5} M; (○) Anaerobic determinations, (■) aerobic determinations.

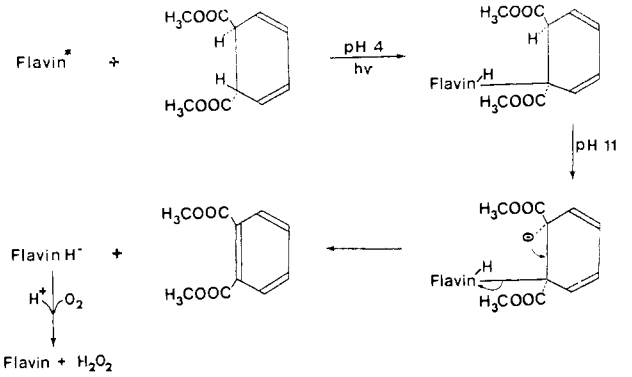

FIGURE 5: Proposed reaction of flavin with dimethyl *trans*-1,2-dihydrophthalate. Flavin* is photoexcited flavin. Flavin H^- is the anion of flavin hydroquinone.

pendent of the initial riboflavin concentration and, as would be expected, the maximal rate did change proportionately.

Figure 4 illustrates that an inhibition of the reactions occurred at high 1,2-dihydrophthalate concentrations. It was felt that this inhibition could be due to quenching of the riboflavin fluorescence by the dihydrophthalates. Calculations based upon the percent inhibition at the several points resulted in the inhibition constants (K_I) given in Table IV. Also, given in the table are dissociation constants for riboflavin–reductant complexes as determined by fluorescence quenching studies. The agreement is very good considering the different methods used in obtaining the constants.

Irreversible Reduction of Flavin. As mentioned in the section on reduction of the flavins, when solutions of riboflavin and the dimethyl ester of either *cis*- or *trans*-1,2-dihydrophthalic acid were irradiated at pH 4.0 under anaerobic conditions, a decrease in the flavin absorbance at 445 nm was observed. However, upon opening the Thunberg tube and bubbling air through the reaction solution for 1 min, no reoxidation of the riboflavin occurred. Under these conditions any flavohydroquinone which was present would have been oxidized to the flavoquinone. Under the same conditions, control experiments which lacked only the diesters, resulted in only a negligible decrease in the flavin absorbance at 445 nm. It was therefore felt that the irreversible photoreduction of the riboflavin was due to an interaction between the diester and the riboflavin. Two possibilities were considered: (a) The diester might labilize the riboflavin toward photodecomposition, or (b) an alkylated flavohydroquinone which is resistant to reoxidation is formed, as has been reported by Hemmerich *et al.* (1967).

If an alkylated flavohydroquinone were indeed formed in this system, it was felt that either the 1 or 2 carbon of the 1,2-dihydrophthalate diesters would serve as the carbon bonded to the flavin. The alkylation could occur by attack of the photoexcited flavin on either the 1 or 2 hydrogen; the resulting alkyl radical and flavosemiquinone would then pair to give the alkylated flavohydroquinone. Models, constructed from CPK space-filling atomic models, of the alkylated flavohydroquinone indicate that the 5 position of the flavin could not be the site of attachment on the flavin due to steric hindrance. The oxygen on the 4-position carbon of the flavin is a doubtful site of attachment. Walker *et al.* (1967) have

TABLE IV: Dissociation Constants for the Interactions of Riboflavin with the Dihydrophthalates.

Reductant	K_I[a] ($\times 10^{-2}$ M)	K_S[b] ($\times 10^{-2}$ M)	K_{diss}[c] (M)
cis-1,2-Dihydrophthalic acid	4.6	6.3	5.8×10^{-5}
trans-1,2-Dihydrophthalic acid	3.2	5.5	5.6×10^{-5}
Methyl hydrogen *cis*-1,2-dihydrophthalate	5.4	3.8	2.5×10^{-4}

[a] K_I = dissociation constant determined from the inhibition shown in Figure 4. [b] K_S = dissociation constant determined from fluorescence quenching experiments. [c] K_{diss} = dissociation constant determined from the kinetic results shown in Figure 4.

shown that such an O-alkylated flavohydroquinone is easily oxidized by air, while the alkylated flavohydroquinone formed in this study was resistent to air oxidation. The nitrogen at position 1 and the carbon atoms (1a and 4a) at the ring juncture could serve as sites of alkylation for the postulated alkylated flavohydroquinone (Walker *et al.*, 1967).

It was felt that it should be possible to displace the alkyl group from the alkylated flavohydroquinone by raising the pH to 11.0. Appreciable ionization of the hydrogen which is α to the ester group would occur at this pH. Figure 5 illustrates this postulated reaction. Dimethyl phthalate and the reduced flavin would be the products of this displacement. The reduced flavin which is formed should then be easily reoxidized by the oxygen which is dissolved in the reaction solution. Table V shows the results of experiments designed to test this hypothesis.

Table V demonstrates that only the postulated alkylated flavohydroquinone which was formed from the dimethyl ester of the *trans*-1,2-dihydrophthalic acid resulted in displacement of the alkyl group at pH 11.0. At 11.0, the flavin was quantitatively reoxidized, which indicated that the displacement had occurred and the dissolved oxygen could reoxidize the displaced flavohydroquinone. The postulated alkylated flavohydroquinone which was formed from the *cis*-diester did not undergo the displacement. No increase in the flavin absorbance at 445 nm was observed when the aerated reaction solution was adjusted to pH 11.0. This result would be expected if the mechanism of the displacement is by a *trans* elimination. Only in the case of the *trans* adduct would the flavin moiety be *trans* to the carbanion which would be formed from the ionization of the hydrogen which is α to the second ester functional group.

This is not conclusive evidence for the formation of the alkylated flavohydroquinones from the *cis*- and *trans*-dimethyl esters. More conclusive proof would be the isolation of the alkylated flavohydroquinone and characterization of its structure as was done by Walker *et al.* (1967). Meager attempts have not yet been successful.

Other Isomers. Not all the isomeric dihydrophthalates have

TABLE V: Anaerobic Photoreduction (Alkylation) of Riboflavin and Subsequent Reoxidation.[a]

Conditions	Absorbance at 445 nm		
	trans-Diester[b]	*cis*-Diester[b]	Control
Zero time	0.350	0.360	0.360
After 10 min of irradiation	0.170	0.175	0.355
After 1 min of aeration	0.170	0.175	0.360
After pH was adjusted to 11.0	0.350	0.180	0.360

[a] Irradiations were performed at pH 4.0, 0.05 ionic strength phosphate. The pH was adjusted to 11.0 by adding 2 N sodium hydroxide. [b] The diesters were dimethyl esters of *cis*- or *trans*-1,2-dihydrophthalic acid.

been synthesized. However, 4,5-dihydrophthalic acid has been synthesized and found to be unreactive in the systems described.

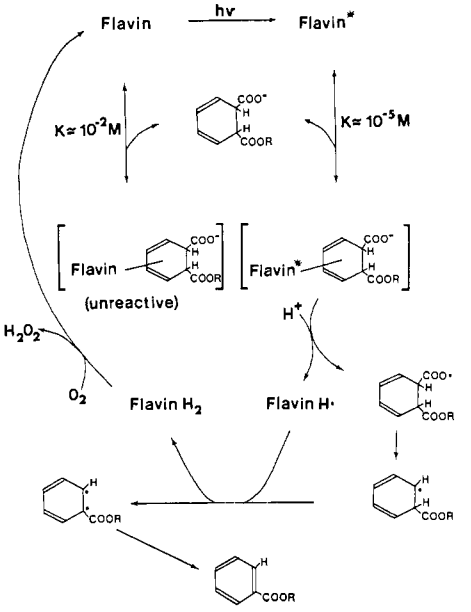

FIGURE 6: Proposed mechanism for the flavin-catalyzed photooxidation of the dihydrophthalates. Flavin* is photoexcited flavin. Flavin H· is the flavin semiquinone. Flavin H_2 is flavin hydroquinone. R is $-CH_3$ or $-H$. • is an unpaired electron.

Discussion

The results of this study clearly establish that the photocatalyzed flavin oxidations of *cis*- and *trans*-1,2-dihydrophthalic acids or methyl hydrogen *cis*-1,2-dihydrophthalate yield 1 mole of benzoic acid or methyl benzoate for every mole of flavin reduced. Analyses of the kinetics, thermodynamics studies, and fluorescence studies all suggest the formation of flavin–dihydrophthalate complexes—some are necessary for reaction and others inhibit the reactions. Figure 6 outlines a proposed pathway for the interactions between flavin and dihydrophthalates that best fits the current information.

When one considers the mechanism for these oxidative decarboxylations, it must be remembered that previous studies have shown that flavin may be reduced by accepting a hydride ion (Suelter and Metzler, 1960; Fox and Tollin, 1966) or alternatively by one electron radical mechanism (Radda and Calvin, 1964; Penzer and Radda, 1967; Byrom and Turnbull, 1967; Penzer and Radda, 1968; Byrom and Turnbull, 1968). In the case of the free acids, a hydride-transfer mechanism would appear to be very attractive. The abstraction of a hydride ion by the flavin from carbon 1 of the dihydro acids would form a carbonium ion at this position. This full positive charge would be in the β position to the carboxyl group which is bonded to carbon 2. This carbonium ion could thus provide the driving force for the decarboxylation. Decarboxylation would result in neutralization of the carbonium ion and establishment of an aromatic ring system. Further, the planar nature of the carbonium ion would explain the observation that both the *cis*- and *trans*-1,2-dihydrophthalic acids give benzoic acid as the sole product. However, with the *cis*-half-ester, this is not the case one would expect the hydride ion to be abstracted from the carbon which is alpha to the free carboxyl group. Decarboxylation cannot occur because there is an ester function which is β to the carbonium ion and the observed product, methyl benzoate, would not be formed. Therefore, a common hydride mechanism would be unlikely.

The results of this study are consistent with previous investigations where the reactions were found to proceed by radical mechanism. Figure 6 presents the mechanism which is proposed for the photocatalyzed flavin oxidations of the 1,2-dihydrophthalates. In the mechanism the photoexcited flavin (triplet state) acts as an electrophilic radical, as suggested by Penzer and Radda (1968), and attacks the ionized carboxyl group. The carboxyl radical which is produced decarboxylates in a reaction similar to the Kolbe electrolysis of carboxylic acids (Weedon, 1952). The alkyl radical which is formed by the decarboxylation has a hydrogen atom abstracted by the flavosemiquinone; this results in the formation of the fully reduced flavin and a diradical. The lone electrons of the diradical can then pair to form the benzenoid ring system.

The effect of pH on the reactions suggested that the ionized free carboxyl group was an important factor since the oxidations closely paralled the ionization of the carboxyl groups. The thermodynamic data suggest that heat energy is necessary to break intramolecular hydrogen bonds to form a fully ionized carboxyl group. The enthalpies of activation for the oxidations of the three dihydrophthalates parallel the degree of intramolecular hydrogen bonding which would be expected for each of the dihydrophthalates. Finally, the fact that the dimethyl esters of the *cis*- and *trans*-1,2-dihydrophthalic acids would not reversibly reduce riboflavin at any pH below 9.0 further supports the ionized free carboxyl group as the center of attack by the photoexcited flavin.

The mechanism which is presented also explains the lack of stereospecificity of the reaction. The planar nature of the alkyl radical (Stewart, 1966) which is formed after the decar-

boxylation would result in a loss of stereochemistry just as a carbonium ion would.

The formation of a N^5-benzylflavohydroquinone from phenylacetic acid (Hemmerich *et al.*, 1967) and of N^5-acetylflavohydroquinone from pyruvate (Bruestlein and Hemmerich, 1968) can also be explained by the mechanism which is presented in Figure 6. In these cases the photoexcited flavin would attack the ionized carboxyl group to yield the flavosemiquinone and the carboxyl radical. The alkyl radicals which would be formed by decarboxylation are not labile to further oxidation by the flavosemiquinone. Radical pairing occurs between the alkyl radicals and the flavosemiquinone with the formation of the N^5-substituted flavohydroquinones.

The photooxidation of amino acids, reported by Frisell *et al.* (1959), can also be encompassed by this mechanism. They demonstrated that the susceptibility of substituted glycines to photooxidation by flavins was increased when the electron density on the amino group was increased. The factors which increased the electron density on the amino group would also increase the electron density of the ionized carboxyl group and attack by the photoexcited flavin on the ionized carboxyl group could occur as proposed by Frisell *et al.* (1959). After the initial formation of the carboxyl radical, decarboxylation would yield the amino-substituted alkyl radical. The flavosemiquinone would now behave as an electrophilic radical and attack the amino group. The resultant Schiff's base would hydrolyze to yield formaldehyde and an amino compound. Frisell *et al.* (1959) also proposed the initial attack of the photoexcited flavin on the amino nitrogen and α carbon. This would undoubtedly result in the formation of the α-keto acid as shown by Byrom and Turnbull (1967) who suggested that the α-keto acid could decarboxylate to yield the aldehyde and CO_2. No experimental evidence was presented to support the latter suggestion.

The irreversible photoreduction of flavin by the dimethyl esters of *cis*- and *trans*-dihydrophthalic acids has suggested the formation of alkylated flavohydroquinones. These alkylated flavohydroquinones may provide an opportunity to correlate the photocatalyzed reactions which have been discussed with the flavin oxidation of carbon–carbon bonds that occur in the dark. These dark reactions will be discussed in a subsequent paper (Weatherby and Carr, 1970).

The extrapolation of the results of this study to a biological system would be quite tenuous. While a radical mechanism has been proposed for the oxidative decarboxylations in this flavin model system, biological oxidative decarboxylations utilize pyridine nucleotides as cofactors. It is generally felt that pyridine nucleotides are reduced by hydride-transfer mechanisms. However, one-electron reductions of nicotinamide analogs have been observed (Sund, 1968). In view of these observations and considering the results of this study, a careful consideration should be given to the possibility that radical mechanisms are operative in such enzymatic systems as malic dehydrogenase (decarboxylating), isocitric acid dehydrogenase, and 6-phosphogluconic acid dehydrogenase.

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