PHOTO-INDUCED REACTIONS OF FLAVINS

by

Maurice Green

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1967
I hereby recommend that this dissertation prepared under my direction by Maurice Green entitled PHOTO-INDUCED REACTIONS OF FLAVINS be accepted as fulfilling the dissertation requirement of the degree of DOCTOR OF PHILOSOPHY.

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Louis Ramsey 12/19/66
Jimmy M. Anderson 12/19/66
J. A. Pote 12/19/66

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SIGNED: Maurice Green
To

GISELE

sine qua non!
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The light-induced reactions of riboflavin, FMN (riboflavin-5'phosphate), and lumiflavin have been investigated using the technique of flash photolysis spectrophotometry. A flash photolysis kinetic spectrophotometer having a response time of 1.4 milliseconds and a sensitivity of 0.001 OD units was constructed for use in these studies. The instrument configuration was that of a double-beam spectrophotometer using a 5 kc modulated monochromatic source and phase-sensitive detection. Actinic light for photolysis was obtained from a pair of GE FT-230 argon flash lamps operated at a maximum of 330 joules. Digital computer analysis was employed for data reduction and preliminary determination of the reaction kinetics.

The degradative photobleaching of FMN in anaerobic aqueous solution, and the flavin-sensitized photooxidation of EDTA, alcohols, glycols, and glycerol, have been
examined. Quenching of these reactions can be produced by addition of KI, by successive flashes, and by increasing the flavin concentration (self-quenching). Studies of these quenching processes indicated that the lowest excited triplet is the photoreactive species. The successive flash effect is due to quenching by a reaction product, while the concentration quenching may be due to either triplet-triplet annihilation or reaction between a ground state flavin and the triplet.

A comparison of the yields for the photooxidation of several alcohols and esters by lumiflavin demonstrated that the hydroxyl hydrogen of the alcohol is abstracted preferentially, and an examination of the flavin spectrum in a series of hydroxylic solvents corroborated the proposal that hydrogen bonding of the flavin was a dominant factor in the reaction.

The use of glycerol as both solvent and reductant provided direct evidence that the initial reaction proceeds by a one-electron oxidation-reduction via the flavin semiquinone. In the case of FMN (and riboflavin) the kinetic results are consistent with an initial intramolecular hydrogen atom transfer, analogous to photo-bleaching in aqueous solution, followed by a reaction of the semiquinone (presumably a biradical) with the reductant which prevents degradation of the ribityl side-chain.
The reoxidation of photoreduced FMN (in aqueous solution with EDTA) by oxygen (autoxidation) was examined. In the tertiary system glycerol:lumiflavin:p-benzoquinone a sequential one-electron transfer at the semiquinone level was demonstrated.
INTRODUCTION

It is common knowledge that the ultimate source of all of our energy and negative entropy is the radiation of the sun. When a photon interacts with a material particle on our globe, it lifts one electron from an electron pair to a higher level...... Life has learned to catch the electron in the excited state, uncouple it from its partner, and let it drop back to the ground state through its biological machinery, utilizing its excess energy for life processes.

ALBERT Szent-Györgyi (1961)

The elucidation of the basic aspects of flavin chemistry in the late 1920's and early 1930's was a significant stride forward in our understanding of the role of vitamins in metabolic processes. Flavoprotein enzymes are involved in a large portion of the oxidation-reduction reactions occurring in biological systems. Much of the interest in flavin chemistry has arisen from the postulated role of flavins as the "cross-over point" from two-electron to one-electron reaction mechanisms in these redox systems.

The second major area of interest is the large number of photochemical processes involving flavins, functioning either directly or as photo-sensitizers. It has been shown (vide infra) that flavins can catalyze the
photooxidation of a wide variety of substrates, including many of biological importance. Flavins have also been implicated in biological processes such as phototropism.

The work to be described here lies wholly within this second realm. Its purpose is to expand the present understanding of the photochemical reactions of riboflavin and its derivatives. In a larger sense, it is an attempt to increase our understanding of the processes involved in the conversion of light energy to chemical energy in biological systems.
Flavin Chemistry

History

Riboflavin (I) (6,7-dimethyl-9-(D-1'-ribityl) isoalloxazine, Rb, vitamin B2, lactoflavin) was first crystallized by Kuhn (Kuhn, Györgyi, and Wagner-Jauregg, 1933). Kuhn and Wagner-Jauregg (1933) observed that in alkaline solution the lactoflavin was photochemically degraded to lumiflavin (II) (6,7,9-trimethylisoalloxazine, Lf).
In acid solution the major photoproduct is the alloxazine derivative, lumichrome (III) (6,7-dimethylalloxazine).

\[ \text{Lumichrome (III)} \]

The discovery of lumiflavin as the photoproduct of Warburg's "old yellow enzyme", a redox enzyme from yeast (Warburg and Christian, 1932, 1933) led to the identification of flavin as the active redox moiety in the yeast enzyme. The naturally occurring forms of flavin are the 5'-phosphate ester (IV) (flavin mononucleotide, FMN, R-5'-P) and the nucleotide of FMN with adenosine monophosphate (V) (flavin adenine dinucleotide, FAD).

\[ \text{FMN (IV)} \]
Approximately fifty flavoprotein enzymes have now been reported (see e.g. Dixon and Webb, 1964, pp. 407-9; Mahler and Cordes, 1966, pp. 572-580). The chemistry and physical properties of the flavins and flavoproteins have been extensively reviewed (Beinert, 1960, 1961; Dudley, et al., 1964; Geissman, 1949; Hemmerich, Veeger and Wood, 1965; Massey and Gibson, 1964; and Strittmatter, 1966).
Role of Free Radicals

Kuhn and Wagner-Jauregg (1934) observed the formation of a red intermediate upon reduction of flavin in HCl. Michaelis and his co-workers confirmed this result using potentiometric titration and magnetic susceptibility measurements. At alkaline or neutral pH the intermediate was green (Michaelis, Shubert, and Smythe, 1936; Michaelis and Schwarzenbach, 1938). These colored intermediates were attributed to the formation of a semiquinoid free radical intermediate (VI). Upon addition of a second electron, the fully reduced colorless "leucoflavin" (VII) is formed (see Figure 1). Michaelis explained these observations in terms of his "principle of compulsory univalent oxidation" which stated that all bivalent oxidation-reductions proceeded by two successive one-electron steps (Michaelis, 1932, 1935, 1946, 1951). Exceptions have now been found to disprove the generality of this theory (Westheimer, 1954, 1962), in particular, the pyridine nucleotides, NAD (DPN) and NADP (TPN), which probably function by a bivalent hydride ion transfer mechanism (Fox and Tollin, 1966). In the mitochondrial electron transport system these coenzymes are reoxidized by a flavoprotein, and the electrons are transferred ultimately to molecular oxygen via the cytochrome system as depicted in Figure 2. Recent studies (Fox and Tollin, 1966) have shown that the
Figure 1. Oxidation States of Flavin
Figure 2. Mitochondrial Electron Transport System
reaction of oxidized flavin with reduced NADH is a two-electron transfer process. The hemoprotein cytochrome system functions via one-electron transfer mechanisms. Thus the flavin is the logical candidate to serve as the transition point from bivalent to univalent oxidation.

In the fifteen year period following 1940 the field of flavin chemistry was relatively quiescent. As is frequently true, it was an advance in technology, in this case two such advances, which led the way to a better understanding of flavin reaction mechanisms. These advances were the development of electron paramagnetic resonance spectrometry (EPR) by Zavoisky in 1945 and flash photolysis by Norrish and Porter (1949). Both of these methods are powerful tools for the study of rapid reactions involving free radicals.

New evidence for the occurrence of free radicals in flavoproteins was provided by Beinert (1956a) who employed a rapid-scanning spectrophotometer (American Optical Co., vide infra) to study reactions of the fatty acyl CoA dehydrogenases. He observed a band in the 500-650 nm region which he proposed was due to a semiquinoid intermediate of the flavin prosthetic group. Later (Beinert, 1956b), in studies of the reoxidation of FMNH2 with air, two bands were seen, one at 565 nm as before, and a new band at 880 nm which was assigned to a semi-
quinone dimer. The occurrence of the 500-650 nm band was also reported in porcine liver yellow acyl dehydrogenase, snake venom L-amino acid oxidase, and "old yellow enzyme" by Beinert (1957).

Using stopped flow techniques, Burn and O'Brien (1959) reported that the 900 nm dimer band could be observed in the reduction of riboflavin by dithionite. Mixtures of FMN and FMNH₂ were examined using both optical spectroscopy and EPR (Beinert, and Sands, 1961; Gibson, Massey and Atherton, 1962). A direct stoichiometric correlation of the 565 nm band and the formation of free radical as measured by EPR was found. However, a plot of [900 nm] vs [560 nm]² was not linear as expected, thus making the assignment of the 900 nm band to semiquinone dimer doubtful. Massey and Palmer (1962) had reported that complexes could be formed between oxidized pyridine nucleotide and reduced flavin, with the appearance of a spectral peak in the 900 nm region. On this basis, Gibson, et al. (1962) proposed that the 900 nm band in flavin mixtures was due to a charge transfer complex of the oxidized and reduced flavin, (FMNH₂-FMN), rather than to a semiquinone dimer. The mechanism suggested was:

\[ \text{FMN} + \text{FMNH}_2 \rightleftharpoons (\text{FMNH}_2\text{-FMN}) \rightleftharpoons 2 \text{FMNH}^* \]
Swinehart (1965, 1966), using temperature jump methods, examined the kinetics of the FMN-FMNH₂ system. The rate constants reported support the mechanism proposed by Gibson. Direct confirmation of these results has been reported by Fox and Tollin (1966). Using a stopped flow apparatus with both optical measurements at 445, 570 and 900 nm, and EPR measurements, they examined the NADH-FMN system. It was observed that (1) the rates of oxidized flavin disappearance (445 nm) and complex appearance (900 nm) were identical, (2) the appearance of the 900 nm band preceded the appearance of the 570 nm band, (3) the kinetics of the optical band at 570 nm and the EPR signal were identical, (4) the 900 nm band decay was first order, and (5) the stoichiometry of the overall reaction was 2 FMN reduced per NADH oxidized. No isotope effects were observed when the reaction was run in D₂O although other workers had reported an isotope effect using deuterated pyridine nucleotide analogs. It was concluded that "reduction of oxidized flavin (by NADH) thus occurs via a hydride ion transfer, or an indistinguishable equivalent." The proposed mechanism was:

\[
\begin{align*}
H^+ + \text{NADH} + \text{FMN} & \rightarrow \text{FMNH}_2 + \text{NAD}^+ \quad (445 \text{ nm}) \\
\text{FMNH}_2 + \text{FMN} & \rightarrow (\text{FMNH}_2-\text{FMN}) \quad (900 \text{ nm}) \\
(\text{FMNH}_2-\text{FMN}) & \rightarrow 2 \text{ FMNH}^* \quad (570 \text{ nm})
\end{align*}
\]
Detailed examinations of the flavin semiquinone have been conducted by EPR (Ehrenberg, 1962; Eriksson and Ehrenberg, 1964; Guzzo and Tollin, 1963a, 1963b, 1964). A complex hyperfine splitting pattern was observed and interpreted in terms of a delocalization of the unpaired electron over the pi system of the isoalloxazine nucleus, with the major portion of the spin density at N(9), N(10) and the benzenoid ring, while the spin density at N(1) and N(3) was negligibly small.

EPR studies have also been reported on a variety of flavo-enzymes. In studies with dihydroorotic dehydrogenase, Beinert (1962) again confirmed the assignment of the 600 nm region absorption band to flavin semiquinone, and stated that flavoproteins do not necessarily become fully reduced during catalysis. The flavin moiety may simply alternate between the fully oxidized and semiquinone states. Rapid freezing techniques were employed to study milk xanthine oxidase (Bray, Palmer and Beinert, 1964; Palmer, Bray and Beinert, 1964). This enzyme contains 1 Mo atom, 4 Fe atoms, and 1 FAD per active site. It was observed that all of these components underwent changes during catalysis at a rate greater than the turn-over rate of the enzyme. The redox scheme proposed for this enzyme was:
Baudras (1965) has measured the reduction of the flavin and cytochrome groups of L-lactate dehydrogenase during anaerobic titration of the LDH with L-lactate. The proposed reaction mechanism includes both a bivalent and a univalent step with the flavin as the intermediate.

\[
\begin{align*}
2e^- & \quad \text{L-lactate} \quad \longrightarrow \quad \text{FMN} \quad \longrightarrow \quad \text{cytochrome}
\end{align*}
\]

Beinert and Hemmerich (1965) have presented evidence that in metalloflavoproteins (e.g. xanthine oxidase, NADH dehydrogenase) the semiquinone of the flavin interacts with the metal ion. All metalloflavoenzymes studied showed an EPR signal. The field has recently been reviewed by Beinert and Palmer (1965).

**Photochemistry of Flavins**

**General Considerations**

(For reviews see: Beinert, 1960; Oster, Bellin and Holmstrom, 1962; Holmstrom, 1964b, 1964c)

The photoreactivity of riboflavin was one of the first recognized properties of this vitamin. Its identification as the cofactor in "old yellow enzyme" was based on
the photoproducit obtained upon irradiation of the enzyme (vide supra). Riboflavin and FMN are identical with regard to their photochemical behavior and can undergo two types of reactions: photoreduction, occurring in the presence of reducing agents, and photobleaching or photolysis. The photoreduction reaction is similar to that observed for methylene blue, eosin, acridine orange, and many other dyes. The photobleaching reaction, occurring in the absence of any added electron donors, leads to a degradation of the ribityl side chain, yielding either lumiflavin or lumichrome, depending on the pH. It is this latter reaction which makes the flavins unique among the photochemical dyes.

The spectral properties of the various flavin derivatives have been tabulated by Beinert (1960) and are given in Table 1. Riboflavin and FMN exhibit a strong fluorescence in solution. In aqueous solution at pH 7 the fluorescence maximum occurs at 527-534 nm with a quantum yield of 0.23-0.26 (Koziol, 1965; Oster, et al.,

1. Some confusion exists in the literature because of the arbitrary use of the terms photobleaching, photoreduction, photolysis, and photofading by various authors. We shall use the term "photobleaching" to indicate the degradative process occurring in the absence of added reducing agents, "photoreduction" for the reversible process which occurs with external reductants, and "photofading" for the loss of color upon illumination, regardless of the mechanism.
### TABLE 1

Spectral Properties of Flavins

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<th>Compound</th>
<th>λmax (nm)</th>
<th>log e</th>
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<tr>
<td>Riboflavin (Rb)</td>
<td>266</td>
<td>4.512</td>
</tr>
<tr>
<td></td>
<td>373</td>
<td>4.025</td>
</tr>
<tr>
<td></td>
<td>445</td>
<td>4.097</td>
</tr>
<tr>
<td>Flavin mononucleotide (FMN)</td>
<td>266</td>
<td>4.502</td>
</tr>
<tr>
<td></td>
<td>373</td>
<td>4.017</td>
</tr>
<tr>
<td></td>
<td>445</td>
<td>4.097</td>
</tr>
<tr>
<td>Flavin adenine dinucleotide (FAD)</td>
<td>263</td>
<td>4.580</td>
</tr>
<tr>
<td></td>
<td>373</td>
<td>3.978</td>
</tr>
<tr>
<td></td>
<td>450</td>
<td>4.053</td>
</tr>
</tbody>
</table>

Solvent: Phosphate buffer, pH 7.0

after Beinert (1960)
1962). In the case of FAD, the molar absorption coefficients at 373 and 450 nm are lowered and the fluorescence yield is decreased compared to FMN and Rb, presumably due to interactions between the adenine moiety and the isoalloxazine nucleus. FAD also exhibits considerably reduced photoreactivity, presumably for the same reason. The reduced flavins are non-fluorescent. Lumiflavin is reported to have the same absorption and fluorescence spectrum as riboflavin (Beinert, 1960), but the fluorescence quantum yield is greater than 0.5 (Tether and Turnbull, 1962). Fluorescence measurements are commonly used as an assay for riboflavin. Koziol (1965, 1966a, 1966b) has examined the absorption and fluorescence spectra of riboflavin, lumiflavin, lumichrome, and riboflavin tetrabutyrate in a series of organic solvents.

In addition to fluorescence, flavins can also exhibit phosphorescence (Szent-Györgyi, 1957) and chemiluminescence (Vorhaben and Steele, 1965; Williams and Steele, 1965). Reduced FMN has been identified as a key participant in bioluminescence (Hastings, Gibson and Greenwood, 1965). An intriguing aspect of this last reaction is that the \( \text{FMNH}_2 \) can be replaced by light. This has been interpreted in terms of an energy storage mechanism in a protein via charge separation, with the reoxidation of \( \text{FMNH}_2 \) by \( O_2 \) supplying the energy to
populate the higher energy states of the protein. Cilento (1965) has stated, "Generation of electronically excited species is a distinct possibility in biological systems; the most likely ones at present appear to be flavins, metallocporphyrins, oxygen, and certain conjugated carbonyl compounds."

**Photobleaching**

When an anaerobic solution of riboflavin or FMN is illuminated, the yellow color disappears indicating reduction of the isocalloxazine nucleus. Upon admission of air to the sample, the original color is partially restored, and the major products found are hydrogen peroxide, and lumiflavin and/or lumichrome, depending on the pH. In aerobic solution the yellow color fades slowly. This is the photobleaching reaction first observed by Kuhn and Wagner-Jauregg (1933b). The mechanism of this reaction, and in particular, the nature of the reductant for the initial conversion to the leuco form, has been the subject of controversy for the past decade.

One group of workers (Vernon, 1959; Nickerson and Strauss, 1960; Strauss and Nickerson, 1961) proposed that water was oxidized to hydrogen peroxide by excited flavin, and the ribityl side chain was degraded by reaction with the hydroxyl free radicals formed. Recently, Enns and
Burgess (1965a, 1965b) invoked the "photofission of water" as an explanation of the photoreduction of riboflavin by amines (vide infra).

An alternative explanation, based on earlier work by Koschara (1934), was offered by Holmstrom and Oster (1961) who proposed that the photobleaching was an intramolecular oxidation-reduction with the ribityl side chain acting as the hydrogen donor. Abstraction of the hydrogen atoms would lead to cleavage of the side chain with the formation of "deuteroflavin" (vide infra), as proposed by Koschara. The deuteroflavin is even more sensitive to light than the original riboflavin, and decomposes to lumichrome upon illumination in anaerobic solution, or to lumiflavin by alkaline hydrolysis in the dark. They proposed the following series of reactions.

Absorption of light produces a singlet excited state

\[ \text{Rb} + \text{light} \rightarrow \text{Rb}^* \]  \hspace{1cm} (H1)

which can then decay by fluorescence or a non-radiative process,

\[ \text{Rb}^* \rightarrow \text{Rb} + \text{light/heat} \]  \hspace{1cm} (H2)
or by intersystem crossing

\[
Rb_T^* \rightarrow Rb_T^* \quad (H3)
\]

to the triplet state. This in turn may phosphoresce or decay by a non-radiative process

\[
Rb_T^* \rightarrow Rb + \text{light/heat} \quad (H4)
\]

or may be quenched

\[
Rb_T^* + Q \rightarrow Rb + Q \quad (H5)
\]

by added quenchers such as potassium iodide. It may also undergo a buffer dependent intramolecular hydrogen transfer reaction

\[
Rb_T^* \rightarrow DfH_2 \quad (H6)
\]

to "leucodeuteroflavin" which in turn may react with additional oxidized flavin

\[
DfH_2 + Rb \rightarrow RbH_2 + Df \quad (H7)
\]

to yield the fully reduced riboflavin (dihydroriboflavin) and deuteroflavin. Photolysis of the deuteroflavin would yield lumichrome and side-chain fragments.

\[
Df + \text{light} \rightarrow Lc + \text{side-chain fragments} \quad (H8)
\]
Upon reoxidation by air

\[
\text{DfH}_2 + O_2 \rightarrow \text{Df} + H_2O_2 \quad (H9)
\]

\[
\text{RbH}_2 + O_2 \rightarrow \text{Rb} + H_2O_2 \quad (H10)
\]

and both steps H9 and H10 were postulated to proceed via free radical intermediates. At high pH and low light intensity

\[
\text{Df} + OH^- \rightarrow \text{Lf} + \text{side chain fragments} \quad (H11)
\]

and lumiflavin is formed as the major product in place of lumichrome. At intermediate values of pH, the mixture of products obtained would result from competition between steps H8 and H11.

This explanation was given additional support by the work of Smith and Metzler (1963) who identified deuteroflavin as 6,7-dimethyl-9-(2'-formylmethyl)-isoalloxazine (VIII), and by the polarographic studies of Moore, et al. (1963), who showed that lumichrome was formed in the anaerobic sample, and hence did not arise from an attack on the side chain by hydroxyl radicals formed during air reoxidation of the photoreduced flavin as had been proposed by Strauss and Nickerson (1961). All three groups rejected the "photofission of water" hypothesis.
Moore and his co-workers proposed an alternative set of reactions for the formation of Lc and Lf from the triplet.

\[
Rb^*_{T} \rightarrow \cdot Rb^* \quad (Ml)
\]

A diradical (IX) was postulated as the first product of the intramolecular transfer of a hydrogen atom from the 2' carbon position of the ribityl side chain to the N(1) position of the isoalloxazine nucleus.
The diradical could decompose to form lumichrome

\[ \cdot Rb \rightarrow \text{Lc + fragments} \quad \text{(M2)} \]

or it could cyclize to form (X) which Moore suggested was the structure of the "leucodeuteroflavin".

\[ \cdot Rb \rightarrow \text{DfH}_2 \quad \text{(M3)} \]

As before, the oxidation of (X) would yield the formylmethyl derivative (VIII). However, Moore proposed that the formation of lumiflavin occurred by the same reaction (M2) as that for lumichrome, but utilizing the 3', rather than the 2', position of the side chain, by virtue of some unspecified change in the reactive site on the isoalloxazine nucleus. This controversy over the pathway for lumichrome and lumiflavin remains unresolved. The data of Smith and Metzler (1963) indicate that during illumination of an anaerobic solution of riboflavin, lumichrome formation is slow and parallels formation of fully
reduced riboflavin (RbH₂), as might be expected from step H7 of Holmstrom's mechanism. But using chemical reduction by H₂/Pd and reoxidation, less of the original color is recovered than in the case of photobleaching, implying a greater production of lumichrome. It is difficult to explain this latter result using Holmstrom's scheme which requires illumination of Df to produce Lc. Recent work by Song, Smith, and Metzler (1966) showed that both lumiflavin and lumichrome could be formed by alkaline hydrolysis of Df in the dark. It appears that both pathways for the formation of lumichrome may be operative.

Further evidence for participation of the ribityl side chain in the photobleaching comes from the work of McCormick (Chassy and McCormick, 1965b; Yang and McCormick, 1965) who showed that the presence of a free 2'-hydroxyl group, although not obligatory, accelerated the photobleaching. These workers also confirmed the earlier observation of Kostenbauder and DeLuca (1963) that the acetylation of the side chain completely inhibited the photobleaching reaction.

Radda and Calvin (1964) demonstrated that Rb, FMN, and 6,7-dimethyl-9-hydroxyethylisoalloxazine are photobleached, but that lumiflavin is unreactive. Halwer (1951) had previously shown that 9-(2'-hydroxyethyl)- and
9-(2'-hydroxy-2'-methyl-n-propyl)-isoalloxazine would react in a manner analogous to riboflavin.

**Photoreduction**

Riboflavin, FMN, lumiflavin, and to a lesser extent, FAD are capable of mediating the photooxidation of a large variety of substances, including amino acids, amines, ascorbic acid, EDTA, indole acetic acid, pyridoxine, xanthine, pterins, and estrogens. Flavin can also sensitize photopolymerizations. In the presence of external reducing agents, the photofading of the flavin is reversible and non-destructive. Holmström (1964c) estimated that the photoreduction with EDTA was $10^{14}$-10$^5$ times more efficient than the degradative photobleaching.

One of the early examples of a flavin-mediated photoreaction was the photoreduction of 2,3,5-triphenyl tetrazolium chloride (TTC) and blue tetrazolium (BT) in the presence of riboflavin and ethylene diamine tetraacetic acid (EDTA) (Nickerson and Merkel, 1953; Merkel and Nickerson, 1954). Reduction also occurred if a previously irradiated solution of EDTA and Rb was added to the TTC. These authors considered that the EDTA was functioning as a chelating agent rather than as a reductant for the flavin.
Vernon and Ihnen (1957) observed that the photo-oxidation of the 2,6-dichlorophenolindophenol (DPIP) - ascorbic acid couple by sugar beet leaf homogenate was stimulated by FMN. In aqueous solution the FMN could photooxidize reduced DPIP and reduced cytochrome c. Kolesnikov (1958) reported that riboflavin could photosensitize the oxidation of a series of phenols including tyrosine, p-cresol, resorcinol, pyrogallol, phloroglucinol, and tannin, as well as ascorbic acid and dihydroxymaleic acid. Rutter (1958) studied the reactions of ferric and ferrous ions with Rb, FMN, and FAD. He found that all three flavins catalyzed both the oxidation of Fe(II) and the reduction of Fe(III). The oxidation of Fe(II) was activated by oxygen, while the reduction of Fe(III) was inhibited by the presence of oxygen.

Frisell, Chung and Mackenzie (1959) found that Rb and FMN catalyzed the photooxidation of sarcosine and dimethyl glycine yielding CO₂, formaldehyde, and the corresponding amine. Glycine, alanine, N-methylalanine, EDTA, and a series of other amines were also photooxidized by Rb and FMN, generally in the order tertiary > secondary > primary > quaternary. FAD was only 10-20% as active as FMN, and thyroxine and I⁻ quenched the reaction. Later, Frisell and Mackenzie (1959) reported the reversible photo-oxidation of NADH by FMN.
The photooxidation of NADH and EDTA, and the photo-reduction of cytochrome c by FMN, Rb, and FAD under anaerobic conditions was reported by Vernon (1959). Assaying enzymatic activity, Uehara found that NAD and coenzyme A were inactivated by light in the presence of flavin and oxygen (Uehara, Mizoguchi and Okada, 1964), but, in contrast to Vernon's results, no inactivation was found under anaerobic conditions. The reaction was pH dependent, with a maximum at pH 4 and no reaction at pH 7 (Uehara, et al., 1966). NADP was also inactivated, and it was proposed that the inactivation was due to destruction of the adenine moiety. Methylene blue, which was known to selectively photooxidize guanine, had no effect.

The reaction of Rb with EDTA, and also with methionine which was oxidized to the sulfoxide, was interpreted in terms of a water photolysis mechanism (vide supra) (Nickerson and Strauss, 1960; Strauss and Nickerson, 1961). Radda and Calvin (1964) found that the rate of photoreduction with EDTA was the same for Rb, FMN, and Lf, but much slower with FAD. A ten-fold variation in EDTA concentration did not affect the rate. NADH reacted with FMN in the dark and in the light under anaerobic conditions. The initial quantum yield of the light reaction was 0.25.
Habermann and Gaffron (1962) reported the oxidation of ascorbic acid to dehydroascorbic acid by flavin upon illumination with white or blue light. If MnCl₂ and catalase were present in the reaction mixture, the ascorbate was oxidized to two-carbon and four-carbon fragments in a three-step reaction. When chloroplasts were also included, red light, which is absorbed by the chlorophyll, but not by flavin, could initiate the reaction. The oxidation of ascorbate by riboflavin was also observed by Holmström (1964e). He reported that a flavin semiquinone was formed which was then apparently reoxidized by the dehydroascorbate.

The products of the flavin photooxidation of N,N'-dimethyl-N'-benzylethylenediamine (NBD), EDTA, and methionine were identified by Enns and Burgess (1965a, 1965b). For NBD the products were benzaldehyde and N,N'-dimethylethylenediamine; for EDTA, glyoxylic acid was found in the anaerobic systems, and glyoxylic acid and formaldehyde in aerobic systems. In the case of methionine the product was methional (3-(methylthio)propionaldehyde). Goodspeed, Scott and Burr (1965) obtained similar results for the photooxidation of diethylglycine and EDTA with methylene blue as the sensitizer. Diphenylamine is photooxidized by flavin in pyridine or glycerol (Terenin, Tachin and Shakhverdov, 1965). Enns and Burgess
proposed that the additional oxygen needed to yield the observed product (glyoxylic acid in the case of EDTA) was supplied from the water by "photofission". It appears more plausible, however, to suggest that the mechanism involves oxidation of EDTA to an imine followed by solvolysis to yield the glyoxylic acid in a manner analogous to that proposed for the flavin photooxidation of sarcosine (Frisell, et al., 1959). A similar mechanism was suggested for the reaction of flavin with NBD in pyridine (Kurtin, et al., 1966).

The presence of an adsorbant or polymer which can bind the flavin enhances the photoreactivity. Kostenbauder and DeLuca (1963) found that both photoreduction and photobleaching were enhanced in the presence of micellar (i.e. > 2x10^{-2}M) sodium decyl sulfate (SDS). Acetylated riboflavin would not photobleach, but could be photoreduced in the presence of ascorbate and SDS. These studies were extended to polyvinylpyrrolidone (PVP) and several non-ionic surfactants (Kostenbauder, DeLuca and Kowarski, 1965). Photoreduction with ascorbate was observed under aerobic conditions for bound flavin. The fluorescence was unaffected by the macromolecules. It was proposed that the flavin is bound in the excited state (triplet), and that binding to the polymer protects the flavin triplet from oxygen quenching. Photochemical
reactions of bound dyes are of practical importance as was noted by Egerton (1964) who reported the degradation of textile fibres, especially nylon, by photosensitizing dyes.

**Phototropism and Photodynamic Action**

Aside from the purely photochemical aspects, there is a great deal of interest in these flavin-sensitized photoreactions with regard to biological processes. (For a description and review of the field see: Thomas, 1965; Seliger and McElroy, 1965). Phototropism is a directional growth of a plant in response to light. Galston (1949) showed that riboflavin could catalyze the photooxidation of the plant growth hormone auxin (indole acetic acid, IAA) and other indoles. There are similarities in the absorption spectrum of riboflavin and the action spectrum of phototropism, and Galston postulated that Rb was the photoreceptor for phototropism. Thimann (1964) has reviewed this field, and points out that the carotenoid lutein is also a possible photoreceptor, and that there may be an energy transfer from flavin to lutein.

IAA did not affect the growth of *Schizosaccharomyces pombe* or *Escherichia coli*, but the products of the flavin-sensitized photooxidation of IAA, 3-hydroxymethylindole and 3-methylindole, had a marked effect on growth (Fukuyama and Moyed, 1964).
Zalokar (1955) observed that the action spectrum for the photoactivation of carotenoid biosynthesis in *Neurospora* was the same as the flavin spectrum. He calculated a quantum yield of ca. 4 molecules of carotenoid per quantum based on the flavin concentration in the mycelium.

The photochemical deiodination of iodothyronine and triodothyronine by FMN was reported by Lissitzky, Benevent and Roques (1961). Suzuki, et al., (1961) found that the deiodination of thyroxine by FMN was suppressed by EDTA, cytochrome c, NADH, Fe(II), and Fe(III), and by anaerobiosis. Similar results were reported by Reinwein and Rall (1966) who noted that if metal ions were added in trace amounts with EDTA, the reaction was enhanced. Ferrous ion plus EDTA, however, was inhibitory, as were quinoline-8-carboxylic acid and 8-hydroxyquinoline.

The photodynamic inactivation of trypsin by FMN has been found to be dependent on dye concentration, with a maximum quantum efficiency of ca. $2 \times 10^{-3}$ at $1.5 \times 10^{-4} \text{ M}$ FMN (Spikes and Glad, 1964). The inactivation rate reached a maximum at pH 7.5-8.0 and trypsin inhibited the fading of flavin at 445 nm, but did not affect the fluorescence (Ghiron and Spikes, 1965a). Tomita and Kim (1966) reported that the riboflavin-sensitized photoinactivation of Taka-amylase A was inhibited by the presence of soluble
starch. They proposed that the inactivation was due to the attack of either $O^*$ or $H_2O_2^*$ radicals formed by the reoxidation of photobleached flavin.

A listing of the various substrates which have been reported to undergo light-induced reactions with flavins is presented in Table 2.
### TABLE 2

**Light-induced Flavin Reactions**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Photoproducct</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Photooxidations:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenine</td>
<td>Adenine</td>
<td>21</td>
</tr>
<tr>
<td>Alanine</td>
<td>Alanine</td>
<td>3</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>Dehydroascorbic acid</td>
<td>6,7,9</td>
</tr>
<tr>
<td>p-Cresol</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Cytochrome c (red.)</td>
<td>Cytochrome c (oxid.)</td>
<td>23</td>
</tr>
<tr>
<td>Dichlorophenolindophenol (DPIP)</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Dihydroxymaleic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N,N'-Dimethyl-N'-benzyl-ethylenediamine (NBD)</td>
<td>Benzaldehyde and N,N'-Dimethyl-ethylenediamine</td>
<td>2,10</td>
</tr>
<tr>
<td>Dimethylglycine</td>
<td>CO₂, formaldehyde, amine</td>
<td>3</td>
</tr>
<tr>
<td>Diphenylamine</td>
<td></td>
<td>19</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid (EDTA)</td>
<td>Glyoxylic acid and Ethylenediamine triacetic acid</td>
<td>2,3,13,14,22</td>
</tr>
<tr>
<td>Ferrous ion</td>
<td>Ferric ion</td>
<td>16</td>
</tr>
<tr>
<td>Glycine</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Indole acetic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-Methylalanine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotinadenine dinucleotide (NADH)</td>
<td>NAD⁺</td>
<td>4,14,21,23</td>
</tr>
<tr>
<td>Phloroglucinol</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Pyrogallol</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Resorcinol</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Sarcosine</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Tannin</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Tyrosine</td>
<td></td>
<td>32</td>
</tr>
</tbody>
</table>
TABLE 2 (Continued)

B. Photoreductions:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue tetrazolium (BT) (in the presence of EDTA)</td>
<td>12</td>
</tr>
<tr>
<td>Cytochrome c (oxid.)</td>
<td>22</td>
</tr>
<tr>
<td>Ferric ion</td>
<td>16</td>
</tr>
<tr>
<td>Silver ion</td>
<td>8</td>
</tr>
<tr>
<td>2,3,5-Triphenyl tetrazolium chloride (TTC)</td>
<td>12</td>
</tr>
</tbody>
</table>

C. Photodeiodinations:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iodotyrosine</td>
<td>11</td>
</tr>
<tr>
<td>Thyroxine</td>
<td>15, 18</td>
</tr>
<tr>
<td>Triiodothyronine</td>
<td>11</td>
</tr>
</tbody>
</table>

D. Photoinactivations:

<table>
<thead>
<tr>
<th>Substance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coenzyme A (presumably by adenine destruction)</td>
<td>21</td>
</tr>
<tr>
<td>Nicotinic adenine dinucleotide, oxid. (NAD')</td>
<td>21</td>
</tr>
<tr>
<td>Pimaricin (an herbicide)</td>
<td>1</td>
</tr>
<tr>
<td>Taka-amylase A</td>
<td>20</td>
</tr>
<tr>
<td>Trypsin</td>
<td>17</td>
</tr>
</tbody>
</table>

Excited State and Semiquinone

Two other aspects of flavin photochemistry which have received considerable attention are the nature of the reactive excited state and the role of the flavin semiquinone. Present evidence indicates that the lowest excited triplet is the photoreactive species. (For general reviews see: Hochstrasser and Porter, 1960; McGlynn, Smith and Cilento, 1964). Much of the evidence is indirect and based on quenching experiments. Weber (1950) reported that the fluorescence of riboflavin (which involves the lowest excited singlet state) could be quenched by a number of substances including iodide ion, purines and pyrimidines, and phenols. Riboflavin fluorescence was quenched 50% by 20 mM I\(^-\), from which Weber calculated a singlet lifetime of ca. 10\(^{-8}\) seconds. The purines and pyrimidines quenched by complex formation and this accounts for the decreased fluorescence of FAD in which the adenine is presumably complexed to the isoalloxazine. Holmström and Tegner (1966) reexamined the iodide quenching and found that for FMN, the quenching constant \(k_{SV}\) (calculated from the Stern-Volmer equation) varied with pH and ionic strength. The values found were: pH 5, \(k_{SV} = 23\ \text{M}^{-1}\); pH 8, \(k_{SV} = 16\ \text{M}^{-1}\). This was explained in terms of counter-ion repulsion of the ionized phosphate group, and
riboflavin ($k_{\text{sv}} = 30 \text{ M}^{-1}$) showed no variations with pH or ionic strength, as expected.

In contrast to this, it has been found that ca. 500-1000 times less KI is required to quench the photoreactions of riboflavin than to quench its fluorescence. Holmstrom and Oster (1961) observed 50% quenching of anaerobic flavin photobleaching with $4 \times 10^{-6} \text{M KI}$. (This value was later reported as $6.5 \times 10^{-6} \text{M}$ (Holmstrom, 1964c).) From their data they calculated an excited state lifetime of 1 millisecond. They also observed a decrease in both quantum yield and $k_{\text{sv}}$ as the reaction proceeded under steady illumination. This effect was ascribed to inhibition by photoproducts, and they calculated a quenching rate constant of $1.06 \times 10^{10} \text{M}^{-1} \text{sec}^{-1}$. Similar results were reported by Kostenbauder, et al. (1965).

The riboflavin-sensitized destruction of the herbicide pimaricin is also retarded by $I^-$ and by paramagnetic ions which quench phosphorescence (Berends and Posthuma, 1962; Berends, et al., 1965). The fluorescence of riboflavin was quenched 40% by $3.3 \times 10^{-2} \text{M KI}$, whereas, 40% retardation of pimaricin destruction required only $4 \times 10^{-5} \text{M KI}$ (Posthuma and Berends, 1966). On the basis of this indirect evidence, it was concluded that the photo-reactive state of riboflavin is the triplet.
Shiga and Piette (1964a, 1964b, 1965) have examined the triplet state of flavins in frozen solutions at 77°K by EPR. They reported the half-life of the FMN triplet in 1N HCl to be 15 msec., increasing to 27 msec. as the free radical, FMNH⁺, was formed. In 50% methanol-water at 77°K, the triplet yield and lifetime was a maximum at pH 7, indicating that the neutral form is the most stable. With oxygen present, the lifetime was 17.6 msec. In 50% glycerol-phosphate buffer at pH 7, the lifetime was 160-190 msec. KI and tryptophan were effective quenchers. The triplet state of FAD was partially quenched by the same adenine-iscalloxazine interaction which quenches FAD fluorescence, and, as in the case of fluorescence, addition of urea disrupted the complex and increased the triplet yield.

Concentration quenching (self-quenching) of flavin photoreactions has been observed (Holmstrom, 1964b; Kostenbauder, et al., 1965; Yang and McCormick, 1965). Gibson, et al. (1962) found that FMN fluorescence was decreased 50% at a concentration of 7x10⁻³ M flavin, from which we can calculate a \( k_{sv} \) of 140 M⁻¹. This is 6-9 times larger than the \( k_{sv} \) (16-23 M⁻¹) for KI quenching of fluorescence given by Tegner and Holmstrom (1966), indicating that flavin itself is a more effective quencher.
of the singlet state than KI. Gibson proposed that this effect was due to formation of FMN-FMNH$_2$ or FMN-FMNH$^+$ complexes.

A different interpretation was offered by Shiga and Piette (1965) who observed that as the flavin concentration was increased, the triplet yield was decreased, but the triplet lifetime was unaffected. Based on agreement of their observed value (ca. $10^{-4}$ M flavin) for the onset of concentration quenching with the reported value for the beginning of ground state dimer (FMN-FMN) formation (Radda and Calvin, 1964) and the fact that the apparent rate constant for the singlet-triplet conversion (intersystem crossing) was also unaffected, they proposed that concentration quenching was due to ground state dimerization which quenched the excited singlet state. However, their use of low temperature glasses (at 77°K) precluded any bimolecular reactions which might be operable in solution at room temperature.

The formation of charge-transfer complexes (CTC) of various substances with flavin is well documented (Szent-Gyorgyi, 1957; Isenberg and Szent-Gyorgyi, 1958; Harbury and Foley, 1958; Harbury, et al., 1959; Fleischman and Tollin, 1965a, 1965b, 1965c). The inhibition of flavin photoreactions by many of these same substances has been observed and ascribed to CTC formation between the donor

A tentative direct observation of the triplet of lumiflavin in aqueous solution was reported by Knowles and Roe (1964). Upon flash illumination they observed two transients with similar spectra; one which decayed with a bimolecular rate constant of ca. $10^9 \text{ M}^{-1}\text{sec}^{-1}$ and which they identified as the semiquinone, and the alleged triplet which decayed with a first order rate constant of 60 sec$^{-1}$. Identification of this second transient was based upon an energy transfer experiment with acridine. Using excitation at a wavelength absorbed only by lumiflavin, they reported that the lifetime of the triplet and the yield of the semiquinone both decreased, while the acridine triplet, which was absent in the absence of lumiflavin, now appeared.

Further studies of the lumiflavin triplet were reported by Tegner and Holmstrom (1966). Using flash photolysis, a transient was seen in anaerobic lumiflavin solutions at pH 6.5. The apparent absorption maximum was 650 nm. The absorption coefficient was pH dependent and was estimated to be in the range of 3600-7200 M$^{-1}$cm$^{-1}$ at 700 nm. This transient disappeared by successive second order and first order processes, the second order step being diffusion-controlled with a rate constant of
The first order rate constant was $3 \times 10^9 \text{M}^{-1} \text{sec}^{-1}$. Iodide ion reacted with the transient with a rate constant of ca. $7 \times 10^9 \text{M}^{-1} \text{sec}^{-1}$.

The nature of the reactions of the triplet and the mechanism for the formation of the semiquinone free radical are of course directly related to the question of whether flavins react photochemically via one-electron or two-electron mechanisms. Once again, as in the case of the dark reactions (vide supra), one must distinguish between two possible sequences for the initial reaction:

(I) \[
\begin{align*}
F + \text{light} & \rightarrow F^* \\
F^* & \rightarrow F^*_S \\
F^*_S & \rightarrow F^*_T \\
F^*_T + \text{AH}_2 & \rightarrow \text{FH}^* + \text{AH}^* \\
2 \text{FH}^* & \rightarrow F + \text{FH}_2
\end{align*}
\]

(II) \[
\begin{align*}
F^*_T + \text{AH}_2 & \rightarrow \text{FH}_2 + A \\
\text{FH}_2 + F & \rightarrow 2 \text{FH}^*
\end{align*}
\]

(In the case of photobleaching, \text{AH}_2 would be the ribityl side chain.)

Merkel and Nickerson (1954) suggested that the semiquinone was the intermediate, based on their polarographic data. Tether and Turnbull (1962) also postulated a reaction mechanism consistent with (I), but none of these groups could unequivocally differentiate between
Flash photolysis is the method of choice, and Holmstrom (1962) observed a transient upon flash illumination of an anaerobic riboflavin solution at pH 7. Addition of KI decreased the yield of the transient while added EDTA increased the yield, but neither affected the second order decay kinetics. Addition of oxygen (in the presence of EDTA) or DPI changed the decay to an apparent first order process. On this evidence, the transient was identified as the semiquinone. Holmstrom calculated an absorption coefficient at 560 nm of $700 \text{M}^{-1}\text{cm}^{-1}$. Previous work had yielded values of 1000-1500 by comparison of potentiometric and spectral evidence (Beinert, 1960), and 8000-13,600 from EPR (Gibson, et al., 1962). Subsequent work by Holmstrom (1964a) gave a revised value of $\varepsilon_{560} = 3050 \text{M}^{-1}\text{cm}^{-1}$ for FMNH$^\cdot$ at pH 6, and also a value of 800 $\text{M}^{-1}\text{cm}^{-1}$ for the molar absorption coefficient of the fully reduced FMNH$_2$ at 450 nm. In this latter work the time resolution of 0.02 msec. for the flash photolysis system allowed direct observation that the semiquinone is formed before the fully reduced species, in agreement with mechanism (I). Similar results have been obtained for the flash photoreduction of other dyes such as eosin (Kashe and Lindquist, 1964; Kato and Koizumi, 1959; Kato, et al., 1964) and fluorescein (Lindquist, 1960, 1963).
Autoxidation

The reaction of fully reduced flavin with oxygen (autoxidation) has been proposed as the first step in bacterial bioluminescence (Hastings, et al., 1965) and is involved in certain flavoenzyme systems (e.g. amino acid oxidase). The role of oxygen reoxidation with regard to flavoprotein enzymes has been discussed by Gibson (1965). Hydrogen peroxide is a product of the autoxidation, and this fact has been used to explain some of the flavin-sensitized photooxidations (e.g. Tomita and Kim, 1966).

Gutfreund (1960) studied the reoxidation of dithionite-reduced FMN using rapid mixing. He reported first order kinetics with a maximum rate constant of 20.8 sec\(^{-1}\) for FMN. The effect of D\(_2\)O on this reaction led him to suggest that the oxygen was bound to the flavin prior to the reoxidation step.

Gibson and Hastings (1962), reexamined this system and found, in addition to confirming Gutfreund's results, that a transient intermediate was formed, and that the reaction was autocatalytic. The proposed sequence, beginning with FMNH\(_2\), was

\[
\begin{align*}
\text{FMNH}_2 + O_2 & \rightleftharpoons J \quad (1) \\
J & \rightarrow \text{FMN} + H_2O_2 \quad (2) \\
\text{FMN} + \text{FMNH}_2 & \rightleftharpoons I \quad (3) \\
\frac{1}{2} I + \frac{1}{2} O_2 & \rightarrow \text{FMN} + \frac{1}{2} H_2O_2 \quad (4)
\end{align*}
\]
The maximum first order rate constant for step (2), obtained by extrapolating a plot of $1/k_{\text{first order}}$ vs. $1/[O_2]$ to $1/[O_2] = 0$, was 24 sec$^{-1}$, and for the second order reaction, step (1), the rate constant, obtained from the slope, was $1.7 \times 10^6$ M$^{-1}$ sec$^{-1}$.

Using flash photolysis on air saturated solutions of FMN and EDTA, Holmstrom (1964d) reported formation of FMNH$^+$ semiquinone during reoxidation, and observed that the rate of reoxidation was quite pH dependent. He reported a range of 10-100 sec$^{-1}$ for the first order step.

Mager and Berends (1965, 1966) have reported the formation of a transient hydroperoxide (presumably the J intermediate of Gibson and Hastings) at the N(1)-N(9) bridgehead carbon. They proposed that the hydroperoxide arises from reaction of the semiquinone with oxygen, and that this compound could be the species responsible for many aerobic flavin-sensitized oxidations.

**Summary**

In reviewing the work on flavin photochemistry, it becomes evident that there are several areas which require further elucidation:

(1) the nature of the hydrogen abstraction in the photobleaching reaction,
(2) the mechanism for the photooxidation of external hydrogen donors,

(3) the mechanism of the product quenching observed in photobleaching, and of the concentration quenching seen in both photobleaching and photoreduction,

(4) the nature of the autocatalysis seen in the autoxidation reaction, and of the reactive intermediate, I, proposed by Gibson and Hastings.

One of the objectives of the present work was to reexamine several of the various light-initiated reactions of flavins to provide evidence bearing upon these questions.

**Instrumentation**

The rapidity and complexity of the reactions which can occur in illuminated flavin systems places fairly stringent requirements on the techniques which can be used to study these systems. This final section of the Introduction will examine some of the techniques which have been employed for studies of fast reactions. This is done to permit comparison with the instrument constructed for use in the present investigation. (For reviews see: Allen, 1964; Cauldin, 1964; Claessson, 1964; and Gibson, 1966).

Rapid reactions may be studied in one of several ways:
(1) by slowing the reaction rate to a level which is measurable with conventional techniques; e.g. by using very dilute concentrations for second order processes, by lowering the temperature, or by increasing the viscosity.

(2) by allowing the reaction to proceed at its normal rate and then quenching or stopping it rapidly; e.g. rapid freezing technique (Palmer, et al., 1964).

(3) by initiating the reaction rapidly and then observing the reaction course with suitable fast response devices. The techniques included in this category may be further subdivided into two classes:

(a) flow methods: the reagents are mixed rapidly and observed. This may be either continuous or stopped flow. (For review see: Roughton and Chance, 1963; Chance, et al., 1964)

(b) perturbation methods: The quiescent system, either at equilibrium or non-reactive, is perturbed rapidly by light, heat, sound waves, or pressure, etc., and then the return to a quiescent state (relaxation) is measured.

The techniques employed to observe the rapid reactions may be spectrophotometric, electric (potentiometric, polarographic), calorimetric, or magnetic (EPR, NMR). For the study of the photochemical reactions of flavins, the obvious method of choice is flash photolysis
combined with kinetic spectrophotometry. The technique of flash photolysis, first developed by Norrish and Porter (1949, 1954) (see also: Porter, 1950, 1959, 1960), produces a high instantaneous concentration of photoreactive species by use of a gas discharge flashtube. Several large systems have been constructed, including an 8000 joule unit operating at 50 KV (Claesson, Lindquist, and Strong, 1964). (For recent reviews of this technique, see: Karyakin and Duzhenkov, 1962; Grossweiner, 1966). The flash system used by Holmstrom for many of his studies on flavins used 60 cm. flashtubes capable of 3600 joules per flash (Lindquist, 1960). Using dilute solutions (ca. $10^{-6}$ M) of flavin in 60 cm. cuvettes, Holmstrom could produce essentially quantitative conversion of the flavin in one flash. The facilities required for such a "brute force" technique would obviously be infeasible for all but the most specialized laboratories. Pulsed illumination, combined with EPR, has also been used for flavin photoreactions (Shiga and Piette, 1964a), while the dark reactions have been examined by temperature-jump (Swinehart, 1965, 1966) and by rapid mixing (Gibson and Hastings, 1962).

The measuring systems must also meet a second requirement: high sensitivity. This arises from the fact that for many biological materials (a) there may be a
very limited supply of material, (b) use of concentrated samples may be complicated by secondary effects such as dimerization, and (c) results from dilute solutions are more easily correlated to in vivo systems. In general, high sensitivity is achieved through an improvement in the signal-to-noise ratio. Two methods to achieve this goal are in popular use: (1) modulation of the carrier signal at a frequency higher than the desired response time and use of a tuned detection system, and (2) time averaging of the signal for repetitive events.

One of the early sensitive rapid response instruments was constructed by Chance (1942). This device used prism beam splitters and glass filters to obtain difference spectra at several wavelengths. The sensitivity was 0.2% with a response time of less than 10^-2 seconds. Subsequent improvements on this instrument (Chance, 1947, 1951) incorporated a vibrating mirror in place of the beam splitters and provided a sensitivity of 2x10^-4 OD (optical density units). This system was used in conjunction with stopped flow and continuous flow mixing systems, and is

---

2. To avoid ambiguity, we shall use the term "difference" for the technique of measuring two wavelengths simultaneously and subtracting one from the other (e.g. to correct for overlapping absorption bands) and "differential" for the subtraction of sample and blank (either solvent or a second sample) at the same wavelength as in a normal double-beam spectrophotometer.
now commercially available (American Instrument Co., Silver Springs, Md.).

Rikmenspoel (1965) has described a spectrophotometer with a detectability limit of $2 \times 10^{-4}$ OD suitable for use as either a scanning or dual wavelength device. Another stopped flow system for combined calorimetric and spectrophotometric measurements was described by Berger and Stoddart (1965). The optical portion had a response time of 0.2 milliseconds. Gloersen (1958) has constructed a time-resolved spectrograph using a rotating slit wheel and a television camera in place of the photographic plate. The sensitivity is $10^6$ photons with a resolution time of 4 microseconds when running the slit wheel at 15,000 RPM in air. The author suggested that the response may be improved to 0.5 microseconds by placing the wheel in a vacuum.

The modulation technique was used by Borisov (1960), who modulated the photomultiplier dynode voltage at ca. 20 cps, and used an amplifier tuned to this frequency. This yielded a 10-fold gain in sensitivity over an unmodulated system. DeSa and Gibson (1966) recently reported a dual-beam stopped flow apparatus using xenon arc lamps modulated at 5 kc and using fibre optics. The response time of 2-3 msec. is limited by the mixing device, while the noise limits the sensitivity to ca. $10^{-3}$ OD.
The techniques of modulating light at rates as high as $10^{10}$ cps have been reviewed by Jones (1964).

Time averaging has been used by Kuntz, Loach and Calvin (1964). In this technique, the signal is subdivided into time (or wavelength) blocks in a digital memory. A repetitive signal will always add to the same memory addresses, whereas, the noise will be randomly distributed over the entire memory. The signal-to-noise ratio increases as the square root of the number of repetitions. The device constructed by Kuntz, et al., (1964) used the Computer of Average Transients (CAT computer, Mnemotron Div., Technical Measurements Corp., White Plains, N. Y.). The rise time was less than 0.1 msec. and the noise level was equivalent to $10^{-4}$ OD, with approximately 2500 scans. In a second system (Kuntz, 1965), a boxcar integrator was used to obtain a detectability limit of $10^{-6}$ OD with a response time of 10 microseconds. Ke, Treharne and McKibben (1964) have also used the CAT computer on a flash photolysis system with a sensitivity of $10^{-4}$ OD and a time resolution of 50 microseconds.

The use of a scanning spectrophotometer rather than a single wavelength system allows one to observe several different species in a reaction sequence simultaneously, and also precludes any possible errors due to wavelength shifts which might otherwise be undetected.
Beinert (1965a) employed a rapid scanning instrument (American Optical Co., Model 10175) in some of the early work on flavoprotein free radicals. This instrument scans the 400-700 nm range at 60 cps and displays the absorption curve on an oscilloscope.

More recently there has been a revival of interest in rapid scanning instruments, particularly in the infra-red region. Bethke (1960) modified a Perkin-Elmer monochromator and added a 180 notch slotted disc at 7000 RPM to obtain 21,000 scans per second. D'yachenko (1960) used an oscillating mirror at 400 scans per second in the 400-900 nm region. A rotating spiral slot was used for scanning by Kolt'sov (1960) and by Gurevich and Kolyadin (1960). Niesel, et al., (1964) used 3 vibrating mirrors at 12 kc and time averaging in a double beam instrument which could scan a 350 nm range at 100 cps with a sensitivity of $10^{-3}$ OD. Three counter-rotating mirrors at 3000 RPM were used by Dmitrievskii (1964) to scan 450-740 nm in 40 microseconds with a resolution of 4 nm.

Herr and Pimentel (1965) used a 10,000 RPM rotating Littrow mirror in a modified Perkin-Elmer monochromator for a scan rate of 10 cm$^{-1}$ per microsecond in the infra-red. Dye and Feldman (1966) constructed a stopped flow system using a commercially available rapid scan monochromator (Perkin-Elmer Corp., Norwalk, Conn., Model 108).
capable of scanning the 0.2-10 micron region at 3-150 scans per second. Data were recorded on an FM tape recorder. Another commercially available system is the Model 501 Millisecond Wavelength Scanning Spectrometer (Warner and Swasey Co., Flushing, N. Y.) which can scan a 340 nm range with a 1.25 millisecond period. The system employs a rotating corner mirror in a Czerny-Turner monochromator.

In the case of flavin reactions, however, there is a third requirement which must be met. Because of the photobleaching and product quenching phenomena, the photo-reactions are not truly repetitive, and hence time-averaging can not be used. In order to successfully investigate these reactions, a second goal of the present work was the design and construction of an instrument system capable of the necessary high sensitivity and rapid response for a single isolated event.

3. It should be emphasized that while the results are not repetitive from flash to flash on a given sample, as would be the case in a truly reversible system, they are reproducible from sample to sample.
EXPERIMENTAL

Chemicals

Riboflavin (U.S.P.) and FMN (Riboflavin-5'-phosphate dihydrate, Na salt, B grade) were obtained from Calbiochem and used without further purification. A single lot of each was used for all experiments. Lumiflavin was synthesized by the method of Guzzo and Tollin (1963a). Mallinckrodt potassium iodide (A.R.) was used. EDTA (ethylene diamine tetraacetic acid, disodium salt) was obtained from Eastman Organic Chemicals. "Baker grade" p-benzoquinone (J. T. Baker Co.) was purified by sublimation prior to use. Phosphate buffer (0.1 M, pH 6.8) was prepared from equimolar quantities of monobasic and dibasic potassium hydrogen phosphate (Mallinckrodt A. R.) dissolved in distilled water. No difference in results was observed when the buffer was prepared using conductivity water obtained from a Hereaus quartz Bi-distiller.

Glycerol ("spectroquality"), ethylene glycol, 1,2-propanediol, iso-propyl acetate and tert-butyl acetate were obtained from Matheson, Coleman, and Bell. Methanol (anhydrous) and iso-propanol were Mallinckrodt A. R. grade. Ethanol (U.S.P. absolute and 95%) was obtained from U. S.
Industrial Chemicals. $n$-Propanol and tert-butanol were J. T. Baker "Analyzed Reagent" grade. 1,3-Propanediol was obtained from Eastman Organic Chemicals and redistilled prior to use. Antara Chemicals 1,4-butanediol (anhydrous) was used. Oxygen, nitrogen, and compressed air were obtained from Airco.

**Control of Oxygen Concentration**

For studies of anaerobic systems, the samples were freed of oxygen by purging the solution for 20-45 minutes with nitrogen gas. The nitrogen was prepurified by passing it over hot copper turnings in a furnace (E. F. Sargent Co., Chicago, Ill., Cat. nos. S-36517 and S-36518) and was presaturated with solvent to minimize loss of solvent from the sample. The longer deoxygenation times were necessary for the alcoholic solvents which show a greater affinity for dissolved oxygen than does water or glycerol. In the case of glycerol, the bubbles produced by purging the sample with nitrogen were removed by warming the sample during the last few minutes of purging, and then cooling under a nitrogen atmosphere. Diffusion of air into the glycerol samples was extremely slow, requiring minimal precautions for maintenance of anaerobicity.

As a test of the efficiency of deoxygenation by nitrogen purging, control samples were deoxygenated on a
high vacuum line. In this procedure, the sample is frozen to liquid nitrogen temperature in a degassing bulb of the type shown in Figure 3, evacuated to $10^{-6}$ Torr, and then thawed. This cycle is repeated until an initial pressure reading of less than $10^{-5}$ Torr is obtained upon first opening the frozen sample to the vacuum line (usually 4-5 cycles for aqueous samples). The cuvette is then evacuated, the main stopcock closed, and the degassing bulb removed from the vacuum line. The bulb is tipped to transfer the sample to the cuvette, the adapter stopcock is closed, and the adapter and cuvette are detached from the bulb and placed in the spectrophotometer.

No difference in results was observed between nitrogen-purged and vacuum-degassed samples, so that in most cases the simpler nitrogen purging routine was used.

For studies of the autoxidation reaction, where it was desired to maintain a controlled concentration of dissolved oxygen, the sample was purged with a mixture of nitrogen and oxygen. For very low oxygen concentrations, a mixture of nitrogen and air was used. The flow of each gas was controlled with a 1/8 inch needle valve and measured with a flowmeter (Matheson Gas Co., Model 201B). The two gases were mixed in a glass Y-tube, the mixed gas was presaturated with solvent, and then bubbled through the sample in the cuvette. Moderate gas flow rates
Figure 3. Vacuum Degassing Apparatus and Cuvette
(900-5000 cc/min) were required for accurate regulation of the composition of the mixture, but because the maximal flow rate in the sample was limited to 100 cc/min by the size of the cuvette, most of the gas mixture was vented to the atmosphere through a third needle valve. At the end of the purging time, the bubbler tube was raised ca. 1 cm. above the surface of the sample in order to maintain an atmosphere of the gas mixture over the sample.

The dissolved oxygen concentration for a series of gas mixtures was measured polarographically with a dropping mercury electrode and a Sargent Model XV Recording Polarograph. The results showed a direct proportionality between the dissolved oxygen concentration and the percent oxygen in the gas mixture. The value for the concentration of dissolved oxygen in air-saturated water at 25°C was taken as $2.8 \times 10^{-4}$ M (Stephen and Stephen, 1963).

Absorption Spectra

Absorption spectra were measured with the Cary 14M or Cary 11 spectrophotometer. Flavin concentrations were determined from the complete absorption spectra or by measuring the absorbance at 445 nm on a Bausch and Lomb "Spectronic 20".
For kinetic measurements of flavin photoreactions, we originally estimated that a spectrophotometer with a sensitivity of at least 1% and a response time of ca. 1 millisecond would be required. To achieve this, the use of a dual-beam differential system was indicated. By using aliquots of the same solution for both sample and blank (rather than sample vs. solvent blank as in conventional dual-beam spectrophotometry) and amplifying the signal difference between them, one can obtain a scale expansion. To obtain a high signal-to-noise ratio, an intensity-modulated light source and a tuned detection system were used. The modulation frequency selected was 5 kc in order to achieve the desired 1 msec. response time.

The original concept involved splitting the monochromatic light into sample and blank beams with a prism, modulating the two beams 180° out of phase with a mechanical chopper, and, after passing them through the respective cuvettes, recombining the two beams onto a single photomultiplier tube. For identical samples and matched optical paths, this would produce a constant light intensity on the photocathode and a steady D.C. output signal. By simple zero suppression, this would be set at zero as
the baseline. A change in absorbance of the sample with respect to the blank would then produce a 5 kc signal whose amplitude was proportional to the difference in transmittance of the sample and blank. Preliminary testing of this system indicated that obtaining the null condition required that the two light beams be exactly matched in intensity, wave shape, modulation frequency, spectral purity, and polarization, as well as exactly 180° out of phase. It soon became apparent that achieving these conditions would be impractical.

The final design adopted utilized separate photomultiplier tubes for the sample and blank paths, and modulation prior to beam splitting. In this manner, the two beams are identical in frequency, phase and wave shape at all times and only intensity matching is required to achieve the null condition. The difference between the two photomultiplier outputs is obtained in a differential amplifier. An added feature is the inclusion of a phase sensitive detection system which provides additional signal-to-noise enhancement by rejecting all signals except those which are either in phase or exactly 180° out of phase with the reference signal, and of the same frequency as the reference. In addition, this system provides a D.C. signal with automatic polarity indication of the transmittance change, rather than an A.C. envelope as
in the original design. The details of the various sub-systems are described in the following sections.

**Optical System**

The optical system is shown in Figure 4. The lamp is a 250 W projection lamp (type DLR or DKM) operated at 21.5 V from an autotransformer. The grating monochromator is a 250 mm Bausch and Lomb Model 33-86-40 used with a 5000 Å blaze grating. A 2.58" focal length cylindrical lens (American Science Center, Chicago, Ill., cat. no. 40,210) concentrates the monochromatic light on the entrance of the first collimator tube.

The monochromatic light is modulated at 5 kc by a 13" diameter chopper disc of 3/16" black anodized aluminum. This disc has 100 radial notches around its circumference. The width of these notches, the width of the intervening spaces, and the diameter of the circular aperture at the terminus of the first collimator tube are all equal. This produces a sinusoidal light intensity variation with 100% modulation depth. The disc and its 1/3 HP A.C. synchronous drive motor are dynamically balanced at the operating speed of 3000 RPM. A timing belt drive is used for constant speed, and the entire optical system (except the monochromator and lamp), the chopper disc, and the drive motor are mounted on a 3/4 ton reinforced concrete pedestal to
Figure 4. Flash Photolysis Spectrophotometer: Optical System
minimize vibration. The chopper assembly is enclosed within a 5/16" thick hardened aluminum shield for protection in the event of mechanical failure of the disc and to minimize the noise level in the laboratory.

The modulated beam passes through a second collimator tube and is split by the primary beam splitter prism (Edmund Scientific Co., Barrington, N. J.). The reflected portion of this beam is split again by a second prism. This produces three light paths of identical modulation frequency, wave shape, and phase for the sample and the blank, and for the reference channel of the phase sensitive detection system.

To obtain a differential null, the same light intensity should be incident on the sample and blank detectors, and, ideally, on both cuvettes. Intensity matching is accomplished by means of a neutral density attenuator system comprised of a set of perforated metal screens for coarse adjustment, and a grey film wedge. The wedge is a 60" length of 16 mm Kodak Fine Grain Positive film which has been fogged to yield a continuous density gradient of 0.05 to 0.55 OD units.

The detectors used are 2", 10 stage, end window photo-multiplier tubes, selected for the wavelength region being studied; EMI Type 9536B for the 400-650 nm region and DuMont Type 6911 for the 650-1000 nm region. The EMI
tubes were selected in preference to competitive types because of their low noise, low drift, and fast recovery from overload. For the less critical phase detector reference path, a DuMont 6292 was used for the visible region. The tubes were fully shielded, and the power supply resistor chains and preamplifiers are mounted directly on the tube base. The photomultiplier tubes are normally operated at -640 volts D.C., supplied by a Keithley Model 242 power supply. Using a 1 megohm load resistor, the anode current normally drawn is approximately 1 microampere.

The cuvettes are made from a 1.5" length of 10 mm i.d. precision bore pyrex square tubing (Fisher and Porter Co.) sealed to a length of 15 mm pyrex tubing with a 10/30 standard taper joint for connection to the vacuum system.

To provide the actinic light, a pair of General Electric FT-230 argon arc flashtubes are mounted in the sample compartment parallel to the cuvette. Appropriate complementary optical filters are interposed between the flashtube and the sample cuvette, and between the cuvette and the photomultiplier tube to reduce the transmission of scattered actinic light to the photomultiplier. For the present study of flavin photoreactions, Corning low-pass glass filters were used between the flashtube and the
cuvette. Between the cuvette and the photomultiplier, a multi-layer interference filter of the desired wavelength was used (Baird Atomic or Bausch and Lomb). The interference filter was chosen in preference to the Corning high-pass filters for three reasons. First, the low-pass flashtube filters have a near-IR pass band and there is sufficient flashtube output in this region to overload the photomultiplier. Second, the high-pass glass filters do not protect the photomultiplier from the intense flavin fluorescence, and third, the high-pass filters themselves exhibit a fluorescence when excited by scattered light from the flash. The calculated spectral distribution of the actinic light obtained with the various low-pass filters is shown in Figure 5.

**Electronic System**

Figure 6 shows a block diagram of the electronic circuitry.

The photomultiplier output signals from the cathode follower preamplifiers go through a pair of gain adjust potentiometers and a phase matching circuit and into a Keithley Model 103R differential amplifier. The output of the 103R is filtered by a low-Q 5 kc filter to remove large transients caused by the flashtube discharge and to improve the signal-to-noise. A high-Q filter would provide much better filtering, but would have a slower
Figure 5. Spectral Distribution of Actinic Flash

- with Corning CS 1-64
- with Corning CS 7-59
- with Corning CS 7-51
- with Corning CS 7-59 + 3-74
Figure 6. Flash Photolysis Spectrophotometer: Electronic System (Block Diagram)
response time. The filtered signal is then fed to the Model RJB Lock-in Amplifier (Electronics, Missiles, and Communications, Inc., Mount Vernon, N. Y.). The reference signal for the lock-in amplifier phase detection bridge is obtained from the reference photomultiplier tube through a Keithley Model 102 isolation amplifier. In order to improve the response time characteristics, the RJB amplifier was modified by shunting the 5 kc signal channel input filter with a 15,000-ohm resistor to lower its Q and increase its bandwidth, and by reducing the RC time constant in the final output integrator circuit. The results of these modifications are summarized in Table 3.

The phase-detected rectified output of the RJB is recorded on a Tektronix type 533 oscilloscope and a Sanborn series 150 strip chart recorder. The oscilloscope traces are recorded photographically with a Honeywell Pentax 35 mm SLR camera mounted on a Tektronix C-27 camera housing or with a Hewlett-Packard type 196A Polaroid oscilloscope camera.

Energy for the flash tubes is provided by a specially constructed 2.5 kV power supply and energy storage capacitor unit. The input energy to the flash tubes is selectable by varying the capacitance and/or the voltage, and the maximum available input energy is 360 joules (115 MFD at 2.5 kV). The two flash tubes are connected in
<table>
<thead>
<tr>
<th>RJB modification</th>
<th>Response time* (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>none</td>
<td>5.1</td>
</tr>
<tr>
<td>5 kc signal filter shunted by 15K resistor</td>
<td>2.8</td>
</tr>
<tr>
<td>RC integrator shunted to a calculated value of 0.2 msec.</td>
<td>2.2</td>
</tr>
<tr>
<td>Both filter and integrator shunted</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*time required for decay of a step-function input to 1/e of its initial value, measured for the complete instrument system.
series for more reliable operation and to allow use of higher voltages.

In addition to the use of complementary optical filters to prevent overloading of the photomultiplier tubes by stray actinic light or sample fluorescence, the sample and blank photomultiplier tubes are gated off during the flash period. This is accomplished by injecting a negative square wave pulse of several hundred volts at the second and fourth dynodes of the tubes. By driving these elements negative with respect to the surrounding dynodes, electron conduction in the multiplier chain is stopped. The gating pulse is obtained from a Tektronix type 162 waveform generator and a single stage triode amplifier. The gate duration is selected such that the flash output decays to zero before the photomultipliers are reactivated. Using a flash input energy of 330 joules, the light output decays to $1/e$ of the peak value in 85 microseconds and to less than 1% of the peak value in 250 microseconds. The gate period normally used for the present work was 1 millisecond, and the photomultiplier output signal was reduced to less than 0.1% (60 db attenuation) in less than 100 msec.

The 162 generator also provides the trigger pulse for starting the oscilloscope sweep and the sawtooth waveform for synchronizing the flash circuit. The delayed
trigger pulse for the flash tube discharge is provided by a Tektronix 161 pulse generator, a thyratron pulse circuit, and a pair of high voltage trigger transformers. Power for the gating and flash trigger systems, and also for the cathode follower preamplifiers, is provided by a Tektronix 160A power supply.

**Instrument Operation**

Aliquots of the same solution are placed in both cuvettes, if necessary the sample is deoxygenated by purging, and the neutral density attenuator and the phaser are adjusted for null. The flash tube capacitors are charged and the pulse sequence (gate on, scope sweep, trigger flash, gate off) is initiated.

If there is a photo-induced change in the sample, there will be a 5 kc differential signal from the 103R amplifier whose amplitude will be proportional to the change in transmittance of the sample. This signal will be filtered, phase-detected, and rectified by the RJB, and the proportional D.C. signal will be displayed on the oscilloscope and recorder. An increase in transmittance will produce a 5 kc differential signal in phase with the reference, whereas, a decrease in transmittance produces a 5 kc signal 180° out of phase with the reference. These will produce negative-going and positive-going D.C. signals, respectively, from the RJB amplifier. A summary of the
instrument operation for a single flash producing a decrease in absorbance of the sample is illustrated by the output waveforms in Figure 7. The small transient pulse observed on the RJB output is due to stray pick-up from the flash discharge and serves as a convenient time marker for the initiation of the flash. Using a non-photoreactive sample or pure solvent a flat baseline is obtained following the flash.

Instrument Performance

The photometric accuracy of the flash photolysis spectrophotometer was compared to that of a Cary Model 14M spectrophotometer (Applied Physics Corp., Monrovia, Calif.), using a series of FMN solutions with absorbances at 445 nm in the range of 0.04 to 1.8 OD. The optical density of each solution was measured at 445 nm on the flash spectrophotometer under normal operating conditions (but without the actinic flash) and then on the Cary Model 14M. The results are shown in Figure 8 and Table 4. The average error was 0.75%. The monochromator slit width used for the present work was 2 mm. This produces a bandwidth of 13 nm, and for the relatively broad flavin peaks, the slit width error was found to be negligible.

The sensitivity of the instrument for measurement of absorbance changes is defined as the absorbance change
Figure 7. Output Waveforms
Figure 8. Optical Density Calibration

Comparison of measured optical densities using a series of FMN samples at 445 nm. Cary Model 14 (ordinate) vs flash spectrophotometer (abcissa). Solid line (slope = 1.0) represents exact agreement of readings.
### TABLE 4

**Optical Density Calibration**

<table>
<thead>
<tr>
<th>Sample #</th>
<th>OD (Cary 14M)</th>
<th>Flash spectrophotometer OD (445 nm)</th>
<th>% error</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.040</td>
<td>0.040</td>
<td>0.0</td>
</tr>
<tr>
<td>1</td>
<td>0.105</td>
<td>0.108</td>
<td>2.8</td>
</tr>
<tr>
<td>2</td>
<td>0.200</td>
<td>.201</td>
<td>0.5</td>
</tr>
<tr>
<td>3</td>
<td>.303</td>
<td>.306</td>
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<td>4</td>
<td>.392</td>
<td>.399</td>
<td>1.7</td>
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<tr>
<td>5</td>
<td>.490</td>
<td>.495</td>
<td>1.0</td>
</tr>
<tr>
<td>6</td>
<td>.582</td>
<td>.580</td>
<td>-0.3</td>
</tr>
<tr>
<td>7</td>
<td>.670</td>
<td>.670</td>
<td>0.0</td>
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<td>8</td>
<td>.770</td>
<td>.777</td>
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<tr>
<td>9</td>
<td>.860</td>
<td>.876</td>
<td>1.8</td>
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<tr>
<td>10</td>
<td>.960</td>
<td>.967</td>
<td>0.7</td>
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<td>0.8</td>
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<td>1.119</td>
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<td>1.705</td>
<td>1.721</td>
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<tr>
<td>20</td>
<td>1.800</td>
<td>1.824</td>
<td>1.3</td>
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</table>

**average error** 0.75\%
equivalent to the peak-to-peak value of the noise (i.e. a signal-to-noise ratio, S/N, of 1.0). This has been determined at several different wavelengths and at several different absorbance levels. For the purpose of comparison with other instruments, the calibration was also repeated for several integrator settings (response times). The results are given in Table 5. The measurements were made under normal operating conditions with 3 pairs of perforated metal screens used to produce the base absorbance levels. Figure 9 shows that the sensitivity of the instrument parallels the normal S-11 spectral response curve of the 9536B photomultiplier tubes but is shifted to longer wavelengths. The red shift in the instrument sensitivity curve can be accounted for by the fact that the monochromator grating is blazed at 500 nm. The output of the tungsten filament projection lamp is increasing linearly with wavelength and the signal-to-noise ratio increases with light intensity. The effect of the various RJB amplifier modifications on the sensitivity at 450 nm are summarized in Table 6.

Data Processing

In the present work, the flash photolysis spectrophotometer was used exclusively as an expanded-scale double-beam instrument with a sample aliquot as the blank
## TABLE 5

**Instrument Sensitivity**

<table>
<thead>
<tr>
<th>Response time (sec)</th>
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<th>0.0100</th>
<th>0.1000</th>
<th>1.000</th>
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<tr>
<td>*<em>Base OD</em> Sensitivity (minimum ΔOD)**•</td>
<td></td>
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<td><strong>405 nm</strong></td>
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<td>0.0015</td>
<td>0.0003</td>
<td>0.0002</td>
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<tr>
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<td>0.0069</td>
<td>0.0017</td>
<td>0.0004</td>
<td>0.0003</td>
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<td>0.0032</td>
<td>0.0006</td>
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<tr>
<td>1.5</td>
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<td>0.0039</td>
<td>0.0011</td>
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<tr>
<td><strong>450 nm</strong></td>
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<tr>
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<td>0.0035</td>
<td>0.0015</td>
<td>0.0003</td>
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<tr>
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<td>0.0024</td>
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<td>1.5</td>
<td>0.0108</td>
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<td><strong>500 nm</strong></td>
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<td><strong>562 nm</strong></td>
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<td>0.0015</td>
<td>0.0003</td>
<td>0.0002</td>
</tr>
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<td>0.0069</td>
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<td>0.0032</td>
<td>0.0009</td>
<td>0.0003</td>
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<tr>
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<td>0.0025</td>
<td>0.0004</td>
<td>0.0002</td>
</tr>
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<td>0.0026</td>
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<td>0.0176</td>
<td>0.0047</td>
<td>0.0013</td>
<td>0.0006</td>
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</table>

* adjusted by addition of perforated metal discs

** at a signal-to-noise ratio, S/N, of 1.
Figure 9. Flash Photolysis Spectrophotometer: Spectral Response
**TABLE 6**

**Effect of RJB Modifications**

<table>
<thead>
<tr>
<th>RJB modification</th>
<th>Sensitivity at 450 nm</th>
<th>max*</th>
<th>0.010</th>
<th>1.00</th>
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<td><strong>Base OD: 0.0</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0.0011</td>
<td>0.0009</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>RC shunted</td>
<td>0.0016</td>
<td>0.0011</td>
<td>0.0001</td>
<td></td>
</tr>
<tr>
<td>Filter and RC shunt</td>
<td>0.0035</td>
<td>0.0015</td>
<td>0.0002</td>
<td></td>
</tr>
<tr>
<td><strong>Base OD: 0.5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0.0017</td>
<td>0.0011</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RC shunt</td>
<td>0.0026</td>
<td>0.0012</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filter and RC shunt</td>
<td>0.0035</td>
<td>0.0013</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Base OD: 1.0</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0.0030</td>
<td>0.0017</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RC shunt</td>
<td>0.0045</td>
<td>0.0021</td>
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</tr>
<tr>
<td>Filter and RC shunt</td>
<td>0.0086</td>
<td>0.0024</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Base OD: 1.5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>none</td>
<td>0.0047</td>
<td>0.0022</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RC shunt</td>
<td>0.0075</td>
<td>0.0024</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Filter and RC shunt</td>
<td>0.0108</td>
<td>0.0033</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* maximum response value varied with modification (see Table 3).
to measure changes in absorbance. The signal obtained ($\Delta S$) is proportional to the change in transmittance ($\Delta T$) of the sample, and the corresponding absorbance change ($\Delta \text{OD}$) is calculated according to the following equation:

$$\Delta \text{OD} = \log \frac{S}{S + \Delta S}$$

where $S$ is the single channel output (nominally 10 volts under normal operating conditions) from the sample at null prior to flashing. For small signals ($\Delta S < 1 \text{ V}$), $\Delta \text{OD}$ is proportional to $\Delta S$.

In the course of a normal working day it is possible to obtain as many as 5000 points of data to be measured and converted to optical density units. The $T$ to $\text{OD}$ conversion could, of course, be eliminated by the addition of a pair of logarithmic amplifiers to the sample and blank channels. The major portion of the manual data processing time is consumed in measuring and recording the voltages from the kinetic traces. In order to process the large volume of data required in this study of flavin reactions, the kinetic traces from the oscilloscope were recorded on 35 mm film. The $x$ and $y$ coordinates of the desired points were measured on the NRI film reader (Nuclear Research Instruments Div., Houston Fearless Co., Berkeley, Calif.) which automatically records the coordinates on standard IBM punch cards.
A computer program was written for the IBM 7072 computer to process the film reader data. A flow chart of this program is shown in Figure 10, and a listing of the Fortran II source program is given in the Appendix.

The program is directed through the various operations by the three control cards and the use of "go to" statements. The control cards specify the number of data sets (one set equals one kinetic trace or fraction thereof), the number of operations per set, the number of repetitions per set, the sequence of operations, and the treatment of the data. Several runs may be processed together by inserting additional control cards. In a typical run the film reader data is reduced to a set of optical density vs. time data. The computer then performs a linear least squares analysis of the natural logarithm of the optical density vs. time (first order kinetics) and of the reciprocal of the OD vs. time (second order kinetics), calculates the respective slopes and intercepts and the percent deviation of the data from the best fit straight line in each case, and prints out this information and a graph of the original data and the first and second order kinetic data. In addition to this, a new set of punched cards is obtained with the reduced data which may be used for subsequent computer runs, as desired.
Figure 10. Computer Program Flow Chart
The program is quite general in nature in that many kinds of data may be processed by simply changing the control cards, and additions or alterations of routines in the program library may be made at any time. It is estimated that manual processing of 100 kinetic curves (a normal day's output) would require 1-2 months, whereas, film reader-computer processing yields the final kinetic plots in one day. It should be noted that the computer processing is not as flexible as the manual method, and that the kinetic graphs obtained are fairly crude. Better graphs could be obtained by use of more sophisticated devices such as the Benson-Lehner off-line plotter, but for the present study the additional expense and time involved were not justifiable. Without the availability of the NRI film reader, or some other analog-to-digital conversion device, however, manual processing would have been the method of choice.
RESULTS

Using the flash photolysis kinetic spectrophotometer, we have examined the anaerobic photobleaching of flavins in phosphate buffer (pH 6.8) with regard to the questions of reaction mechanism and the nature of the observed product inhibition and self-quenching. In addition, the anaerobic photofading with EDTA in phosphate buffer, and with alcohols, glycols, and glycerol, has been studied in an effort to determine the details of the hydrogen abstraction process. The effect of these various hydroxylic compounds on the spectral properties of riboflavin and lumiflavin was also measured. The reoxidation of reduced flavin by oxygen (autoxidation) was reexamined, and reoxidation by p-benzoquinone in a three component system was studied. Computer analysis was employed for data reduction and for preliminary kinetic evaluation. The final kinetic data (reaction order and rate constants) were obtained manually using the reduced data from the computer and the integrated rate expressions for first order \((\ln \Delta \text{OD} = kt + \ln \Delta \text{OD}_0)\) and second order \((1/\Delta \text{OD} = (k/\epsilon)t + 1/\Delta \text{OD}_0)\) reactions.
Anaerobic Photobleaching

The photobleaching of riboflavin and FMN in 0.1M phosphate buffer, pH 6.8, was studied as a function of flavin concentration and number of successive flashes (sample history). Figure 11 shows a typical oscilloscope trace observed at 450 nm following flash excitation of the flavin. As expected, one observes a rapid bleaching of the flavin absorption with no observable regeneration of the oxidized flavin.

Figure 12a shows the flavin concentration dependence of the relative quantum yield, $\Phi'$, as given by

$$\Phi' = \Phi \cdot I_{\text{actinic}} \cdot e_1 = \Delta OD/(1-T),$$

where $\Phi$ is the absolute quantum yield, $I_{\text{actinic}}$ is the total incident actinic flash intensity, $T$ is the transmittance of the solution ($= I/I_0$ for the monochromatic monitoring beam), and $(1-T)$ is the fraction of the incident actinic light absorbed. The quantum yield decreased with increasing flavin concentration for a given actinic light intensity.

For a series of successive actinic flashes on the same sample, shown in Figure 12b, the relative quantum yield decreased, in agreement with the results reported by other workers (Holmström and Oster, 1961; Moore, et al., 1963; Holmström, 1964b; Yang and McCormick, 1965).
Figure 11. Anaerobic Photobleaching of FMN at 450 nm

8 x 10^{-5} M FMN in 0.1 M phosphate buffer, pH 6.8, 25°. Actinic flash: 330 joules with Corning CS 7-51 filter.
Figure 12. Photobleaching Quantum Yield

(a) FMN in 0.1 M phosphate buffer, pH 6.8, 25°, 330 joules per flash with CS 7-51 filter.

(b) 9 \times 10^{-5} M FMN in 0.1 M phosphate buffer, 330 joules per flash with CS 7-59 filter.
The kinetics of the initial photobleaching step, and of the photofading process in the subsequent work on photoreduction, could not be measured because of the instrument response time limitations and because no measurements could be made during the actinic excitation period.

With lumiflavin, no anaerobic photobleaching was observed, in agreement with previous work by others (Radda and Calvin, 1964; Tegner and Holmström, 1966).

The effect of potassium iodide on the photobleaching of FMN was examined. The Stern-Volmer constant, $k_{sv}$, was determined at several concentrations of flavin and of iodide, as illustrated in Figure 13, using the expression:

$$\frac{\Phi}{\Phi'} = \frac{\Phi_0}{\Phi'_0} = \frac{\Delta OD_0}{\Delta OD}$$

$$= 1 + k_{sv}[KI]$$

where $\Phi$ and $\Phi'$ are the relative quantum yields of photobleaching in the absence and in the presence of iodide, respectively. As seen in Figure 14, $k_{sv}$ varied inversely with flavin concentration.

Photoreduction and Solvent Effects

The anaerobic photoreduction of riboflavins, FMN, and lumiflavin by EDTA was measured in aqueous 0.1 M phosphate buffer, pH 6.8. The results for all three flavins were identical within the limits of experimental error.
Figure 13. Effect of Iodide on Photobleaching (Stern-Volmer Plot)

2 x 10^{-5} M FMN in phosphate buffer, pH 6.8, 25°, 330 joules per flash with CS 7-59 filter.

Slope = k_{SV} = (8.10 \pm 2.02) \times 10^{4} M^{-1}

(The vertical bars represent the uncertainty in each value as estimated from the noise level in the signal.)
Figure 14. Reciprocal of Stern-Volmer Constant with Respect to Flavin Concentration FMN in phosphate buffer, pH 6.8, 25°, 330 joules per flash with CS 7-59 filter. (The vertical bars represent the uncertainty in each value as estimated from the noise level in the signal.)
The quantum yield at 450 nm was constant at low flavin concentrations and decreased for a series of successive flashes, as shown in Figure 15.

The photoreduction process was examined further using a series of hydroxylic compounds as both solvent and hydrogen donor. In each case, the flavin was dissolved in the donor solvent, diluted to the final concentration as determined colorimetrically at 445 nm, and deoxygenated. The effect of the various solvents on the visible absorption spectrum of riboflavin and lumiflavin is shown in Figures 16 and 17. The position of the 445 nm band is virtually unaffected in all solvents, but there is a pronounced bathochromic shift of the near UV band. Qualitatively, the extent of this blue shift was inversely related to the flavin solubility in the given solvent. Thus, a solution of lumiflavin in isopropyl acetate which showed a blue shift of ca. 45 nm (Figure 17) was saturated at $2 \times 10^{-5}$ M Lf, whereas, a $10^{-4}$ M solution of lumiflavin could be prepared easily in aqueous buffer or glycerol.

The extent of photofading, caused by a single flash, with and without added EDTA, in the various hydroxylic solvents is summarized in Table 7. The increase in the extent of photofading of riboflavin in going from the monohydroxylic to the polyhydroxylic alcohols is attributed to the difference in the amount of actinic
Figure 15. Photoreduction Quantum Yield

(a) FMN in 0.1 M phosphate buffer, pH 6.8, 25°, with 2 x 10^{-3} M EDTA. 330 joules per flash with CS 7-51 filter.

(b) Lumiflavin (9 x 10^{-5} M) in 0.1 M phosphate buffer with 5 x 10^{-3} M EDTA. 330 joules per flash, CS 7-51 filter.
Figure 16. Visible Spectra of Riboflavin in Various Solvents

$[\text{Riboflavin}] \approx 2.5 \times 10^{-5} \text{M}$. 
- Phosphate buffer, 0.1M, pH 6.8;
- Methyl alcohol;
- Ethyl alcohol;
- iso-Propyl alcohol;
- tert-Butyl alcohol;
- Ethylene glycol (other glycols and glycerol showed essentially the same spectrum.)
Figure 17. Visible Spectra of Lumiflavin in Various Solvents

[Lumiflavin] \( \approx 2.5 \times 10^{-5} \text{M} \). ——— Phosphate buffer, 0.1 M, pH 6.8; ——— Ethyl alcohol; ——— iso-Propyl alcohol; ——— tert-Butyl alcohol; ——— Ethylene glycol; ——— Glycerol; ——— iso-Propyl acetate.
### TABLE 7

Photofading in Hydroxylic Solvents

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<tr>
<th>SOLVENT</th>
<th>SPECTRAL MAXIMA (nm)</th>
<th>( \Delta \text{OD}_{\text{max}} ) at 450 nm</th>
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<td></td>
<td></td>
<td>Flavin only</td>
</tr>
<tr>
<td>A. RIBOFLAVIN</td>
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</tr>
<tr>
<td>Phosphate buffer, 0.1M</td>
<td>373 445</td>
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<td>Methyl alcohol</td>
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<td>Ethyl alcohol</td>
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<td>iso-Propyl alcohol</td>
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<tr>
<td>tert-Butyl alcohol</td>
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<td>0.010</td>
</tr>
<tr>
<td>Ethylene glycol</td>
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<td>1,2-Propanediol</td>
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<td>0.023</td>
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<td>1,3-Propanediol</td>
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<td>0.018</td>
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<td>Glycerol</td>
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<td>0.016</td>
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<tr>
<td>B. LUMIFLAVIN</td>
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<tr>
<td>Phosphate buffer, 0.1M</td>
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<td>0.000</td>
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<td>0.010</td>
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<td>iso-Propyl alcohol</td>
<td>337 443</td>
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<tr>
<td>tert-Butyl alcohol</td>
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<td>---</td>
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<tr>
<td>Ethylene glycol</td>
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<td>0.016</td>
</tr>
<tr>
<td>Glycerol</td>
<td>361 443</td>
<td>0.018</td>
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Flavin concentration ca. 2.5x10^{-5} M

Actinic flash: 330 joules with CS 7-51 filter
light absorbed caused by the shift of the UV peak (cf. Figures 5 and 16). Addition of EDTA to riboflavin produced no change in the extent of photofading in the non-aqueous solvents, while similar addition to lumiflavin solutions produced a two- to three-fold increase in fading. This would suggest that these two flavins have different reaction paths in the non-aqueous solvents.

A comparison of the relative quantum yields in iso-propanol, tert-butanol, iso-propyl acetate, and tert-butyl acetate was made to determine the site of hydrogen atom abstraction. Lumiflavin was used to exclude the intramolecular photobleaching reaction, and the CS 7-59 + 3-74 actinic filter combination was used to excite only the 443 nm band in order to eliminate spectral shift effects. The relative yields are given in Table 8.

Reactions in Glycerol

The use of glycerol as a solvent for studying flavin reactions in vitro has several notable advantages. Owing to the low solubility of oxygen in this solvent (0.238 mM O₂ at 25°C for 92.5% glycerol compared to 1.28 mM O₂ for water and 9.63 mM O₂ for ethanol; Stephen and Stephen, 1963) and the high viscosity which retards oxygen diffusion, the maintenance of anaerobic conditions requires only minimal precautions. For example, it was observed
<table>
<thead>
<tr>
<th>SOLVENT</th>
<th>H atoms</th>
<th>$\Phi'$ at 450 nm</th>
</tr>
</thead>
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<tr>
<td>iso-propyl alcohol</td>
<td>-OH, -α, -CH$_3$</td>
<td>0.0719 ± 0.0113</td>
</tr>
<tr>
<td>tert-butyl alcohol</td>
<td>-OH, -CH$_3$</td>
<td>0.0651 ± 0.0113</td>
</tr>
<tr>
<td>iso-propyl acetate</td>
<td>-α, -CH$_3$</td>
<td>0.0295 ± 0.0113</td>
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<tr>
<td>tert-butyl acetate</td>
<td>-CH$_3$</td>
<td>0</td>
</tr>
</tbody>
</table>

[Lumiflavin] = 5×10$^{-6}$M

Actinic flash: 330 joules, CS 7-59 + 3-74 filters
that a glycerol solution of FMN which had been bleached by exposure to a 250 W projection lamp did not reoxidize when allowed to stand in an open cuvette for 72 hours, after which time purging with oxygen effected essentially quantitative reoxidation.

Comparison of the spectra of flavoproteins with those of free flavin in aqueous and non-aqueous solvents indicates that in many flavoproteins the bound flavin is in a hydrophobic environment (Massey and Ganther, 1965). The flavoprotein spectra and the spectrum of flavin in glycerol exhibit a shoulder on the long wavelength side of the 400-500 nm region band, whereas, for flavin in phosphate buffer, this band is structureless. On the basis of the spectral similarities, the glycerol medium may be regarded as a better approximation of in vivo conditions than aqueous buffer solutions. The use of hydroxylic reductants, especially the polyhydroxylic ones such as glycerol, with lumiflavin may also be considered as a model system for intramolecular photobleaching in riboflavin.

The most important advantage of the glycerol system, of course, is the fact that the high viscosity slows down many of the bimolecular redox reactions to measurable rates. Thus, in aqueous buffer solution, the decay of the flavin semiquinone at 560 nm is immeasurably fast or
Figure 18 shows some typical results for the anaerobic photoreduction of lumiflavin in glycerol. The 560 nm species was observed to decay completely, whereas, only about half of the initial absorption loss at 450 nm was recovered under anaerobic conditions. The half-times for these two processes were the same. Kinetic analysis of these data, as shown in Figure 19, indicated that the 560 nm decay was a second order process. The return of the 450 nm absorption appeared to be mixed order (i.e. between first and second order), but a more precise determination could not be made from the data available. The quantum yield measured at both 450 and 560 nm did not decrease with successive flashes. This is in contrast to the results in phosphate buffer with EDTA (Figure 15b). The rate of decay of the 560 nm species, however, was inversely proportional to the light intensity, as seen in Figure 19. The measured rates, determined from the optical density data directly, are expressed as $k/e$, where $k$ is the apparent bimolecular rate constant and $e$ the molar absorption coefficient. The addition of EDTA to the lumiflavin-glycerol system produced an increase in photofading measured at 450 nm (see Table 7). The rate of 560 nm
Figure 18. Anaerobic Photoreduction in Glycerol Lumiflavin (10^{-4} M) in glycerol. Actinic flash: 220 joules with Corning CS 7-59 filter. (a) 560 nm (b) 450 nm (insets show corresponding data at longer times from the Sanborn recorder.)
Figure 19. Lumiflavin in Glycerol: 560 nm Decay Kinetics

10⁻⁴M lumiflavin in glycerol, 25°. Actinic flash as indicated:

- ▲ 110 joules, CS 7-59, k/ε = 1700 sec⁻¹cm
- ♦ 330 joules, CS 7-51, k/ε = 1700 sec⁻¹cm
- □ 220 joules, CS 7-59, k/ε = 1260 sec⁻¹cm
- ○ 330 joules, CS 7-59, k/ε = 800 sec⁻¹cm
decay was increased, as shown in Figure 20, and the apparent reaction order became mixed. It was also noted that the relative quantum yield at both 450 and 560 nm now decreased with successive flashes, in a manner similar to that shown in Figure 15b.

With FMN, the situation was somewhat more complex. In the absence of added EDTA, the rate of 560 nm decay was slower than that seen with lumiflavin, and the rate did not vary significantly with actinic light intensity, as shown in Figure 21. At low flavin concentrations (less than $10^{-4}$M) the relative quantum yield at 450 nm was approximately the same as for lumiflavin in glycerol, and the yield at both 450 and 560 nm was constant with concentration (Figure 22). The yields at both 450 and 560 nm decreased with successive flashes, and, as seen in Figure 23, the rate of this decrease was proportional to the glycerol concentration. Increasing the flavin concentration or decreasing the light intensity abolished this successive flash effect.

Unlike lumiflavin, the relative quantum yield at 450 nm for FMN-glycerol did not increase upon addition of EDTA (Table 7). This indicates a fundamental difference in reaction mechanism in the two cases. However, in the FMN-glycerol-EDTA system, as with lumiflavin, the rate of 560 nm decay increased, the reaction order became mixed,
Figure 20. Effect of EDTA on 560 nm Decay Kinetics
$10^{-4}$M lumiflavin in glycerol, anaerobic, 25°.
(a) Oscilloscope traces. (b) Recorder data at longer times. Curve 1: lumiflavin alone. Curve 2: $5 \times 10^{-3}$M EDTA added.
Figure 21. FMN in Glycerol: 560 nm Decay Kinetics

FMN (10^{-4} M) in glycerol, anaerobic, 25°. Actinic flash as indicated.

- △ 110 joules, CS 7-59, k/ε = 425 sec^{-1} cm
- □ 220 joules, CS 7-59, k/ε = 400 sec^{-1} cm
- ○ 330 joules, CS 7-59, k/ε = 385 sec^{-1} cm
Figure 22. Quantum Yield in Glycerol with Respect to Flavin Concentration

FMN in glycerol, anaerobic, 25°, 330 joules per flash with Corning CS 7-59 + 3-74 filters.
(a) 450 nm   (b) 560 nm
Figure 23. Successive Flash Effect in Glycerol

9 x 10^{-5} M FMN, 330 joules per flash with CS 7-59 filter. Quantum yield normalized to 1.0 for first flash of each curve.

- O 0.1 M phosphate buffer, pH 6.8;
- □ 0.13 M glycerol; △ 2.3 M glycerol;
- □ 4.5 M glycerol.
and the successive flash effect was observed at low light intensity.

The successive flash effect does not occur at low flavin concentration (2x10^{-5} M) and low actinic light intensity (e.g. with CS 7-51 filter), for riboflavin, FMN, and lumiflavin, with or without added EDTA, in the mono-hydroxylic alcohols, and for lumiflavin in ethylene glycol. In ethylene glycol, Rb and Rb plus EDTA showed a decrease in quantum yield with successive flashes. Addition of KI decreased the extent of the successive flash effect, and, for a given flavin system, it was noted that the extent of the flash effect was proportional to the ratio of the light intensity to flavin concentration.

The ratio of the maximum AOD at 560 nm (extrapolated to zero time) to the final AOD at 450 nm provides a measure of the extent of formation of fully reduced flavin from the semiquinone. For lumiflavin in glycerol, this ratio was 1.83. In the case of lumiflavin plus EDTA, and for both FMN alone and FMN plus EDTA, this ratio was approximately half of the lumiflavin value (0.89-0.96). This is taken as further evidence of a difference in reaction mechanisms for these two flavins.

Addition of KI to the glycerol systems produced a quenching of the reaction as shown in Figure 24, but the value of the Stern-Volmer constant was reduced from that
Figure 24. Stern-Volmer Plot for Flavin in Glycerol

- Lumiflavin ($10^{-4}$M) in glycerol, 25°, 450 nm, 330 joules per flash with CS 7-59 + 3-74 filters.
  $k_{SV} = 1.48 \times 10^4 \text{ M}^{-1}$

- 450 nm and △ 560 nm, FMN ($9 \times 10^{-5}$M) in glycerol, 25°, 330 joules per flash with CS 7-59 filter.
found for FMN in aqueous solution (Figure 13). A summary of the results obtained for FMN and lumiflavin in glycerol and in the other solvents previously discussed is given in Table 9.

Autoxidation of Reduced Flavin

The reoxidation of photoreduced FMN-EDTA by molecular oxygen in 0.1 M phosphate buffer, pH 6.8, was measured at 450 nm. Figure 25a shows a typical oscilloscope trace. The oxygen concentration was varied from 0.22 to 1.4 mM by varying the ratio of oxygen and nitrogen in the purging gas. In this range of $O_2$ concentrations, the autoxidation was an apparent first order reaction as seen in Figure 25b. This is in agreement with the results of Gibson and Hastings (1962) and Holmström (1964d). The reciprocal-reciprocal plot of Figure 26 (after Gibson and Hastings) yields a maximum first order rate constant of 83.3 sec$^{-1}$ at infinite $O_2$ concentration, and a second order rate constant of $5.95 \times 10^5$ M$^{-1}$sec$^{-1}$ from the slope.

Three Component System

As an extension of the preceding work on reoxidation, an electron acceptor, p-benzoquinone, was added to the flavin-glycerol system. It was also intended that this three component system would serve as a model for sequential
**TABLE 9**

<table>
<thead>
<tr>
<th>Reaction parameter</th>
<th>Lf</th>
<th>Lf + EDTA</th>
<th>Rb (or FMN)</th>
<th>Rb + EDTA</th>
</tr>
</thead>
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<tr>
<td>Relative quantum yield</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in 0.1 M phosphate buffer</td>
<td>0</td>
<td>3.5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>in non-aqueous solvents</td>
<td>1</td>
<td>3.5</td>
<td>1-2</td>
<td>1-2</td>
</tr>
<tr>
<td>560 nm decay in glycerol</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>apparent reaction order</td>
<td>2</td>
<td>mixed*</td>
<td>mixed*</td>
<td>mixed*</td>
</tr>
<tr>
<td>k/ε, 2nd order rate constant (sec⁻¹ cm⁻¹)</td>
<td>1700</td>
<td>5600</td>
<td>425</td>
<td>2600</td>
</tr>
<tr>
<td>light dependence of k/ε</td>
<td>inverse</td>
<td>---</td>
<td>constant</td>
<td>---</td>
</tr>
<tr>
<td>Successive flash effects</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in 0.1 M phosphate buffer</td>
<td>---</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>in glycerol</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>in alcohols</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>in glycols</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>ΔOD₅₆₀/ΔOD₄₅₀</td>
<td>1.83</td>
<td>0.96</td>
<td>0.94</td>
<td>0.89</td>
</tr>
</tbody>
</table>

* The reactions were apparent 2nd order initially and became mixed order as the reaction progressed (see Discussion).
Figure 25. Autoxidation of FMN

FMN (10^{-4}M) with EDTA (10^{-2}M) in 0.1 M phosphate buffer, pH 6.8, 25°, equilibrated with 80% air - 20% nitrogen ([O_2] = 2.2 x 10^{-4}M). 450 nm. Actinic flash: 330 joules with CS 7-59 filter. (a) Oscilloscope trace. (b) First order kinetic plot.
Figure 26. Autoxidation Kinetics

FMN (10^{-4}M) with EDTA (10^{-2}M) in 0.1 M phosphate buffer, pH 6.8, 25°, 450 nm, 330 joules per flash with CS 7-59 filter. Slope = 5.95 x 10^5 M^{-1} sec^{-1}; intercept = 83.3 sec^{-1}. 
mitochondrial electron transport. Again, lumiflavin was selected to avoid the added complications of the intramolecular reaction. Kinetic measurements were made at 450 and 560 nm for the flavin, and at 430 nm for the p-benzo-semiquinone radical. The results obtained at these three wavelengths are shown in Figures 27, 28, and 29. Addition of quinone to the lumiflavin-glycerol system increases the rate of decay at 560 nm and changes it from second to mixed order. Although the quinone itself exhibits a light-induced reaction, as evidenced by the 430 nm data, the three component system produces a 2½ fold increase in the yield of the benzosemiquinone radical (cf. Figures 27a and 27b; note scale change in the ordinate). The most striking feature of these data, however, is the biphasic nature (i.e. both negative-going and positive-going components) of the 450 nm curves for the three component system (Figure 28c). Preliminary studies indicated analogous results for FMN and FMN-EDTA in glycerol with p-benzoquinone.
Figure 27. Three Component System at 430 nm

Actinic flash: 330 joules with CS 7-59 + 3-74 filters.

(a) $10^{-3}$M p-benzoquinone in glycerol.

(b) $10^{-4}$M lumiflavin plus $10^{-3}$M benzoquinone in glycerol.

(Note change in ordinate scale.)

(c) Same as (b) at longer times from Sanborn recorder.
Figure 28. Three Component System at 450 nm
Actinic flash: 330 joules with CS 7-59 + 3-74 filters.
(a) $10^{-4}$M lumiflavin in glycerol.
(b) $10^{-3}$M $p$-benzoquinone in glycerol.
(c) $10^{-4}$M lumiflavin plus $10^{-3}$M benzoquinone in glycerol.
(note change in ordinate scale)
Figure 29. Three Component System at 560 nm

Actinic flash: 330 joules with CS 7-59 + 3-74 filters.

(a) $10^{-4}$M lumiflavin in glycerol.

(b) $10^{-4}$M lumiflavin plus $10^{-3}$M p-benzoquinone in glycerol.
DISCUSSION

The Nature of Hydrogen Abstraction

The presently accepted theory for the degradative photobleaching of riboflavin and FMN in anaerobic solution proposes that the reaction proceeds from the lowest excited triplet state of the flavin and involves the abstraction of a hydrogen atom from the ribityl side chain with the subsequent formation of lumiflavin and/or lumichrome. Flavin analogs such as 9-(2'-hydroxyethyl)-isoalloxazine react in a similar fashion. The absence of photobleaching and the larger fluorescence yields (compared to Rb and FMN) of lumiflavin and side-chain acetylated flavins may be cited as further evidence of interaction between the side chain and the isoalloxazine nucleus. The addition of reducing agents such as EDTA to the flavin solution produces a non-degradative photoreduction reaction and hence "protects" the ribityl side chain.

Both the photobleaching and photoreduction reactions appear to proceed by one-electron mechanisms, via the flavin semiquinone free radical. For flash studies of the semiquinone reactions, Holmstrom (1964c) stated that
"a very short light pulse or a very long cell (permitting high dilution) is essential." An alternative method is represented in the present work by the use of a high viscosity solvent such as glycerol. The results shown in Figure 18 represent a direct observation of the disproportionation of the photochemically-produced semiquinone (560 nm) to regenerate approximately half of the oxidized flavin originally bleached (450 nm), and to form a like amount of the fully reduced leuco species. Upon reoxidation with air, greater than 90% of the original absorbance at 450 nm is restored, indicating that this reaction is a reversible photoreduction. These results provide further support for the one-electron theory.

Koziol (1966a) found that the fluorescence of several flavins was increased in solvents of low polarity and hydrogen bonding ability (compared to water). The photoreaction of riboflavin, lumiflavin, and lumichrome, as measured by loss of fluorescence intensity, was much faster in these solvents than in aqueous solution (Koziol and Knobloch, 1965; Koziol, 1966b). He concluded that "Riboflavin and lumichrome (and presumably lumiflavin) were found to be effectively stabilized towards the action of light by hydrogen bonds with solvent molecules, and, consequently, are most light stable in water solutions."
The results of the present investigation with alcohols, glycols, glycerol, and esters indicate a quite different interpretation of these data. As in the case of EDTA photoreduction, the anaerobic photofading of riboflavin and lumiflavin in the hydroxylic solvents (excluding water) is reversed upon reoxidation, with usually greater than 90% recovery of absorbance. Under aerobic conditions, no fading, or occasionally a short transient fading, is seen. The observation that lumiflavin shows no photoreaction in either tert-butyl acetate (a non-hydrogen bonding solvent) or in water (the best hydrogen bond former used) suggests that the relative stability of the flavins in water is not due to the hydrogen bonding ability of the solvent, but rather to its lack of hydrogen (or electron) donating ability. What is seen in these other solvents is a photooxidation of the alcohol, glycol, or glycerol by the flavin, rather than a degradation of the side chain.

The spectral shifts observed for riboflavin and lumiflavin (Figures 16 and 17; Table 7) provide additional information on the nature of the flavin-solvent interactions. The near UV absorption maxima of riboflavin and lumiflavin (373 nm and 367 nm, respectively, in aqueous phosphate buffer) have been assigned to π, π* transitions (Koziol, 1965). The large molar absorptivity and the
observed shift of this band toward shorter wavelengths with a decrease in solvent polarity are consistent with such an assignment (Kasha, 1961), and are in agreement with the results of Koziol for other organic solvents (Koziol and Knobloch, 1965; Koziol, 1966a). Harbury, et al. (1959) reported similar results for 3-methyl lumiflavin. However, the decrease in solvent polarity is not sufficient to account for the observed blue shifts seen in Figure 17. For example, the observed spectral shift for riboflavin in going from water ($\varepsilon = 78.5$ at $25^\circ$) to methanol ($\varepsilon = 32.6$) was 30 nm, whereas, the shifts in going from water to glycerol ($\varepsilon = 42.5$) or ethylene glycol ($\varepsilon = 37.7$) were only 6 nm and 12 nm, respectively. Similarly, in going from water to 1,2-propanediol ($\varepsilon = 32$) the blue shift was only 13 nm. One can rationalize this as follows. For $\pi,\pi^*$ transitions, hydrogen bond formation would be expected to stabilize both the ground state and the excited state. If the energy of the excited state were lowered to a greater extent than that of the ground state, a decrease in hydrogen bonding would produce an increase in transition energy and a blue shift in the spectrum. Thus the largest blue shift (relative to flavin in water, which is the most polar, best hydrogen-bonding solvent used) should be expected in non-polar, non-hydrogen bonding solvents such as the esters, and this is observed.
This interpretation is consistent with the proposal by Koziol (1966a) that hydrogen bonding is responsible for the partial quenching of flavin fluorescence in water, and with the observation by Sakai (1956) and by us that flavin solubility is increased under conditions where hydrogen bonding is favored.

The relative yields shown in Table 8 give an indication of the lability of the various hydrogen atoms of the reductant toward abstraction by photoexcited lumiflavin. The lack of reaction in tert-butyl acetate suggests that hydrogen atom abstraction from methyl groups does not occur. The ratio of yields in tert-butyl alcohol (with only hydroxyl hydrogen available) and iso-propyl acetate (with only α-hydrogens available) is $2.21 \pm 0.40$. It would then appear that the hydroxyl hydrogen atom is approximately twice as labile as the α-hydrogen. This is supported by the large reduction in yield in going from iso-propyl alcohol to iso-propyl acetate.

These results are in contrast to those of Wells (1956, 1961a, 1961b) who found that the hydrogen abstraction from alcohols by anthraquinone-2-sulfonate in aqueous solution is specific for the α-hydrogen. Kendall and Leermakers (1966) reported that benzophenone did not abstract hydrogen from tert-butyl alcohol, while abstraction from methyl-2-octyl ether did occur, as reported by
Cohen and Aktipis (1965). Walsh and Benson (1966) measured the bond strength ($\Delta H^\circ_{298}$) of the $\alpha$-C-H bond in isopropyl alcohol using a gas phase radical reaction, and reported a value of 90.3 kcal/mole. For the same system, they calculated the hydroxyl O-H bond strength to be 102 kcal/mole.

The apparent increased lability of the hydroxyl hydrogen in the flavin reaction can be explained on the basis of hydrogen bonding of the alcohol through the hydroxyl hydrogen to the flavin. The anthraquinone-2-sulfonate and benzophenone reaction involve $n,\pi^*$ transitions, and, in contrast to $\pi,\pi^*$ transitions, excitation would disrupt hydrogen bonding because both phenomena involve the lone pair electrons on the oxygen atom of the quinone or ketone. This explanation assumes hydrogen bond formation at the primary reduction site of the isoalloxazine nucleus, presumably N(1). The favorable steric factor for the abstraction of the hydroxyl hydrogen would probably compensate for the small difference in bond enthalpy cited above.

**Quenching of Flavin Photoreactions**

In aqueous solution, an inhibition of the photo-bleaching of FMN was observed as a result of (a) an increase in the concentration of FMN (Figure 12a), (b)
successive flashes (Figure 12b), and (c) addition of KI (Figure 13). It was also seen (Figure 14) that there was an inverse dependence of the $k_{sv}$ for iodide quenching on the FMN concentration. In order to explain these effects, one can use the following set of reactions:

\[ \text{FMN} + \text{light} \rightarrow \text{FMN}^* \quad \text{(triplet state)} \quad (0) \]

\[ \text{FMN}^* \rightarrow \text{P} \quad \text{(products)} \quad (1) \]

\[ \text{FMN}^*_T + \text{FMN} \rightarrow 2 \text{FMN} \quad (2) \]

\[ \text{FMN}^*_T + \text{P} \rightarrow \text{FMN} + \text{P} \quad (3) \]

\[ \text{FMN}^*_T + \text{Q} \rightarrow \text{FMN} + \text{Q} \quad (4) \]

where step (2) represents the concentration quenching, assuming that ground-state flavin is involved. Step (3) represents quenching with successive flashes, presumably due to photoproducts, and step (4) represents quenching by added substances such as KI. The rationale for including these reactions will be discussed below.

In the absence of added quenchers:

\[ (-d[\text{FMN}]/dt)_0 = k_0[\text{FMN}] - k_2[\text{FMN}][\text{FMN}^*_T] - k_3[\text{P}][\text{FMN}^*] \]

\[ d[\text{FMN}^*]/dt = k_0[\text{FMN}] - k_1[\text{FMN}^*] - k_2[\text{FMN}][\text{FMN}^*_T] - k_3[\text{P}][\text{FMN}^*] \]

All of the reactions listed above (steps 0 through 4) were completed within the period of flash excitation, indicating that the lifetime of the excited triplet must be less than or equal to the duration of the actinic flash. On this
basis, the use of the steady state approximation for the triplet species appears fairly reasonable and is necessary for an analytic solution of the kinetics.

Assuming a steady state for FMN*, \( \frac{d[FMN^*]}{dt} = 0 \), and

\[
[FMN^*] = \frac{k_0[FMN]}{k_1 + k_2[FMN] + k_3[P]}
\]

Hence,

\[
(-\frac{d[FMN]}{dt})_0 = k_0[FMN] - \frac{k_0[FMN](k_2[FMN] + k_3[P])}{k_1 + k_2[FMN] + k_3[P]}
\]

\[
= \frac{k_0k_1[FMN]}{k_1 + k_2[FMN] + k_3[P]}
\]

In a similar manner, it may be shown that in the presence of added quenchers:

\[
\frac{-d[FMN]/dt}{d[FMN]/dt} = \frac{k_0k_1[FMN]}{k_1 + k_2[FMN] + k_3[P] + k_4[Q]}
\]

Then, at a given flavin concentration and actinic light intensity, the ratio of the absolute quantum yields, in the absence and presence of added quenchers, is given by:

\[
\Phi_0/\Phi = \frac{(-d[FMN]/dt)_0}{(-d[FMN]/dt)}
\]

\[
= \frac{k_1 + k_2[FMN] + k_3[P] + k_4[Q]}{k_1 + k_2[FMN] + k_3[P] + k_4[Q]}
\]

\[
= 1 + \frac{k_4[Q]}{k_1 + k_2[FMN] + k_3[P]}
\]
and as in the simple case of a Stern-Volmer plot for iodide quenching, we may write:

\[ k_{sv} = \frac{(\Phi_0/\Phi) - 1}{[Q]} \]

\[ = \frac{k_4}{k_1 + k_2[FMN] + k_3[P]} \]

Finally,

\[ \frac{1}{k_{sv}} = \frac{k_1 + k_3[P]}{k_4} + \frac{k_2}{k_4} [FMN]. \]

A plot of \( \frac{1}{k_{sv}} \) vs. \([FMN]\) yields a straight line as shown in Figure 14, indicating that the inverse variation in \( k_{sv} \) is due to the onset of concentration quenching as the flavin concentration is increased.

**Iodide Quenching**

The data of Figure 13 were obtained at \( 2 \times 10^{-5} \text{M} \) FMN, at which concentration the self-quenching effect is minimal (i.e. \( k_2[FMN] \approx 0 \)). From these data, and the value of \( k_4 \) \( (7 \times 10^9 \text{M}^{-1}\text{sec}^{-1}) \) reported by Tegner and Holmstrom (1966), we obtain a value of \( (8.64 \pm 2.15) \times 10^4 \text{sec}^{-1} \) for \((k_1 + k_3[P])\). These data (Figure 13) were obtained from the results of the first flash on each sample, for which approximately 6% of the flavin is bleached (see Figure 12b). Then the maximum concentration of photoproduct formed by the first flash would be \( 1.2 \times 10^{-6} \text{M} \). Holmstrom
and Oster (1961) reported a value of $1.06 \times 10^{10} \text{ M}^{-1} \text{sec}^{-1}$ for $k_3$, from which we calculate a maximum value ($1.26 \times 10^4 \text{ sec}^{-1}$) for $k_3[P]$. This is within the experimental error ($2.15 \times 10^4 \text{ sec}^{-1}$) for the sum of $(k_1 + k_3[P])$ indicating that, for the data of Figure 13, the photoproduct quenching effect is negligible, and that the value obtained above ($8.64 \times 10^4 \text{ sec}^{-1}$) is a maximum value for $k_1$.

From the intercept of Figure 14 (again neglecting $k_3[P]$ for first flash data) we find that $k_1/k_4$ is $1.02 \times 10^{-5} \text{M}$, and from the slope, $k_2/k_4 = 8.08 \times 10^{-2}$. From this we can calculate $k_1 = 7.17 \times 10^4 \text{ sec}^{-1}$ and $k_2 = 5.65 \times 10^8 \text{ M}^{-1} \text{sec}^{-1}$.

The data of Figure 24 for KI quenching in glycerol were also obtained from first flash results, and since no concentration quenching effect was observed in glycerol, both $k_2[\text{FMN}]$ and $k_3[P]$ are negligible, and $k_{sv} = k_4/k_1$. It was seen that for quenching by KI in glycerol, the $k_{sv}$ for FMN ($7.32 \times 10^3 \text{ M}^{-1}$) and for lumiflavin ($1.48 \times 10^4 \text{ M}^{-1}$) were decreased compared to the $k_{sv}$ for FMN in aqueous solution ($8.10 \times 10^4 \text{ M}^{-1}$) calculated from Figure 13. The quenching of flavin by iodide in aqueous solution is a diffusion-controlled reaction. The rate of a diffusion-controlled process is inversely proportional to the viscosity of the solution. Owing to the hygroscopic nature of glycerol and to variations in the ambient relative
humidity and the atmospheric contact time, the viscosity of the glycerol solutions used here was estimated to be in the range of 200-900 centipoises (corresponding to 92-100% glycerol). The value of $k_4$ in glycerol is then calculated to be $(0.8-3.5) \times 10^7 \text{ M}^{-1}\text{sec}^{-1}$. A decrease in $k_{sv}$ in going from water to glycerol, as observed, would be expected, since the intramolecular reaction, or the solute-solvent reaction (if the reaction is intermolecular), will be less sensitive to viscosity effects than a solute-solute reaction (quenching). Thus, $k_4$ should be decreased to a greater extent than $k_1$.

Concentration Quenching

Using the value for $k_2$ of $5.65 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$ obtained from Figure 14 (vide supra), and the value of $8.64 \times 10^4 \text{ sec}^{-1}$ obtained from the Stern-Volmer plot (Figure 13) as the maximum value of $k_1$, we find that the ratio $k_2/k_1$ (analogous to $k_{sv}$ for iodide quenching) is $6.5 \times 10^3 \text{ M}^{-1}$. This is ca. 60 times larger than the $k_{sv}$ (140 M$^{-1}$) for the concentration quenching of fluorescence calculated from the data of Gibson, et al. (1962) (see Introduction). (It should be noted that a lower value of $k_1$, as might be obtained if photoproduct quenching were not negligible, will only exaggerate this ratio.) On the basis of this evidence, it is concluded that the concentration quenching
of photobleaching in solution occurs via the excited triplet state.

The photoreaction of FMN with external reducing agents proceeds through the excited triplet state (Holmstrom, 1964c). We find that addition of EDTA abolishes the concentration quenching effect at FMN concentrations less than $10^{-4} \text{M}$ (Figure 15). This can be attributed to a competition for the excited flavin species by the EDTA and is consistent with the conclusion that concentration quenching occurs at the triplet level.

A comparison of the values of $k_2$ ($5.65 \times 10^8 \text{M}^{-1} \text{sec}^{-1}$) and $k_4$ ($7 \times 10^9 \text{M}^{-1} \text{sec}^{-1}$) indicates that KI is approximately 10 times more effective than flavin in quenching the excited triplet state, whereas, the reverse was seen to be true for the excited singlet (see Introduction). Since the iodide quenching of the singlet and of the triplet are diffusion-controlled processes and the rate of diffusion is governed primarily by the structure of the diffusing species, we shall assume that $k_f^s$ (for the singlet quenching) $\approx k_4 = 7 \times 10^9 \text{M}^{-1} \text{sec}^{-1}$. For the singlet quenching, the ratio $k_2^s/k_4^s$ was 6-9, from which it follows that $k_2^s = (4.2 - 6.3) \times 10^{10} \text{M}^{-1} \text{sec}^{-1}$. Thus, intrinsically, the concentration quenching of the excited FMN molecule in the singlet state is ca. 100 times more efficient than the quenching of an excited triplet FMN ($k_2^s \text{ vs. } k_2$),
possibly due to a more favorable charge distribution or conformation of the singlet species.

Tegner and Holmstrom (1966) reported a range of 16-23 M$^{-1}$ for the $k_{sv}$ for iodide quenching of fluorescence ($= k_4/k_{f1}$). A value of 20 M$^{-1}$ can be obtained from the data of Posthuma and Berends (1966). From this, the lifetime of the excited singlet state is calculated to be of the order of $10^{-9}$ seconds. For the excited triplet, the lifetime estimated from the value of $k_\perp$ is of the order of one millisecond, in good agreement with the value calculated by Holmstrom and Oster (1961). The observation (vide supra) that, in solution, concentration quenching is more effective at the triplet level than at the singlet level is thus attributable to the longer lifetime of the excited triplet species.

Three possible mechanisms can be envisioned to explain the concentration quenching process in solution at room temperature. Shiga and Piette proposed that in a rigid glass at 77°K the process involved a static quenching of the excited singlet state due to ground state dimer (FMN-FMN) formation. They observed that the triplet lifetime and the rate constant for the singlet-triplet conversion (intersystem crossing) were independent of the FMN concentration. Thus, dimer formation acts at the singlet level and, to the extent that these results can
be extrapolated to the present work, we can exclude the dimer mechanism from consideration.

A collisional (dynamic) quenching of the excited triplet by a ground state flavin molecule as represented by reaction (2) (possibly via excimer formation), is consistent with all of the data. As noted above, the addition of an external reductant such as EDTA or glycerol would provide a competitive pathway for deactivation of the triplet and produce an apparent inhibition or abolition of the concentration quenching, as seen in Figures 15a and 22. Additionally, in glycerol, the increase in viscosity would retard a collisional process.

The third possible mechanism, quenching by triplet-triplet annihilation, has been shown to occur in gases, liquid solutions, rigid glasses, and crystals (McGlynn, et al., 1964). For this process, one can show that a plot of $1/k_{sv}$ vs. [FMN] (Figure 14) would be non-linear. However, this expected non-linearity may be within the experimental error, so that the present data do not allow us to differentiate between one or the other of these last two mechanisms.
Photoproduct Quenching

The decrease in quantum yield with successive flashes has been attributed to quenching by a product or products of the photoreaction (vide supra). Using steady illumination, Holmstrom and Oster reported that the quantum yield for photobleaching of FMN decreased with time. This is analogous to the flash effect, but the use of constant illumination does not provide sufficient evidence to determine whether the quenching occurs by reaction with an excited species (singlet or triplet) or with some later metastable intermediate (e.g., semiquinone). In the present flash studies, it was seen that both the initial loss of absorption at 450 nm and the yield of semiquinone (560 nm) were quenched with successive flashes, indicating that the photoproduct must react with an excited state molecule.

The photobleaching of FMN in aqueous buffer is quenched 50% with two flashes, and if all of the flavin bleached by these two flashes formed quencher, the maximum [P] would be $10^{-5}$ M. This yields a photoproduct quenching $k_{sv} \left( = k_2/k_1 \right)$ of $10^{5}\text{M}^{-1}$, which is approximately equal to the $k_{sv}$ for iodide quenching. If photoproduct quenching occurred at the singlet level, $k_1 \approx k_{fluorescence} \approx 10^{9}$ sec$^{-1}$, and $k_2$ would be $10^{14}\text{M}^{-1}\text{sec}^{-1}$. This is three orders of magnitude larger than the maximum rate constant for diffusion in aqueous solution (ca. $10^{10}\text{M}^{-1}\text{sec}^{-1}$). Hence,
the quenching must occur at the excited triplet level. Using the value of $k_1$ determined above ($8.64 \times 10^4 \text{M}^{-1} \text{sec}^{-1}$), $k_3 = 8.64 \times 10^2 \text{M}^{-1} \text{sec}^{-1}$, in good agreement with the value calculated by Holmstrom and Oster ($1.06 \times 10^4 \text{M}^{-1} \text{sec}^{-1}$) from their steady illumination data on the basis of an assumed triplet state reaction.

For lumiflavin with EDTA in phosphate buffer (Figure 15b), the apparent $k_{sv} = k^3 / k_1$ for photoproduct quenching was decreased compared to that for FMN photobleaching (Figure 12). This might be attributed to the increase in $k_1$ for the more efficient photoreduction reaction. However, in glycerol, lumiflavin with EDTA shows a successive flash effect, whereas, none is seen without EDTA. This would be inconsistent with an explanation in terms of $k_1$.

Addition of glycerol to aqueous solutions of FMN (Figure 23) inhibited the successive flash effect, as might be expected from consideration of the viscosity effect, and addition of EDTA to FMN in glycerol increased the quenching by photoproduct. For the less viscous monohydroxylic alcohols, however, no product inhibition could be demonstrated for any of the flavins (FMN, Rb, Lf), with or without EDTA. Additionally, Rb and Rb plus EDTA, were quenched by product in glycols, whereas, lumiflavin was not. The dominant factor which determines the extent
to which photoproduct quenching is observed would thus appear to be the nature of the product, as determined by the reaction mechanism and the reducing agent involved. The apparent dependence of the quenching effect on the ratio of light intensity to flavin concentration can be attributed to variations in product yield. Further studies of the photoproduct quenching effect, including product identification, will be needed before the nature of the quencher can be specified.

**Mechanisms of Flavin Photoreactions**

It was shown above that the results of the present investigation are consistent with the one-electron theory for flavin photoreactions. Evidence from quenching experiments was cited to support the theory that these reactions proceed from the lowest excited triplet state of the flavin. In contrast to the results in aqueous solution, it was seen that in alcohols, glycols, and glycerol, all three flavins (FMN, Rb, Lf) undergo a non-degradative photofading involving abstraction of a hydrogen from the hydroxyl group of the solvent.

In glycerol, the lack of an EDTA enhancement in the case of FMN (or riboflavin), whereas, an enhancement occurred with lumiflavin, was cited as suggestive of a difference in reaction paths for FMN and lumiflavin.
Further indications of such a difference are the inverse light dependence of the 560 nm decay rate, observed for lumiflavin (Figure 19) and absent in the case of FMN (Figure 21), the difference in the values of $k_{sv}$ for iodide quenching, the difference in the 560/450 ratios for lumiflavin compared to riboflavin and both flavins with EDTA (Table 8), and the difference in photoproduction quenching in glycol for lumiflavin compared to riboflavin and Rb plus EDTA.

The following reaction paths are now proposed to describe the photofading of lumiflavin and FMN (or riboflavin) in glycerol, and to account for the observed differences between these two flavins. From a comparison of the results in Table 7, it may be concluded that these mechanisms will also be applicable to the alcohol and glycol solvent systems.

For lumiflavin, with or without added EDTA

$$\text{Lf} + \text{light} \rightarrow \text{Lf}^*_S \rightarrow \text{Lf}^*_T \quad \text{(L0)}$$

$$\text{Lf}^*_T + \text{AH}_2 \rightarrow \text{LfH}^* + \text{AH}^* \quad \text{(L1)}$$

where $\text{AH}_2$ is the reducing agent, either solvent or added EDTA. The semiquinone can then disappear by disproportionation, with formation of a charge transfer complex intermediate, as proposed by Gibson, et al. (1962).
\[ 2 \text{LfH}^\cdot \leftrightarrow (\text{LfH}_2--; \text{Lf}) \leftrightarrow \text{LfH}_2 + \text{Lf} \quad (L2) \]

In the absence of added EDTA, the rate of semiquinone disappearance is second order, as expected, and the rate constant varies inversely with the actinic light intensity (Figure 19). This latter effect can be accounted for by a secondary dark reaction involving a photoproduct which regenerates the flavin free radical.

\[ \text{LfH}_2 + \text{AH}^\cdot \rightarrow \text{LfH}^\cdot + \text{AH}_2 \quad (L3) \]

Since both steps (L2) and (L3) are bimolecular, a plot of \(1/\Delta\text{OD}\) at 560 nm vs. time (second order kinetic plot) would be expected to yield a straight line for most of the reaction (as is observed), but the apparent rate constant determined from such a plot would be smaller than the intrinsic rate constant for the disproportionation reaction. Increasing the actinic light intensity would increase the yield of \(\text{AH}^\cdot\) and thus the contribution of step (L3), causing a decrease in the apparent rate constant.

The large increase in yield observed upon addition of EDTA to lumiflavin in glycerol (Table 7) shows that the amine is a much better reducing agent than the solvent. This fact can be used to account for the observed increase in the 560 nm decay rate in the presence of EDTA (Figure 20) and the change in decay kinetics from second order to mixed order, by including the following reaction step,
when EDTA is present, in place of reaction L3.

\[ LfH^* + AH_2 \rightarrow LfH_2 + AH^* \quad (L4) \]

With EDTA present in excess compared to LfH*, step (L4) would be pseudo-first order and would become important compared to step (L2). To complete the sequence,

\[ 2 AH^* \rightarrow A + AH_2 \quad (L5) \]

In flash studies on FMN with EDTA in aqueous phosphate buffer, Holmstrom (1964a) observed that as the 560 nm decay proceeds, the second order plot is a straight line for most of the reaction and then curves upward (1/\DeltaOD increasing) at the end, representing an increase in the apparent rate constant. He suggested that this effect was due to back reaction of the oxidized and reduced flavin, i.e. reversal of the semiquinone disproportionation reaction (L2). It would appear, however, that such a back reaction would decrease the net rate of disappearance of semiquinone and produce an apparent decrease in the second order rate constant. Indeed, preliminary results which we have obtained using an analog computer⁴ to solve the rate equations show that such is the case: a decrease, rather

---

⁴ Pace TR-10, Electronic Associates, Inc., Long Branch, New Jersey. The assistance of Dr. John Butcher in programming the computer is gratefully acknowledged.
than an increase, of the slope of the second order plot is observed as the contribution of the back reaction is increased.

Similar increases in slope of the second order plots have been observed in the present work, and two possible explanations are suggested. The disproportionation is known to proceed via consecutive second and first order reactions (Swinehart, 1965, 1966; Fox and Tollin, 1966). Near the end of the reaction there would be very little semiquinone (neglecting back reaction) and one would observe primarily the first order decay of the charge transfer complex. This would produce an increase in slope of the second order plot. In fact, a constantly increasing slope over the entire second order plot can be shown to represent a reaction order less than second (see e.g. Holmstrom, 1962, Figure 3, p. 872). Similarly, a constantly decreasing slope of a first order \( \ln (\Delta \text{OD}) \) vs. time plot can be shown to represent a reaction order greater than first. Those reactions which are referred to in the present work as of mixed order (Table 9) show both types of deviations.

An alternative explanation for the observed increase in slope of the second order plot is the inclusion of the opposing reaction (L3) coupled with the second disproportionation (L5). The reaction rate for the
disappearance of flavin semiquinone would be repressed until the AH* had been consumed by steps (L3) and (L5) at which point the reaction rate would increase to the intrinsic value for step (L2). It is not possible to decide between these alternatives at present.

For FMN (or riboflavin) the following reaction sequence is proposed:

\[
\text{FMN} + \text{light} \rightarrow \text{FMN}_T^* \quad \text{(F0)}
\]

The lack of EDTA enhancement with FMN in organic solvents (Table 7) suggests that interaction occurs preferentially between the side chain and the isocalloxazine nucleus, preventing the EDTA from reacting at N(1). Thus the photofading of FMN is entirely intramolecular, and can be represented by the hydrogen transfer process (from the 2'-hydroxyl group of the side chain) forming a biradical species as proposed by Moore (Moore, et al., 1963; Moore and Baylor, 1966).

\[
\text{FMN}_T^* \rightarrow \text{FMN}' \quad \text{(F1)}
\]

As in the case of lumiflavin, the free radical may disproportionate:

\[
2 \text{FMN}' \leftrightarrow \text{FMN} + \text{FMNH}_2 \quad \text{(F2)}
\]

Reaction of the biradical with the solvent or with added EDTA can occur by
\[
'\text{FMN}^* + \text{AH}_2 \longrightarrow ('\text{FMNH}^* + \text{AH}^*) \longrightarrow \text{FMNH}_2 + \text{A} \quad (\text{F3})
\]

The lack of any inverse light effect (Figure 21) suggests that no significant amount of free \( \text{AH}^* \) is formed to react with the reduced flavin, as in the proposed mechanism, which assumes that the reaction of the biradical with \( \text{AH}_2 \) is essentially a two-electron process.

Among the four glycerol systems (FMN, FMN + EDTA, Lf, and Lf + EDTA), recovery of absorbance at 450 nm upon reoxidation was lowest for FMN alone, indicating that some degradation of the side chain occurs,

\[
'\text{FMN}^* \longrightarrow \text{degradation products} \quad (\text{F4})
\]

In all cases the recovery of absorbance was much higher in glycerol than in phosphate buffer. This indicates that step (F4) is much slower than (F2) and (F3), and also that EDTA is more efficient than glycerol in preventing degradation. Again, as with Lf plus EDTA, the combination of reactions (F2) and (F3) predicts mixed order kinetics for 560 nm decay for FMN-EDTA, and this is observed.

Using the values for \( k_{sv} = (k_4/k_1) \) from Figure 24 and the estimated value for \( k_4 \) in glycerol, \((0.8 - 3.5) \times 10^7 \text{M}^{-1}\text{sec}^{-1}\), we obtain the following values for \( k_1 \) in glycerol:
The difference in $k_{SV}$ for iodide quenching for the two flavins in glycerol (Figure 24), due to the difference in $k_1$, is taken as further evidence supporting the proposed difference in reaction mechanisms. The difference in photoproduct quenching can be rationalized on the premise that reactions (L1) and (L5), or reactions (L1)-(L4)-(L5), will yield different products than reaction (F3).

**Semiquinone Absorption Coefficient**

The stoichiometry of the proposed reaction sequences may be used to predict limits for the ratio of fully reduced flavin to flavin semiquinone, $[\text{FH}_2]/[\text{FH}^+]$. For lumiflavin in glycerol, assuming that $k_3 \geq k_4$ (for steps (L3) and (L4), respectively, no added EDTA), varying the ratio of $k_3/k_2$ leads to the conclusion that

$$0.5 \geq \left( \frac{[\text{FH}_2]}{[\text{FH}^+]} \right) \geq 0.$$

For FMN in glycerol, and for both flavins with added EDTA,

$$1.0 \geq \left( \frac{[\text{FH}_2]}{[\text{FH}^+]} \right) \geq 0.5.$$

This provides an explanation for the observed differences in the 560/450 nm ratio (Table 9). From this we may now estimate limits for the molar absorption coefficient of the flavin semiquinone at 560 nm as given by:

FMN: $1100 - 4800 \text{ sec}^{-1}$

Lf: $540 - 2400 \text{ sec}^{-1}$
\[ \varepsilon_{560 \text{ nm}} = \frac{\Delta \varepsilon(560 \text{ nm}) \times [\text{FH}_2]}{\Delta \varepsilon(450 \text{ nm}) \times [\text{FH}^*]} \times \varepsilon_{450 \text{ nm}} \]

The contribution of the fully reduced flavin (\( \varepsilon_{445} = 800 \text{ M}^{-1}\text{cm}^{-1} \)) to the optical density will be negligibly small owing to its low concentration. Then, using the reported value of 1.22x10^4 \text{ M}^{-1}\text{cm}^{-1} for \( \varepsilon_{450} \) (Beinert, 1960), we obtain a value of 5400 - 11,200 \text{ M}^{-1}\text{cm}^{-1} for \( \varepsilon_{560} \), in good agreement with the latest reported value of 8000 - 10,000 in aqueous solution, determined by correlation of absorption spectra and EPR measurements (Fox and Tollin, 1966). Using the mean value of 8300 \text{ M}^{-1}\text{cm}^{-1}, we can calculate minimum values for the disproportionation rate constant in glycerol: for Lf, \( k_2 = 1.4 \times 10^7 \text{ M}^{-1}\text{sec}^{-1} \); for FMN, \( k_2 = 3.5 \times 10^6 \text{ M}^{-1}\text{sec}^{-1} \). Using a value of 700 \text{ M}^{-1}\text{cm}^{-1} for \( \varepsilon_{560} \), Holmstrom (1962) reported a rate constant of 3x10^8 \text{ M}^{-1}\text{sec}^{-1} for the disproportionation in aqueous buffer. In a more recent paper (Holmstrom, 1964c) he reports the same value of \( k_2 \) using a value of 3050 \text{ M}^{-1}\text{cm}^{-1} for \( \varepsilon_{560} \), and assuming \([\text{FH}_2]/[\text{FH}^*] = 0.5\). On the basis of the value of \( \varepsilon_{560} \) determined in the present work, \( k_2 \) in aqueous solution would be 8.3x10^8 \text{ M}^{-1}\text{sec}^{-1}, somewhat slower than a diffusion controlled process. Knowles and Roe (1964) reported 10^9 \text{ M}^{-1}\text{sec}^{-1} for the lumiflavin semiquinone.

The value for \( k_{\text{diffusion}} \) in glycerol was estimated to be \((0.8 - 3.5) \times 10^7 \text{ M}^{-1}\text{sec}^{-1}\) (vide supra).
Comparing this with the values for $k_2$ found above indicates that, in glycerol, the rate of disproportionation for the lumiflavin semiquinone is diffusion-controlled, whereas, that for FMN is not. The smaller rate constant determined for FMN may be attributable to interactions between the solvent and the ribityl side chain.

Summary

The rate constants for the various reactions of FMN (or riboflavin) and lumiflavin determined in the present work are listed in Table 10. Where available, constants reported by other workers are included for comparison.
### TABLE 10

Rate Constants for Flavin Reactions

<table>
<thead>
<tr>
<th>Reaction</th>
<th>Rate Constant</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>In aqueous solution:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1) $\text{FMN}^*_{T} \rightarrow \text{products (P)}$</td>
<td>$(8.64 \pm 2.15) \times 10^4 \text{ sec}^{-1}$</td>
<td>(Fig. 13)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$7.17 \times 10^4 \text{ sec}^{-1}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$4.55 \times 10^4 \text{ sec}^{-1}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$5.65 \times 10^8 \text{ M}^{-1}\text{sec}^{-1}$</td>
</tr>
<tr>
<td>(2) $\text{FMN}^*_{T} + \text{FMN} \rightarrow 2 \text{ FMN} + \text{heat}$</td>
<td>$8.64 \times 10^9 \text{ M}^{-1}\text{sec}^{-1}$</td>
<td>(Fig. 12b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$1.06 \times 10^{10} \text{ M}^{-1}\text{sec}^{-1}$</td>
</tr>
<tr>
<td>(3) $\text{FMN}^*_{T} + \text{P} \rightarrow \text{FMN} + \text{P} + \text{heat}$</td>
<td>$5.95 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$</td>
<td>(Fig. 25)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$7 \times 10^5 \text{ M}^{-1}\text{sec}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$\text{FMNH}^* + \text{O}_2 \rightarrow \text{FMNOOH}^*$</td>
<td>$80 \text{ sec}^{-1}$</td>
</tr>
<tr>
<td></td>
<td>$\text{FMNOOH}^* \rightarrow \text{FMN} + \text{HO}_2^*$</td>
<td></td>
</tr>
<tr>
<td><strong>In glycerol:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(L1) $\text{Lf}^<em>_{T} \xrightarrow{\text{AH}_2} \text{LfH}^</em> + \text{AH}^*$</td>
<td>$540 - 2400 \text{ sec}^{-1}$</td>
<td>(Fig. 24)</td>
</tr>
<tr>
<td>(L2) $2 \text{ LfH}^* \xleftrightarrow{} \text{Lf} + \text{LfH}_2$</td>
<td>$1.4 \times 10^7 \text{ M}^{-1}\text{sec}^{-1}$</td>
<td>(Fig. 19)</td>
</tr>
<tr>
<td>(F1) $\text{FMN}^<em>_{T} \rightarrow \text{FMN}^</em>$</td>
<td>$1100 - 4800 \text{ sec}^{-1}$</td>
<td>(Fig. 24)</td>
</tr>
<tr>
<td>(F2) $2 \text{ 'FMN}^* \xleftrightarrow{} \text{FMN} + \text{FMNH}_2$</td>
<td>$3.5 \times 10^6 \text{ M}^{-1}\text{sec}^{-1}$</td>
<td>(Fig. 21)</td>
</tr>
</tbody>
</table>
Reoxidation of Reduced Flavins

Autoxidation

The results obtained in the present study are consistent with the theory of flavin autoxidation proposed by Gibson and Hastings (1962) (see Introduction). In contrast to their rapid-mixing studies with fully reduced flavin (FMNH₂), the primary photoprocess in our flash studies will be the flavin semiquinone. The reaction proceeds, in both cases, by consecutive second and first order reactions (Figure 26), presumably with the formation of an intermediate hydroperoxide. The second order rate constant obtained from our data (Figure 26) for the reaction of FMNH⁺ with O₂ (5.95 x 10⁻⁵ M⁻¹ sec⁻¹) is about half of that for FMNH₂ with O₂ (1.7 x 10⁻⁶ M⁻¹ sec⁻¹) obtained by Gibson and Hastings, and is in good agreement with their reported value (7 x 10⁻⁵ M⁻¹ sec⁻¹) for the oxidation of the semiquinone. The first order rate constant for the decomposition of the intermediate, however, is about 3.5 times larger in the case of the semiquinone (83.3 sec⁻¹) as compared to that reported for the fully reduced flavin (24 sec⁻¹). Thus, the faster overall autoxidation rate for the semiquinone compared to the fully reduced species, as observed by Holmstrom (1964d), is due to the instability of the hydroperoxide intermediate rather than to an initially faster reaction with oxygen.
Reoxidation with Benzoquinone

From the results obtained (Figures 27, 28, 29), it is obvious that in the three component redox system, donor: flavin:acceptor (i.e. glycerol:flavin:p-benzoquinone), a complex series of consecutive reactions occur. In the case of lumiflavin, these can be described as follows:

\[
\text{Lf} + \text{light} \rightarrow \text{Lf}^* \quad (S0)
\]

\[
\text{BQ} + \text{light} \rightarrow \text{BQ}^* \quad (S0')
\]

\[
\text{Lf}^* + \text{AH}_2 \rightarrow \text{LfH}^* + \text{AH}^* \quad (S1)
\]

where \(\text{AH}_2\) represents the donor, in this case the solvent, glycerol. Benzoquinone can react in a like manner (Figure 27a):

\[
\text{BQ}^* + \text{AH}_2 \rightarrow \text{BQH}^* + \text{AH}^* \quad (S1')
\]

The three free radicals can disproportionate.

\[
2 \text{LfH}^* \leftrightarrow \text{Lf} + \text{LfH}_2 \quad (S2)
\]

\[
2 \text{BQH}^* \leftrightarrow \text{BQ} + \text{BQH}_2 \quad (S2')
\]

\[
2 \text{AH}^* \leftrightarrow \text{A} + \text{AH}_2 \quad (S2'')
\]

In the three component system, the biphasic nature of the 450 nm curves (Figure 28c), the increased rate of 560 nm decay (Figure 29b vs. 29a), the increased yield of benzosemiquinone radical (Figure 27b vs. 27a), and the change in the apparent reaction order can be accounted for by:
The increased yield of benzosemiquinone excludes the possibility of a radical-radical reaction such as \( \text{LfH}^* + \text{BQH}^* \). The biphasic nature of the 450 nm curve (Figure 28c) cannot be accounted for by the summation of the two component curves (Figures 28a and 28b), even taking into account the 2½ fold increase in benzosemiquinone. Also, this hypothesis (simple summation) would not explain the increased rate of decay at 560 nm and the change in reaction order. It was also observed that when the benzoquinone was present in only slight excess, there was a transition in the 450 nm curves for successive flashes from the biphasic type (Figure 28c) to the monophasic type typical of flavin alone (Figure 28a). This is consistent with a depletion of benzoquinone by steps (S3) and (S2'), and a concomitant increasing production of reduced flavin via step (S2).

Thus, in the presence of excess quinone, the primary reaction sequence would be the sequential electron transfer via steps (S0)-(S1)-(S3)-(S2'). In the case of FMN, although the preliminary results are similar to those obtained for lumiflavin, the reaction sequence may also be further complicated by reaction of the fully reduced flavin (produced in step (F3), vide supra) with the benzoquinone.
The observed sequential electron transfer from glycerol through flavin to quinone is consistent with the postulated role of flavin in mitochondrial electron transport. A subsequent step, the electron transfer from quinone to cytochrome c, has already been reported by Yamazaki and Ohnishi (1966).
APPENDIX

COMPUTER PROGRAM
***

PHOTOFLAVINS MASTER PROGRAM

C LISTING OF FORTRAN II SOURCE DECK

C THE INPUT DECK CONSISTS OF THE THREE CONTROL C CARDS FOLLOWED BY EITHER THE RAW DATA FROM C A PREVIOUS COMPUTER RUN. THE FIRST CONTROL C CARD IDENTIFIES THE RUN BY DATE AND NUMBER, C AND GIVES THE MAXIMUM NUMBER OF SETS OF DATA C (MREP), THE NUMBER OF OPERATIONS PER SET C (MCON), AND THE NUMBER OF ITERATIVE CYCLES C IN THE VARIABLE DEFINITION ROUTINE (MIC). FOR EXAMPLE -

C DEC 06 1966 01 01 07 02

C (IDATE)(JDATE)(KDATE)(IRUN)(MREP)(MCON)(MIC)

C THE SECOND CONTROL CARD LISTS THE OPERATIONS C IN THE ORDER TO BE PERFORMED. FOR EXAMPLE-

C 02 04 07 05 06 08 09

C 02 READ AND REDUCE FILM READER DATA C 04 DEFINE VARIABLES (FIRST CYCLE) C 07 CLEAR GRAPH MATRIX AND STORE DATA C 05 DEFINE NEW VARIABLES C 06 DO LINEAR LEAST SQUARES DATA FIT C 08 STORE DATA IN GRAPH MATRIX C 09 PRINT OUT GRAPH

C THE THIRD CARD TELLS THE COMPUTER HOW TO C DEFINE THE VARIABLES FOR EACH CYCLE.

C 01010103 01010603

C CYCLE 1, GRAPH A PLOT OF REDUCED DATA C 0101 X = TIME C 0103 Y = OPTICAL DENSITY

C CYCLE 2, GRAPH B FIRST ORDER KINETIC PLOT C 0101 X = TIME C 0603 Y = LN(OPTICAL DENSITY)
C MAIN PROGRAM CONTROL

100 DIMENSION JCON(50), JC(10), JD(10), KC(10),
1 KD(10), IXT(100), X(100), Y(100), NUM(100),
2 VAR(3, 100), IP(101, 51), SMAXX(10), SMAXY(10)
101 READ 10100, 9995, IDATE, JDATE, KDATE, IRUN, MREP,
1 MCON, MIC
10100 FORMAT(3A4, 4X, I4, 3(6X, I4))
102 READ 10200, 9995, (JCON(KCON), KCON=1, MCON)
10200 FORMAT(4012)
103 READ 10300, 9995, (JC(IC), JD(IC), KC(IC), KD(IC),
1 IC=1, MIC)
10300 FORMAT(8(4I2, 2X))
110 DO 9990 IR.rep=1, MREP
111 DO 9990 KCON=1, MCON
112 ICON=JCON(KCON)
113 GO TO (11100, 11200, 11300, 11400, 11500, 11600, 11700, 11800, 11900, 12000, 12100, 12200, 12300, 12400, 12500, 12600, 12700, 12800, 12900, 13000, 13100, 13200, 13300, 13400, 13500, 13600, 13700, 13800, 13900, 14000, 14100, 14200, 14300, 14400, 14500, 14600, 14700, 14800, 14900, 15000, 15100, 15200, 15300, 15400, 15500, 15600, 15700, 15800, 15900, 16000, 16100, 16200, 16300, 16400, 16500, 16600, 16700, 16800, 16900, 17000, 17100, 17200, 17300, 17400, 17500, 17600, 17700, 17800, 17900, 18000, 18100, 18200, 18300, 18400, 18500, 18600, 18700, 18800, 18900, 19000, 19100, 19200, 19300, 19400, 19500, 19600, 19700, 19800, 19900, 20000, 20100) ICON

C FILM READER INPUT ROUTINE

C THIS SECTION ACCEPTS DATA FROM THE NRI
C FILM READER, CHECKS FOR OUT OF ORDER CARDS
C AND PUNCHING ERRORS, REDUCES THE DATA TO
C TIME, VOLTAGE, AND OPTICAL DENSITY, AND
C PRINTS AND PUNCHES THE NEW SET OF DATA.

C VAR(1, I) = TIME(I)
C VAR(2, I) = VOLTAGE(I)
C VAR(3, I) = OPTICAL DENSITY(I)

201 READ 20100, 9995, IDENT, BASE, SCLX, SCLY
20100 FORMAT(I9, 1X, 3F10.7)
202 PRINT 20200, IDATE, JDATE, KDATE, IRUN, IDENT, BASE,
1 SCLX, SCLY
20200 FORMAT(1H1, 3A4, 11H RUN NUMBER, I4, //,
1 13H FRAME NUMBER, 1X, I9, //, 6H BASE=, E10.3, //,
2 15H X(FULL SCALE)=, E10.3, //, 15H Y(FULL SCALE)=,
3 E10.3)
203 DO 212 I=1, 100, 5
204 K=I+4
205 READ 20500, 9995, JDE, NT, (IXT(J), X(J), Y(J), J=I, K)
20500 FORMAT(I9, 1X, 5(A1, F4.0, F5.0, 4X))
206 IF (IDENT=JDENT) 207, 209, 207
207 PRINT 20700, IDENT, I
20700 FORMAT(19H CARDS OUT OF ORDER, 1X, I9, 1X, I4)
208 GO TO 9999
DO 211 L=I,K
210 IF (X(L)+Y(L))213,213,211
211 CONTINUE
212 CONTINUE
213 NPT=L-1
214 NTOT=NPT-4
215 PUNCH 21500 IDENT NTOT
21500 FORMAT(19,3X,14)
216 DO 263 I=1,NPT
217 NUM(I)=I-4
218 XT=IXT(I)
219 IF (XT-90.0)220,223,223
220 IF (XT-80.0)221,229,229
221 IF (XT-70.0)229,222,222
222 IF (XT-70.0)225,227,227
223 X(I)=X(I)+(XT-90.0)*10000.0
224 GO TO 231
225 X(I)=X(I)+(XT-70.0)*10000.0+100000.0
226 GO TO 231
227 X(I)=X(I)+(XT-70.0)*10000.0
228 GO TO 231
229 PRINT 22900 IDENT I
22900 FORMAT(17H DATA PUNCH ERROR,1X,19,1X,14)
230 GO TO 9999
231 IF (NUM(I))232,234,236
232 PRINT 23200 X(I),Y(I)
23200 FORMAT(5X,2(F10.0,1X),9H FIDUCIAL,1X,14)
233 GO TO 263
234 PRINT 23400 X(I),Y(I)
23400 FORMAT(5X,2(F10.0,1X),7H ORIGIN,5X,4H NUM,
1 7X,1HX,10X,1HY,7X,4HTIME,6X,7HVOLTAGE,4X,
2 THDENSITY)
235 GO TO 263
236 VAR(1,I-4)=(X(I)-X(4))*SCLX/(X(2)-X(1))
237 VAR(2,I-4)=(Y(I)-Y(4))*SCLY/(Y(3)-Y(2))
238 VAR(3,I-4)=BASE/(BASE+VAR(2,I-4))
239 IF (VAR(3,I-4))229,229,240
240 VAR(3,I-4)=LOGF(VAR(3,I-4))
255 PRINT 25500 NUM(I),X(I),Y(I),VAR(J,I-4),J=1,3
25500 FORMAT(I4,2(X,F10.0),3(1X,E10.3))
260 PUNCH 26000 IDENT,NUM(I),VAR(J,I-4),J=1,3
26000 FORMAT(19,3X,14,4X,3E10.3)
263 CONTINUE
264 GO TO 9990
C REDUCED DATA INPUT ROUTINE
C
C THIS SECTION ACCEPTS REDUCED DATA FROM A
C PREVIOUS COMPUTER RUN FOR FURTHER WORK.

301 READ 30100,9995,IDENT,NTOT
30100 FORMAT(I9,3X,I4)
302 PRINT 30200,IDENT,JDATE,KDATE,IRUN,IDENT
30200 FORMAT(1H1,3A4,11H RUN NUMBER,I4,///,
          13H FRAME NUMBER,1X,I9,///,4H NUM,4X,
          7H TIME,6X,7HVOLTAGE,4X,7HDENSITY)
303 DO 309 I=1,NTOT
304 READ 30400,9995,JIDENT,NUM(I),(VAR(J,I),J=1,3)
30400 FORMAT(I9,3X,I4,4X,3E10,3)
305 IF (IDENT-JIDENT)306,308,306
306 PRINT 30600,IDENT,I
30600 FORMAT(19H CARDS OUT OF ORDER,1X,I9,1X,I4)
307 GO TO 9999
308 PRINT 30800,NUM(I),(VAR(J,I),J=1,3)
30800 FORMAT(I4,3(1X,E10,3))
309 CONTINUE
310 GO TO 9990
C VARIABLE DEFINITION ROUTINE

C THIS ROUTINE CONVERTS THE ORIGINAL DATA AS DESIRED FOR USE IN THE LEAST SQUARES AND GRAPH ROUTINES. FOR EXAMPLE, STATEMENT 417 USED ON VARIABLE (3,1) AND STMNT 408 USED ON VARIABLE (1,1) WHEN COMBINED WITH THE LAST THREE ROUTINES WILL PROVIDE THE FIRST ORDER KINETIC PLOT OF LN OD VS TIME, THE RATE CONSTANT, AND THE EXTRAPOLATED INITIAL OD. IT WILL ALSO SHOW THE ERROR AND PERCENT DEVIATION OF EACH POINT FROM THE BEST FIT STRAIGHT LINE.

400 IC=0
401 IC=IC+1
402 DO 457 I=1,NTOT
403 J=1
404 LC=JC(IC)
405 LD=JD(IC)
407 GO TO (408,409,410,411,413,415,418,419,420,423,424,425),LC
408 Z=VAR(LD,I)
GO TO 450
409 Z=-VAR(LD,I)
GO TO 450
410 Z=1.0/VAR(LD,I)
GO TO 450
411 IF (VAR(LD,I))412,413,414
412 Z=LOGF(-VAR(LD,I))
GO TO 450
413 Z=0
GO TO 450
414 Z=LOGF(VAR(LD,I))
GO TO 450
415 IF (VAR(LD,I))416,413,417
416 Z=LOGEF(-VAR(LD,I))
GO TO 450
417 Z=LOGEF(VAR(LD,I))
GO TO 450
418 Z=VAR(LD,I)**2
GO TO 450
419 Z=1.0/VAR(LD,I)**2
GO TO 450
420 IF (VAR(LD,I))421,423,422
421 Z=SQRTF(-VAR(LD,I))
GO TO 450
422 Z=SQRTF(VAR(LD,I))
GO TO 450
423 Z=EXPF(VAR(LD,I))
GO TO 450
424 Z=EXPF(VAR(LD,I))
GO TO 450
425 GO TO 450
450 GO TO (451,456),J
451 X(I)=Z
452 J=2
453 LC=KC(IC)
454 LD=KD(IC)
455 GO TO 407
456 Y(I)=Z
457 CONTINUE
458 PRINT 45800,IDATE,JDATE,KDATE,IRUN,IDENT
45800 FORMAT(1H3,3A4,11H RUN NUMBER,14//,
1 13H FRAME NUMBER,1X,I9,,//,
2 21H VARIABLE DEFINITIONS)/
459 L=87
460 LC=JC(IC)
461 LD=JD(IC)
462 PRINT 46200,IC
46200 FORMAT(13H CYCLE NUMBER,I5)
463 GO TO (464,465,466,467,468,469,470,471,472)
1 473,474,475),LC
464 PRINT 46400,L,LD
46400 FORMAT(IX,A2,11H = VARIABLE,I4)
GO TO 490
465 PRINT 46500,L,LD
46500 FORMAT(1X,A2,12H = -VARIABLE,I4)
GO TO 490
466 PRINT 46600,L,LD
46600 FORMAT(1X,A2,13H = 1/VARIABLE,I4)
GO TO 490
467 PRINT 46700,L,LD
46700 FORMAT(1X,A2,19H = LOG(10) VARIABLE,I4)
GO TO 490
468 PRINT 46800,L,LD
46800 FORMAT(1X,A2,14H = 0 (VARIABLE,I4,1H))
GO TO 490
469 PRINT 46900,L,LD
46900 FORMAT(1X,A2,14H = LN VARIABLE,I4)
GO TO 490
470 PRINT 47000,L,LD
47000 FORMAT(1X,A2,11H = VARIABLE,I4,2X,8H Squared)
GO TO 490
471 PRINT 47100,L,LD
47100 FORMAT(1X,A2,13H = 1/VARIABLE,I4,2X,8H Squared)
GO TO 490
472 PRINT 47200,L,LD
47200 FORMAT(1X,A2,26H = SQUARE ROOT OF VARIABLE,I4)
GO TO 490
473 PRINT 47300,L,LD
C LINEAR LEAST SQUARES ROUTINE

501 SX=0
502 SY=0
503 SXX=0
504 SXY=0
505 DO 512 I=1,NTOT
506 XX=X(I)*X(I)
507 XY=X(I)*Y(I)
508 SX=SX+X(I)
509 SY=SY+Y(I)
510 SXX=SXX+XX
511 SXY=SXY+XY
512 CONTINUE
513 PRINT 51300, SX, SY, SXX, SXY
514 PTOT=FLOATF(NTOT)
515 SLOPE=(PTOT*SXY-SX*SY)/(PTOT*SXX-SX*SX)
516 YATX0=(SXX*SY-SX*SXY)/(PTOT*SXX-SX*SX)
517 PRINT 51700,NTOT, SLOPE, YATX0
518 DO 525 I=1,NTOT
519 DEVLS=SLOPE*X(I)+YATX0-Y(I)
520 PCDEV=DEVLS*1000/Y(I)
521 PRINT 52100, I, X(I), Y(I), DEVLS, PCDEV
522 CONTINUE
523 GO TO 9990
GRAPH STORAGE ROUTINE

THIS SECTION STORES THE RESULTS IN A 50X100 MATRIX. NORMALIZED TO THE MAXIMUM VALUES OF THE X AND Y VARIABLES FOR GRAPHING.

```plaintext
DO 604 IY=2*50
602 DO 604 IX=2*100
603 IP(IX,IY)=0
604 CONTINUE
6010 DO 6013 IY=2*50
6011 IP(IX,IY)=26
6012 IP((101,IX),IY)=26
6013 CONTINUE
6020 DO 6023 IX=1*101
6021 IP(IX,1)=15
6022 IP(IX,51)=15
6023 CONTINUE
6030 DO 6034 IX=11*91*10
6031 JP=IX/10 +90
6032 IP((IX,1)=JP+190
6033 IP((IX,51)=JP
6034 CONTINUE
6040 DO 6044 IY=6*46*5
6041 KP=IY/5 +90
6042 IP(1,IX)=KP+190
6043 IP(101,IX)=KP
6044 CONTINUE
605 PMAXX=0
606 PMAXY=0
607 DO 616 I=1*NTOT
608 IF (X(I)) 610 612 610
609 IF (X(I)-PMAXX) 611 612 612
610 IF (PMAXX-X(I)) 611 612 612
611 PMAXX=X(I)
612 IF (Y(I)) 613 616 614
613 IF (Y(I)-PMAXY) 615 616 616
614 IF (PMAXY-Y(I)) 615 616 616
615 PMAXY=Y(I)
616 CONTINUE
620 DO 639 I=1*NTOT
621 IF (PMAXX) 622 624 623
622 IF (X(I)) 628 626 626
623 IF (X(I)) 625 625 627
624 IF (PMAXY) 625 643 625
625 IX=1
GO TO 630
626 IX=101
GO TO 630
627 IX=X(I)*100.0/PMAXX+1.5
```
GO TO 630
628 IX=101.5-X(I)*100.0/PMAXX
630 IF (PMAXY)=631.643.632
631 IF (Y(I))=635.633.633
632 IF (Y(I))=634.634.636
633 IY=1
GO TO 637
634 IY=51
GO TO 637
635 IY=Y(I)*50.0/PMAXY+1.5
636 IY=51.5-Y(I)*50.0/PMAXY
637 IP(IY)=IC+60
639 CONTINUE
640 SMAXX(IC)=PMAXX
641 SMAXY(IC)=PMAXY
642 ID=IC+60
643 PRINT 64300,IREP,ID
64300 FORMAT(///,17H SEE GRAPH NUMBER,14.5X,
   13H CURVE NUMBER,A2)
649 GO TO 9990

C GRAPH OUTPUT ROUTINE

651 PRINT 65100,IDENT,IDATE,JDATE,KDATE,IRUN,IREP
65100 FORMAT(1H1,3A4,1X,11H RUN NUMBER,I4,
   1 13H GRAPH NUMBER,I4)
652 DO 655 IC=1,MIC
653 ID=IC+60
654 PRINT 65400,IDENT,ID,SMAXX(IC),SMAXY(IC)
65400 FORMAT(10X,13H FRAME NUMBER ,IX,19,
   1 13H CURVE NUMBER,A2,5X,12H X MAXIMUM =,
   1 2 E10.3,5X,12H Y MAXIMUM =,E10.3)
655 CONTINUE
656 DO 658 IY=1,51
657 PRINT 65700,(IP(IY),IX=1,101)
65700 FORMAT(5X,101(A1))
658 CONTINUE
659 GO TO 9990
9990 CONTINUE
9991 GO TO 101
9995 PRINT 99950
99950 FORMAT(5(23H END OF DATA END OF RUN))
.9996 GO TO 9999

9999 STOP

END
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