1960

Mechanism of riboflavin-catalyzed oxidations

Daniel Oscar Carr
Iowa State University

Mic 61-440

CARR, Daniel Oscar. MECHANISM OF RIBOFLAVIN-CATALYZED OXIDATIONS.

Iowa State University of Science and Technology
Ph.D., 1960
Chemistry, biological

University Microfilms, Inc., Ann Arbor, Michigan

MECHANISM OF RIBOFLAVIN-CATALYZED OXIDATIONS

by

Daniel Oscar Carr

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1960

TABLE OF CONTENTS

INTRODUCTION

Riboflavin in its coenzyme forms (riboflavin-5'-phosphate and flavin adenine dinucleotide), along with the appropriate enzymes, catalyzes many oxidation-reduction reactions. Flavoenzymes which produce carbonyl compounds have such substrates as amines, amino acids, alcohols, α-hydroxy acids, hemiacetals and free aldehydes. Flavoenzymes which form an alkene linkage in the α-β position to a carbonyl, have fatty acyl - coenzyme A derivatives and dihydroorotic acid as substrates. Formation of a double bond also occurs in the A ring of steroids through flavin catalysis. Flavin catalyzed hydroxylation reactions are performed on purine compounds but these may be classified as alcohol oxidations. Flavoenzyme reactions which form only compounds of a higher oxidation state or which cannot be classified above, occur with the pyridine nucleotides, the cytochromes, the nitrogen compounds in various oxidation states, and the sulhydryl-disulfide compounds.

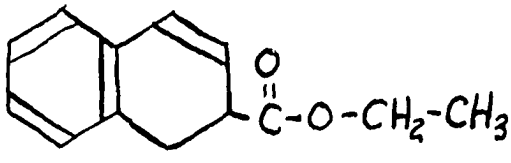
These numerous substrate oxidation-reduction reactions are linked directly to the respiratory chain via flavoenzymes. With the flavin lying distal to the pyridine nucleotides in the respiratory chain, there is an inherent loss in the ability to form one adenosine triphosphate because one adenosine triphosphate is formed in the pyridine nucleotide-flavin

reaction. Since pyridine nucleotide enzymes can oxidize hydroxyl compounds, the question arises as to why an organism should have such a "wasteful" system. The answer may well be that the wide versatility of riboflavin as an oxidizing agent easily compensates for the few kilocalories of energy lost by allowing the organism to be less dependent on what would otherwise be a large number of cofactors.

Very little work has been done on oxidation-reduction reactions using only riboflavin as a catalyst - without the corresponding enzyme systems. The only reversible, single product oxidation-reduction reactions studied have used other respiratory pigments, usually diphosphopyridine nucleotide or its analogs, as substrates. The oxidations of the cytochromes and hemoglobin involve no bond breakage but a change in the oxidation state of the iron.

A systematic attempt to oxidize flavin substrates and their analogs was made using only riboflavin as a catalyst. Success was obtained to a high degree for only one compound. This substrate was ethyl Δ^3-dihydro-β-naphthoate. The oxidation of this compound is the first, single product, non-enzymic, substrate level oxidation reported for riboflavin.

The compound, whose structure is:



is a fatty acid analog which is greatly activated. However, its mode of oxidation may not be too far removed from that of the fatty acyl - coenzyme A derivatives. It was hoped that any mechanism found for this substrate oxidation would help elucidate the mechanism of other reactions. The reaction of the ethyl Δ^3-dihydro-β-naphtoate was characterized as to product, order of reaction, and effects of pH, metal ions (chelation), anions, other compounds (molecular complexes), solvent, ionic strength, heat, and light.

The results of these studies allowed the postulation of a mechanism for the oxidation and raised the question as to whether the oxidation occurred by one or two electron transfer.

Leucoflavin (dihydroriboflavin) was prepared, characterized, and allowed to react with oxidized substrates, riboflavin, and oxygen. Leucoflavin had not been previously analyzed, chemically or spectrally, in a solution free from other substances.

In attempting both the oxidations and reductions, it was necessary that various properties of riboflavin be studied. The results of these studies will also be discussed.

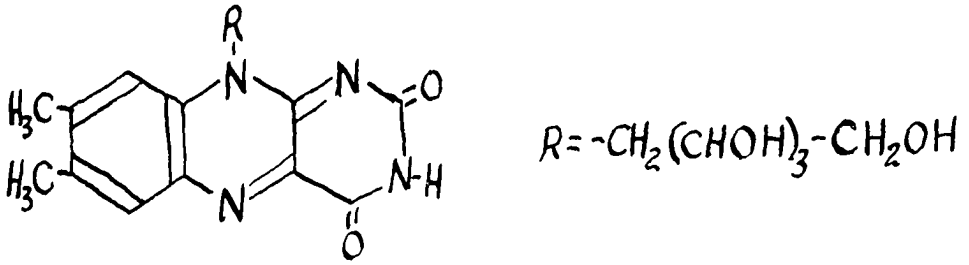
REVIEW OF PERTINENT LITERATURE

The chemical and biological character of the flavins has been quite well studied in the last thirty years. However, the bluish-fluorescent coloration of milk was known long before this period. In 1879, the "alkaloidal coloring matter" of milk was isolated by Blyth (1) and designed "lactochrome." This was proven to be identical to "ovoflavin" as isolated from egg white by Kuhn et al. (2) in 1933. In a short time after the renewed interest developed, riboflavin was structurally analyzed and totally synthesized by Karrer et al. (3) and Kuhn et al. (4). A very thorough review of flavin history and chemistry has been written recently by Beinert (5).

The oxidation-reduction capabilities of riboflavin were soon noted. Catalytic hydrogenation was facile and reversible. The reoxidation occurred very rapidly in air. A flavin enzyme had earlier been isolated by Warburg and Christian (6). This enzyme was the classical old yellow enzyme of yeast. The yellow color was almost immediately attributed to the presence of a flavin coenzyme and was so proven by isolation (7). These results suggested the role of riboflavin in biological systems.

The physical, chemical and biological properties of riboflavin are quite well covered in vitamin texts such as

"The Vitamins" (8). The structure of riboflavin (6,7-dimethyl-9-(D-1'-ribityl)-isoalloxazine) is:



The only properties which will be elaborated upon in this discussion will be the ones involved in the dissertation, viz., solubility, heat stability, light stability, spectral properties, fluorescent properties, oxidation-reduction properties, chelation abilities, and complexation abilities.

A single value for riboflavin water solubility would not be correct for all riboflavin samples. The usual value given for the solubility of riboflavin, U.S.P., is 12 mg/100 ml at 25°. Riboflavin, R.S., U.S.P., has a solubility ten times that of riboflavin, U.S.P. As described by Beinert (5), the various solubilities are apparently due to polymorphism.

Solvents other than water form much less concentrated solutions of riboflavin (9). Anhydrous solvents such as amyl alcohol, cyclohexanol, benzyl alcohol, phenol and ethanol dissolve riboflavin to an extent less than 10^{-5} M. Ninety-five per cent ethanol forms a 10^{-4} M solution of riboflavin. Riboflavin is classified as insoluble in ether, acetone, chloroform and benzene. Basic solutions dissolve riboflavin

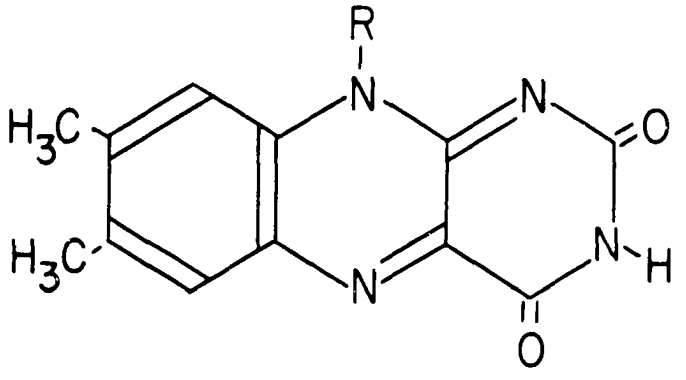
to a large extent due to the flavin ionization. The basic solutions are unstable, however.

It has been reported (10) that riboflavin can be heated to 120° for 6 hours with only slight destruction. A detailed study has been made on pH and buffer anion effects (11). In light, the destruction at high temperatures is very fast (12).

The photodegradation of riboflavin has been studied as to pH effect, optimum wavelengths, and influence of other compounds. The products obtained from this degradation are large in number, with the side chain isomers being investigated most thoroughly. The degradation compounds most often studied are lumiflavin (6,7,9-trimethylisoalloxazine) (6, 13, 14, 15), lumichrome (6,7-dimethylalloxazine) (16, 17), 1'-carboxymethyllumiflavin (18), 1'-carboxylumiflavin (19, 20, 21, 22), and 1'-methyllumiflavin (23) with the by-products formaldehyde, formic acid, glycolic acid, and a four carbon sugar (24, 25). The structures of the flavins are shown in Figure 1.

The photodecomposition is much faster in alkaline solutions. The wavelength range of maximum efficiency is 365-590 mμ (26) with no appreciable decomposition occurring above 610 mμ. Recent reviews (23, 24, 27) on the photodecomposition and mechanism thereof have made this aspect more comprehensible.

Figure 1. Structures of riboflavin photodegradation products



R = $-CH_3$ LUMIFLAVIN

= $-H$ LUMICHROME

= $-CH_2-CH_2-COOH$ 1'-CARBOXYMETHYL-LUMIFLAVIN

= $-CH_2-COOH$ 1'-CARBOXYLUMIFLAVIN

= $-CH_2OH$ 1'-HYDROXYLUMIFLAVIN

Recently, it has been shown by electron spin resonance (28) and sulfite oxidation (29) that light produces long lived radicals in a riboflavin-5'-phosphate solution. This effect is probably a result of light induced oxidation-reduction properties discussed on page 16b.

The spectral properties of riboflavin are given visually in Figure 2. The neutral spectra of all flavins, riboflavin (14, 15), lumiflavin (15), riboflavin-5'-phosphate (30), flavin adenine dinucleotide (30), and others (31), are almost identical. The pH dependency at all pH has been reported (32) and is in agreement with the spectra in Figure 2. The flavin spectrum has been confirmed many times since (33, 34, 35, 36).

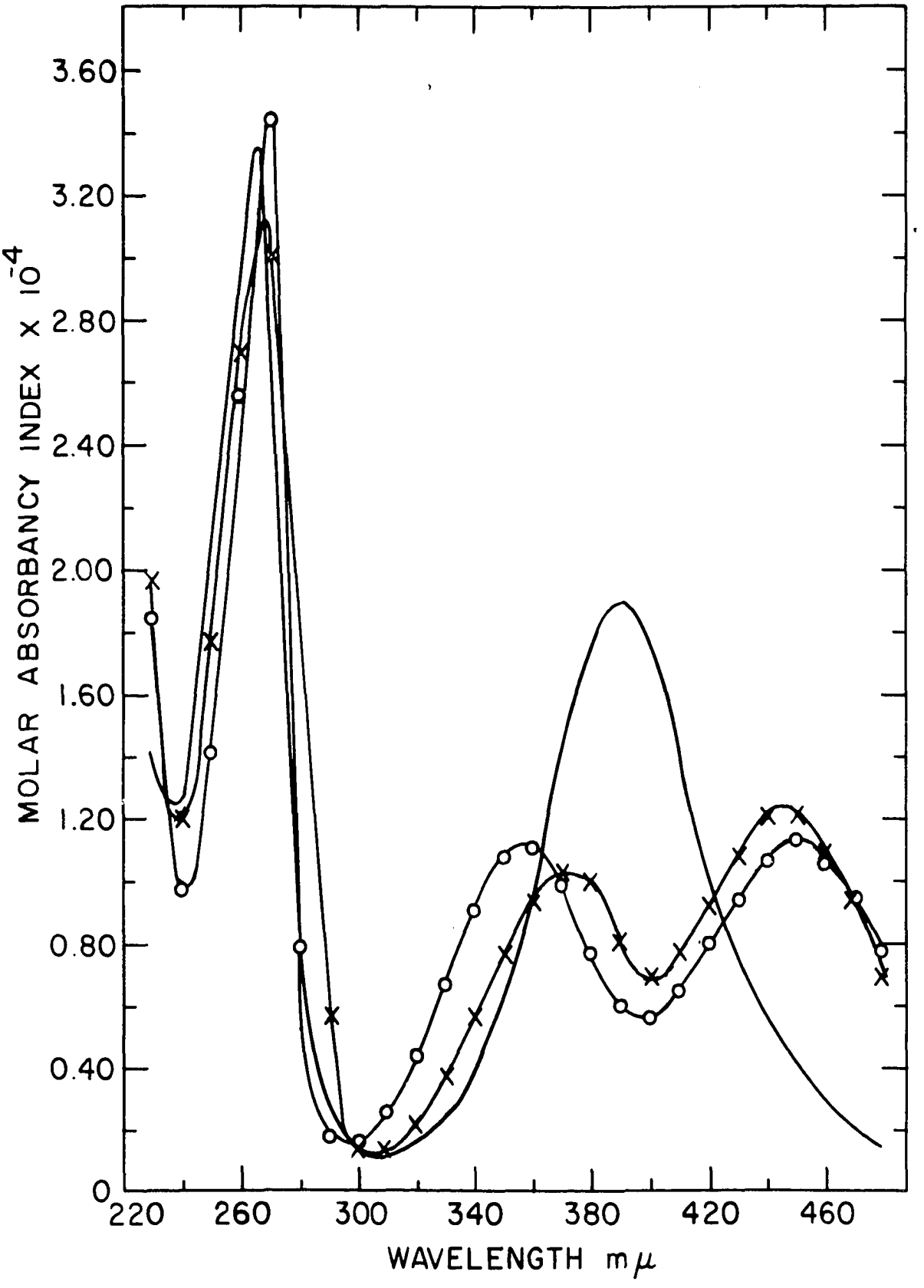
Riboflavin is frequently described as being an amphoteric compound. This statement is a reflection of early fluorometric studies. Because the neutral form of riboflavin has a strong yellowish-green fluorescence, any change in the ionic character of the molecule should alter the fluorescence spectrum and intensity. Thus, from fluorescence studies pK_a values of 10.2 and 1.7 were obtained (37, 38). The higher pK has been redetermined by this method (34) and agrees with the titrimetric value of 9.8 (39), 9.93 (40), and 9.95 (41). However, the lower pK does not agree with the titrimetric value of -0.02 (39) or 0.12 (41). The titrimetric values were obtained by spectrophotometric methods

Figure 2. Riboflavin spectra

o—o—o Riboflavin anion

x—x—x Neutral form

——— Calculated for riboflavin cation


MOLAR ABSORBANCY INDEX X 10^{-4}
3.60
3.20
2.80
2.40
2.00
1.60
1.20
0.80
0.40
0
220
260
300
340
380
420
460
WAVELENGTH mμ

using the shifts as shown in Figure 2. The higher pK is due to the ionization of the hydrogen in the 3 position. The lower pK is attributed to the protonation of either the 1 or 10 position.

Weber (42) explained the discrepancy between the fluorometric value and the spectrophotometric value of the lower pK was due to the much shorter lifetime of the photoexcited state in acid. Another explanation is that there is an actual difference in the pK between the ground state molecule and the photoexcited molecule. Leonhardt and Weller (43) have shown this to be the case with acridine. The pK of the ground state of acridine is 5.45 whereas in the excited state it rises to 10.65. Since photoexcited states favor the more ionic species, the same reasoning can be applied to riboflavin. At low pH, an ionic form of photoexcited riboflavin is preferred and photoexcited riboflavin will therefore accept a proton at a higher pH than will the ground state molecule. Since the protonated form does not fluoresce, the observed pK will be higher than it is in the ground state. Obviously, both of these explanations lie in the older term, "proton quenching".

A number of fluorescence quenchers are operative with riboflavin. Ions and organic compounds are known for their quenching ability (44). Discussion of these will be given with the chelate-complex discussion because fluorescence has

been a tool in determining chelate and complex stability.

With riboflavin functioning as an oxidation-reduction agent in biological systems, much time was spent by the early workers in establishing the process and identifying intermediates and products. With mild reducing agents (H_2/P_t, dithionite, thallous ion, Na/Hg, and active metals in acid), riboflavin is reduced to leucoflavin (dihydrooriboflavin). It was assumed, but has never been proven, that the reduction occurs 1,10. Since both flavins are highly tautomeric systems, where the hydrogens originally bond remains an academic question. The pK of leucoflavin was reported by Michealis et al. to be 6.1 (39). The reduced compound rapidly returns to the oxidized form in air, reacting with oxygen to produce hydrogen peroxide. The solubility of leucoflavin in water is much less than that of riboflavin, forming about 2×10^{-5} M solutions (5). This low solubility is used in the commercial isolation of riboflavin. Also leucoflavin is more light stable than is riboflavin (45).

Catalytic hydrogenation, under high pressure, yields octahydroflavins which in alkaline solution, oxidize to the hexahydroflavins - the benzene ring remaining reduced (46). This hydrogenation has been carried out on 9-arabitylisoalloxazine but not on riboflavin itself.

The most astonishing, spectacular and important thing observed was a red intermediate (λ_{max} 490 mμ) which forms

when riboflavin is reduced in acid (< PH1) (47). Michealis and Hill (48) had observed this phenomenon earlier in connection with some phenazine compounds and, therefore, applied the same interpretation - a semiquinone radical was present. Michealis et al. (39) concluded that the radical existed al all pH but, due to varying degrees of ionization, was only red and visible to the eye below pH 1. Michealis et al. (39) continued their studies on this compound until they isolated large red plate crystals. Soon thereafter, Kuhn and Ströbele (49) reported the isolation of several different partially reduced dimeric products (0.25, 0.5, 0.75 reduced or oxidized). Supposedly, these dimers had accepted one electron at a time until the total of four electrons produced leucoflavin. Michealis and Schwarzenbach (50) reviewed their titration data but could find no evidence for these intermediates. Following the directions of Kuhn and Ströbele, one can prepare the colors they describe. Recently (51), more work has been done on these intermediates and their solubilities have been determined. In conjunction with the above studies, the reduction potentials of riboflavin at pH 7 and 5.9 were found to be -0.186 V and -o.146 V, respectively (50, 52, 53, 54, 55), at 20°. More recent determinations (56, 57) are in agreement with these values. Lumiflavin has a similar value (55, 58). Riboflavin-5'-phosphate and flavin adenine dinucleotide possess Eo' of -0.219 V (pH 7, 20°) (59).

Polarographic determinations result in a half-wave potential [reduction potential versus saturated calomel electrode (E° -0.246 V)] of -0.445 V (pH7). A normal wave is obtained over the pH range 1-13 (56, 60, 61). The wave represents two simultaneous one electron reductions with a slight "prewave" due to absorbed leucoflavin on the dropping mercury electrode. The half-wave value agrees quite well with the potentiometrically determined one. Riboflavin-5'-phosphate and flavin adenine dinucleotide behave similarly (62).

Little more was done on this aspect of flavin chemistry until recently when Beinert (63) investigated the spectrum of riboflavin-5'-phosphate at different oxidation levels using dithionite as a reducing agent. He reported the monomeric semiquinone as having a 565 mμ absorption band between pH 2 and 7. The dimer form of the semiquinone possessed a broad absorption band between 700 and 1100 mμ. He could detect the dimeric form as high as pH 11.8, but observed no other dimeric forms. Recently, another study on the rate of dithionite reduction has been performed (64).

With the possibility that the above observations really do not reflect the mode of riboflavin enzymic action, Beinert et al. (65, 66, 67) studied various flavoenzyme systems spectrophotometrically and found intermediate oxidation states of the flavin. Absorption bands were found which were similar

to bands of the Kuhn and Ströbele compounds (49) and to the enzyme spectrum of Haas (68). To explain the radicals, Beinert et al. employed both flavins of the enzymes and formed radicals, each flavin taking one electron from the substrate. Searls (69, 70) and Massey (71) have recently proposed a similar idea using a sulfhydryl radical and a flavin adenine dinucleotide radical. Reoxidation of xanthine oxidase (72) or L-amino acid oxidase (73) by oxygen shows no 565 mμ peak appearing in the enzyme spectrum as Beinert found for riboflavin-5'-phosphate, but a new spectrum appears due, apparently, to a flavin-oxygen intermediate complex.

Even though the semiquinone is stable, this stability should not suggest that the flavin accepts one electron at a time. The experiment of disproportionation of riboflavin and leucoflavin has not been reported; however, it is the author's opinion that a two electron reduction, employing a hydride ion, followed by disproportionation would be a more acceptable representation. A report of this reaction will be given in this dissertation. This experiment will not solve the age old academic problem of a one electron transfer versus a two electron transfer since in the enzyme-substrate complex, either process may be considered a possibility with no means of differentiating them. Recently, electron spin resonance experiments have shown that radicals are present when riboflavin-5'-phosphate or flavin adenine dinucleotide is half

reduced (74). These experiments are not quite equivalent to the disproportionation experiment since the radicals could have been formed in the reduction process and the half reduced condition may not have been significant.

Karrer et al. (75), Singer and Kearney (76), and Suelter and Metzler (41, 77) have reported systems using only riboflavin as a catalyst in the air oxidation of flavin substrates. Vernon (78) and Frisell and Mackenzie (79, 80) have reported that the reduced diphosphopyridine nucleotide system of Singer and Kearney also requires light. This light catalyzed oxidation is interesting, but the same effects are apparently not operative in the case of the reduced pyridine nucleotide analogs of Suelter and Metzler (41, 77). Perhaps the role of the adenine moiety of the pyridine nucleotide is making an appearance as suggested in the enzyme complex of Dolin (81, 82) even though Dolin could not observe the same phenomenon for reduced diphosphopyridine nucleotide-flavin adenine dinucleotide. In all these cases, a reduced pyridine nucleotide analog or reduced diphosphopyridine nucleotide itself was oxidized.

Isaka et al. (83, 84), Shugar (85), Sakurai and Kuroki (86), Brauner and Brauner (87, 88, 89), Frisell et al. (90, 91), Merkel and Nickerson (92, 93), Vernon (78), Galston (94, 95, 96), Fridovich and Handler (29) and others (97, 98) used riboflavin to catalyze the photooxidation of nitrogen con-

taining compounds. Several workers (78, 92, 93) have suggested that riboflavin was reduced due to its oxidation of water. The work of Frisell et al. (90, 91) vitiates this conclusion and leaves only amine oxidation as an explanation. Fridovich and Handler (29) have recently endorsed this conclusion. The mechanisms of these oxidations have been well studied by Frisell et al. (91). Since the products of these reactions are small molecules (NH_3, CO_2, HCHO) of a degradative nature, these irreversible reactions are of an obscure biological significance. The oxidation of indole acetic acid (85, 87, 88, 89, 94, 96) may be the one case where there is some biological significance - in phototropism.

Also, such compounds as pyruvic acid (99), ascorbic acid (86, 87, 100, 101, 102, 103, 104, 105) sugars (106), estrogens (107), and polyene antibiotics (105, 108, 109) are photooxidized in the presence of riboflavin. Some of these oxidations can be inhibited by oxidizable compounds such as reduced glutathione (102, 103), cysteine (103), hydroquinone (86, 89, 104, 105), pyrogallol (86, 89, 104), guaiacol (89). The phenol derivatives have been photooxidized by riboflavin in the absence of other compounds (111). Also, reduced indophenol (112) and p-toluene diamine (113) have been photooxidized by riboflavin. Even enzymes (95, 96) and bacteriophage (96) are inactivated by riboflavin in the presence of light. The effect probably occurs by oxidation of the amino

acids, primarily the aromatic amino acids.

Riboflavin can be photoreduced anaerobically (23, 28, 78, 89, 93). Whether riboflavin is photolyzing water or oxidizing itself is still a question. The author feels that in light of his own work and that of Kuhn et al. (14, 15), the latter explanation is most probable.

Metal ions show inhibitory effects (84) on the above photooxidations which are in agreement with the fluorescence quenching ability of each ion, i.e., as a rule colored divalent ions (Co^{++}, Cu^{++}, Fe^{++}, Ni^{++}, Mn^{++}) quench fluorescence (44).

Riboflavin (114, 115) and flavin adenine dinucleotide (116) have been reported to carry out oxidation-reduction reactions with the Fe^{+++} -Fe^{++} couple. The Fe^{+++} reduction is inhibited by oxygen. Ferrocytochrome c has been photooxidized by riboflavin-5'-phosphate (112). Mn^{++} is also oxidized (117).

After reviewing the literature, one can only state that these reactions are not unique to riboflavin. Several reviews (118, 119, 120) are valuable introductions; as are the references given in early papers (121, 122, 123), Harden and Norris (124) had noticed in 1915 that light activated the lactate reduction of methylene blue and, similarly, in 1913, Dixon and Tunnicliffe (125) observed the same phenomenon for reduced glutathione reduction of methylene blue.

Gaffron (121) photooxidized serum proteins, enzymes, tyrosine, phenol and uric acid with hematoporphyrins or rose bengale as sensitizers. Harris (122), simultaneously, was photooxidizing tyrosine, tryptophan and taurocholic acid. Lieben (126) followed, using the same sensitizers to photooxidize tyrosine, tryptophan and histidine. The other amino acids, including the sulfur amino acids, showed no appreciable photooxidation. Carter (123) reviewed this phenomenon quite well and went on to conduct this type of experiment on a great number of compounds (too numerous to list) using the sensitizers, hematoporphyrin, uroporphyrin, methylene blue, and eosin. (If a photooxidation is planned, this is the paper to read first to see if the oxidation has been done.) Windaus et al. (127, 128) used similar sensitizers to photooxidize ergosterol to the peroxide in water and to "ergopinakon" (ergopinacol) in alcohol.

Weber (129) has shown the fluorescence of sodium napthionate is quenched by metal ions, hydroquinone, pyrogallol, phenol and diethyl allylthiourea. In similar experiments, Lauth's violet was shown to undergo reduction when exposed to light with these compounds present. Martini (130) observed that methylene blue catalyzed the photooxidation of ascorbic acid. Glucose was also oxidized. The latter oxidation was slow in acid but much faster in alkali.

Weiss has shown that Fe^{++} (131) and other inorganic ions, e.g., $SO_3^=$ and $A_sO_2^=$ (132) are also photooxidized in the presence of the same sensitizers. The Fe^{++}-Fe^{+++} reaction goes much faster and at lower light intensities with sensitizers than without. Rabinowitch (133, 134) has shown that Fe^{++} effect again, with thionin. Recently, this worker (135) has shown that the thionine - Fe^{++} reactive complex does not exhibit an absorption spectrum different from that of the thionin moiety.

Weil and Maher (136, 137) used methylene blue as a sensitizer to photooxidize nicotine and many other alkaloids. Light of the wavelength 660 mμ (peak absorption of methylene blue) was most effective. The product was considered to be an amine oxide of the pyrollidine ring. No carbon dioxide was liberated. Other thiazine dyes funtioned equally as well.

In further studies, Weil et al. (138, 139) used methylene blue to photooxidize phenol, tyrosine, and tyramine with no ammonia being produced but one mole of carbon dioxide per mole of phenol oxidized was obtained. Glycyltyrosine and hordeine (N-methyl tyramine) gave similar results. Histidine, imidazole and histamine photooxidized with 4% ammonia being produced. Tryptophan and indole liberated 16% ammonia. Methionine, allocystathionine, N-acetyl methionine, and other methionine derivatives as well as cystine, cystine disulfoxide,

and cysteic acid absorbed oxygen, liberated no ammonia and produced 26% of the sulfur as sulfate. The other amino acids were resistant to photooxidation.

Ferri (140, 141) found that indole acetic acid was photooxidized in the presence of quinine sulfate, esculin and 2,3,5-triphenyltetrazolium chloride with the only common property of the dyes being that of fluorescence. Brauner (88) oxidized indole acetic acid photochemically with the sensitizers (in order of efficiency): riboflavin-thionin-methylene blue-eosin-rose bengale-fluorescein-nile blue-rhodamine B. Ascorbic acid had a similar response to these sensitizers.

Kutsaya and Dain (142) found that methylene blue was photoreduced in alcohol solutions but not in water. Of the metal ions, only Cu^{++} affected the results. The same effect was found for the eosin dyes (143). Schenck (144) used eosin and methylene blue to photooxidize thiourea and terpenes; the latter forming peroxides.

Koizumi and Obata (145, 146) repeated the reduction of methylene blue in alcohol solutions. Various amines were then checked as to their ability to photoreduce methylene blue as the alcohol does. An intermediate was found with λ_{max} at 800, 850, and 950 mμ. Fe^{+++} stabilized the intermediate as determined at 950 mμ. The heat of activation of the photooxidation of dimethyl amine was calculated to be

8.6 kcal/mole. Trimethyl amine was thought to have a lower value.

Methylene blue will photooxidize ethylenediaminetetraacetic acid with red light (147, 148). Other secondary and tertiary amines were also oxidized. Anethol, allylthiourea and diethyl allylthiourea reduce methylene blue photochemically (149). Millich and Oster (150) postulate for this system a long lived excited state, a transition from first singlet excited state to the long lived state induced by ground state dye molecules, and an induction, which occurs over distances of 500 Å.

Mauzerall (151) has used ethyl acetoacetate and 2-carbethoxypentanone as photoreducing agents for thionin dyes. Broyde and Oster (152) have used polyhydroxy compounds, many were sugars, to photoreduce these dyes. Oster et al. (113) have used numerous fluorescent dyes, including riboflavin, to oxidize p-toluene diamine. Hendriks and Berends (105) found that only fluorescent dyes could oxidize pimaricin, a polyene antibiotic.

Buckley et al. (153) have shown, in the oxidation of tertiary amines that light is not needed if the reduction potential of the p-quinones used is sufficiently positive. This result shows a transition from light catalyzed reactions to dark reactions depending on whether the needed energy is internally or externally furnished. These workers also

oxidized the reduced pyridine nucleotide analog of Karrer et al. (75). Similar reports have been made by Horner and Spietschka (154) using o-quinones and by Treibs et al. (155) and Barclay and Campbell (156) oxidizing substituted indoles.

The studies without light are similar to the ones reported by Dimroth (157), Dost (158, 159) and Braude et al. (160, 161, 162) on the quinone oxidation of dihydropyridines, dihydrobenzenes and dihydronaphthalenes. A recent review by Kenner (163) discusses other oxidations. Diphenylpicrylhydrazyl (164) also oxidizes the dihydrocompounds. However, light, peroxide or other radical producing agents does not catalyze the reactions. All the above reactions were favored by polar solvents, proton activation and more positive reduction potentials for the oxidants. Also related is the menadione oxidation of leucoflavin (165).

Several conclusions can be made about light induced oxidations. In the case of amines, the rate of oxidation follows the order: tertiary > secondary > primary > ammonium > quaternary or aromatic. The dyes are always fluorescent. If no quenching of the fluorescence is observed, the oxidation must not involve the first excited state of the dye. Almost any oxidizable structure can be photooxidized with a fluorescent dye. Depending upon the reduction potential of the oxidant, light may or may not be needed. Consequently, any role of riboflavin in photooxidations must be reviewed quite

carefully before claim is made to its biological function because the fact that riboflavin is fluorescent may be fortuitous.

A very fascinating aspect of riboflavin chemistry, which has been studied quite thoroughly, is the role of various compounds in forming molecular complexes with riboflavin. Weber (42) suggested the formation of complexes when he observed a quenching of riboflavin fluorescence by certain organic compounds. This phenomenon has been known for a long time with flavin adenine dinucleotide which has an intramolecular quencher, adenine. The flavin adenine dinucleotide spectrum has been studied recently (35) and found to show an interaction. Weber's report was a more intensive study of an earlier report by Weil-Malherbe (166). This effect was not the result of the collision phenomenon since a lowering of the temperature increased the degree of quenching. An intermolecular complex fits this thermal study data because the activity due to thermal excitation is less and the molecules have more opportunity to complex and not be shaken apart by collisions. Due to the smooth transition between the mean life of the excited state in a complex formation situation and the mean life in a collision quenching situation, no clear decision can be made as to which phenomenon is most responsible for the observed quenching (167). Many other workers have followed. Yagi and Matsuoka (168, 169,

170) have reported increased absorption between 300 and 500 mμ in the presence of phenol. The phenol concentration was 0.1 to 0.6 M in a 2.66×10^{-5} M riboflavin solution. The variation in concentration showed the reaction to be bimolecular. Hydrogen bonding was postulated as well as complex formation based upon the discrepancy between the dissociation constants obtained by spectrophotometric and fluorometric methods. Yagi et al. (171, 172) has shown phenol and substituted phenols inhibit D - amino acid oxidase and concluded that the effect was due to complexation. Hydroxylamine has been reported to give a shift in the flavin spectrum at 240 mμ but none above 300 mμ (173).

Harbury and Foley (174, 175) have described other systems exhibiting this complexation phenomenon. Their use of 3-methylriboflavin in forming strong complexes lead to the conclusion that a charge transfer mechanism may be the more important feature of complex formation and that hydrogen bonding was not significant. This conclusion is based on the assumption that the riboflavin is donating the hydrogen for hydrogen bonding and would be false if the riboflavin were accepting the hydrogen. The higher electron density due to the induction of the methyl group in 3-methylriboflavin should facilitate hydrogen bonding and make hydrogen bonding more important.

Isenberg et al. (176, 177, 178) also found complexation of riboflavin with other compounds but their evidence is much more convincing. Their complexes are red. Tryptophan, when mixed with riboflavin, gives a red color in concentrated solutions or in more dilute solutions when frozen. Subsequently, other compounds have given similar results when mixed with riboflavin. These complexes exhibit a broad absorption band around 500 mμ which is similar to the region of absorption of the semiquinoid form of riboflavin in acid solutions, pH < 1. Consequently, it was postulated that tryptophan stabilized the semiquinoid species at neutral pH. This is doubtful unless some oxidizable material is present to form the semiquinone. The photooxidation of water or tryptophan might be such a possibility as would riboflavin oxidizing itself photochemically. Since silver forms a similar red color with riboflavin in the dark and this color is independent of light intensity, perhaps some other explanation is more tenable.

Dolin (82) has observed this red color in the enzyme system, reduced diphosphopyridine nucleotide - flavoprotein peroxidase. Here, a reduced diphosphopyridine nucleotide-flavoprotein complex was postulated. The reaction: reduced diphosphopyridine nucleotide + riboflavin = reduced diphosphopyridine nucleotide • riboflavin has been reported by Searls (179) to occur without the enzyme system. Also, this worker

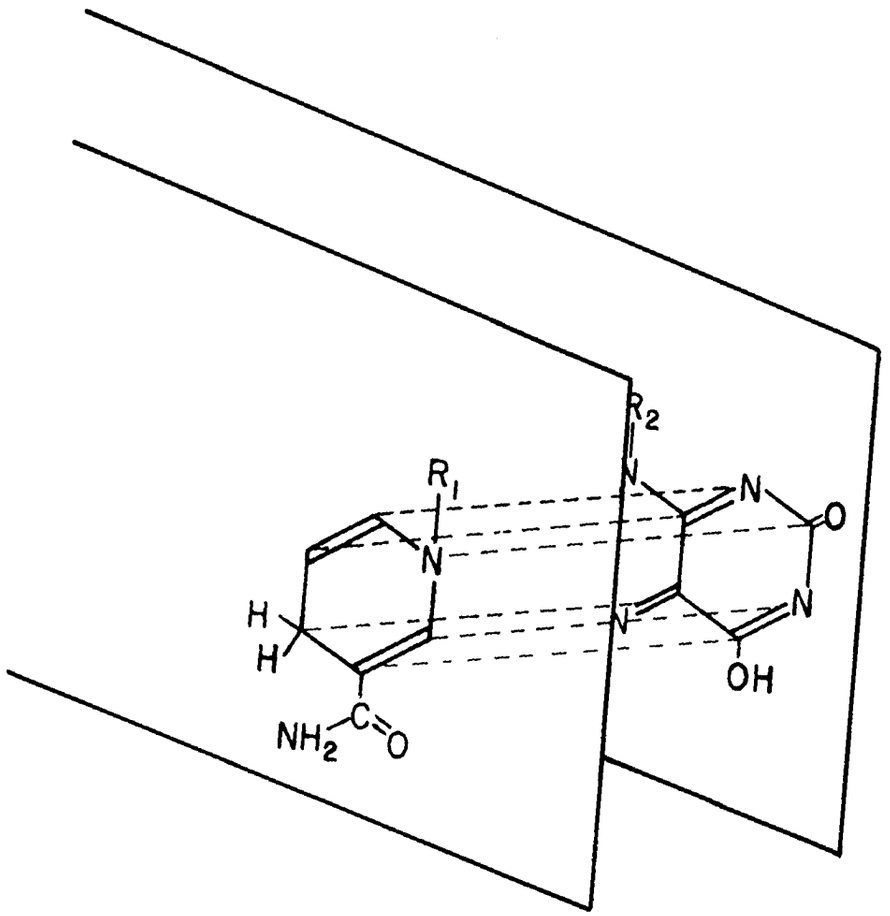
reports a similar red color with the system riboflavin - reduced ∝-lipoic acid (179). The color in the enzyme systems may be due to radicals as reported by Commoner et al. (180, 181, 182, 183, 184) and Ehrenberg (74) using electron spin resonance and Ehrenberg and Ludwig (185) using paramagnetic resonance.

Grabe (186, 187, 188) has postulated a reduced pyridine - riboflavin complex based on theoretical calculations and has used this to predict the direct hydride shift from the 4 position of the dihydropyridine ring of the pyridine nucleotide to the 10 position of riboflavin (Figure 3).

Pullman and Pullman (189, 190, 191) have calculated the highest occupied molecular orbitals and the lowest unoccupied molecular orbitals for many compounds by the linear combination of atomic orbitals method. Complexes predicted by these calculations have been confirmed experimentally in only a few cases. It is only fair to state that no complexes have been found to disagree with their predictions.

Another aspect of these interactions is the role of compounds in solubilizing riboflavin. It was reported (192) that nicotinamide solubilizes riboflavin. However, many compounds solubilize riboflavin (193) particularly water soluble cyclic compounds with one or more -OH, $-NH_2$ or $-CO_2H$ groups (104, 194). These compounds also quench the fluorescence of riboflavin (104). The position of the groups and nature of the

Figure 3. Diagram of Grabe's riboflavin-pyridine nucleotide complex



R_1 = -RIBOSE-PHOSPHATE-PHOSPHATE-RIBOSE-ADENINE

R_2 = $-CH_2-\underset{H}{\overset{OH}{C}}-\underset{H}{\overset{OH}{C}}-\underset{H}{\overset{OH}{C}}-\underset{H}{\overset{OH}{C}}-H$

nucleus also affect the results. The ultraviolet absorption spectrum shows β-hydroxy naphthoic acid (194), 3-hydroxy-2-naphthoic acid (174) and 2-naphthoic acid (174) combine with riboflavin. Any deduction from these studies as to complexation would have to be quite general.

Riboflavin has been reported to form chelates with metal ions. The early work by Albert (40, 195, 196) in itself, suggests the nature of the problem. It was first put forth (195) that riboflavin did not chelate metal ions and one year later riboflavin chelates were reported (196) by the same worker. This work has been quite well reviewed and serious doubts raised as to its validity. A more recent report (197) restates the possibility of an interaction with metal ions with the exception of ferrous ion. The value given in the latter report for the stability constant of the riboflavin - Cu^{++} chelate is not in agreement with the maximum value obtained by Hemmerich and Fallab (198) nor are the published titration curves in agreement with theoretical titration curves.

Silver ion, as suggested above, unmistakably has some interaction with riboflavin. The red riboflavin - Ag^{+} "chelate-complex" has been studied by Kuhn and Bor (199), Weber (42) and recently, more thoroughly, by Baarda (200). It would be erroneous to call this a chelate or a complex. The compound crystallizes out of neutral solution as red

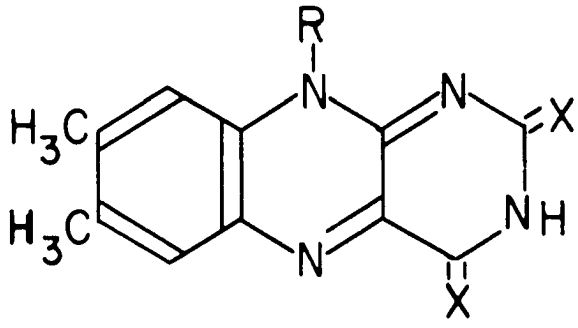
crystals of a 1:1 composition and one proton is liberated per 1:1 unit. However, on standing the solution becomes collodial and eventually the compound precipitates.

The isolated riboflavin - metal ion mixtures of Foye and Lange (201) and the spectral analyses of these by Mahler et al. (202) are thought not to be of significance anymore or, at least, until some new evidence is forthcoming. The work on thionin - Fe^{++} reactive complexes (135) suggests that when no shifts are observed in the absorption spectrum, it should not be concluded that a complex does not exist.

Walaas (203, 204) and Rutter (115) used fluorometric methods on riboflavin chelates and ion exchange on riboflavin phosphate derivatives. The fluorescence measurements showed decreased fluorescence of riboflavin in the presence of Hg^{++}, Fe^{++}, Fe^{+++}, Co^{++} and Au^{+++}. These are all good fluorescence quenchers and the results do not lead to the conclusion of chelation unless the effect increases as temperature is decreased. This was not done; consequently, no new evidence has been revealed on riboflavin chelates. The ion exchange method disagreed with the fluorometric method as to the stability constants and is not considered reliable either.

It would be difficult to write a review and not mention the very informative synthetic work of Hemmerich et al. This group of workers synthesized many flavin derivatives (Figure 4) including the sulfur and nitrogen analogs in-

Figure 4. Structures of flavin analogs prepared by Hemmerich et al. (205, 206, 207, 208, 209, 210)

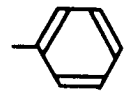


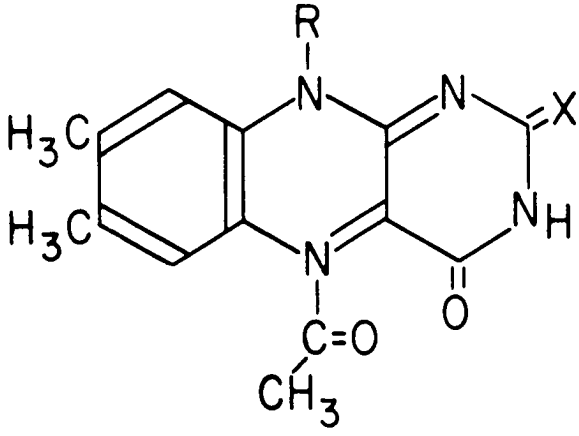
R = $-CH_2(CHOH)_3CH_2OH$

OR $-CH_3$

X = =S, =NH, $<^{H}_{H}$,

$<^{H}_{R'}$ OR $<^{H}_{OR'}$

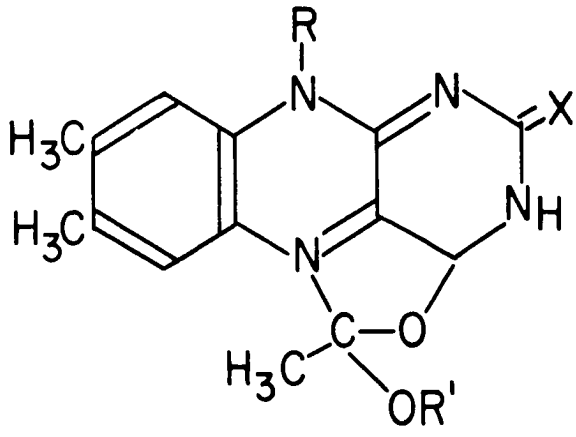
R' = $-CH_3$ OR 



R = $-CH_2(CHOH)_3CH_2OH$

OR $-CH_3$

X = =S OR =NH



R = $-CH_2(CHOH)_3CH_2OH$

OR $-CH_3$

X = =S OR =NH

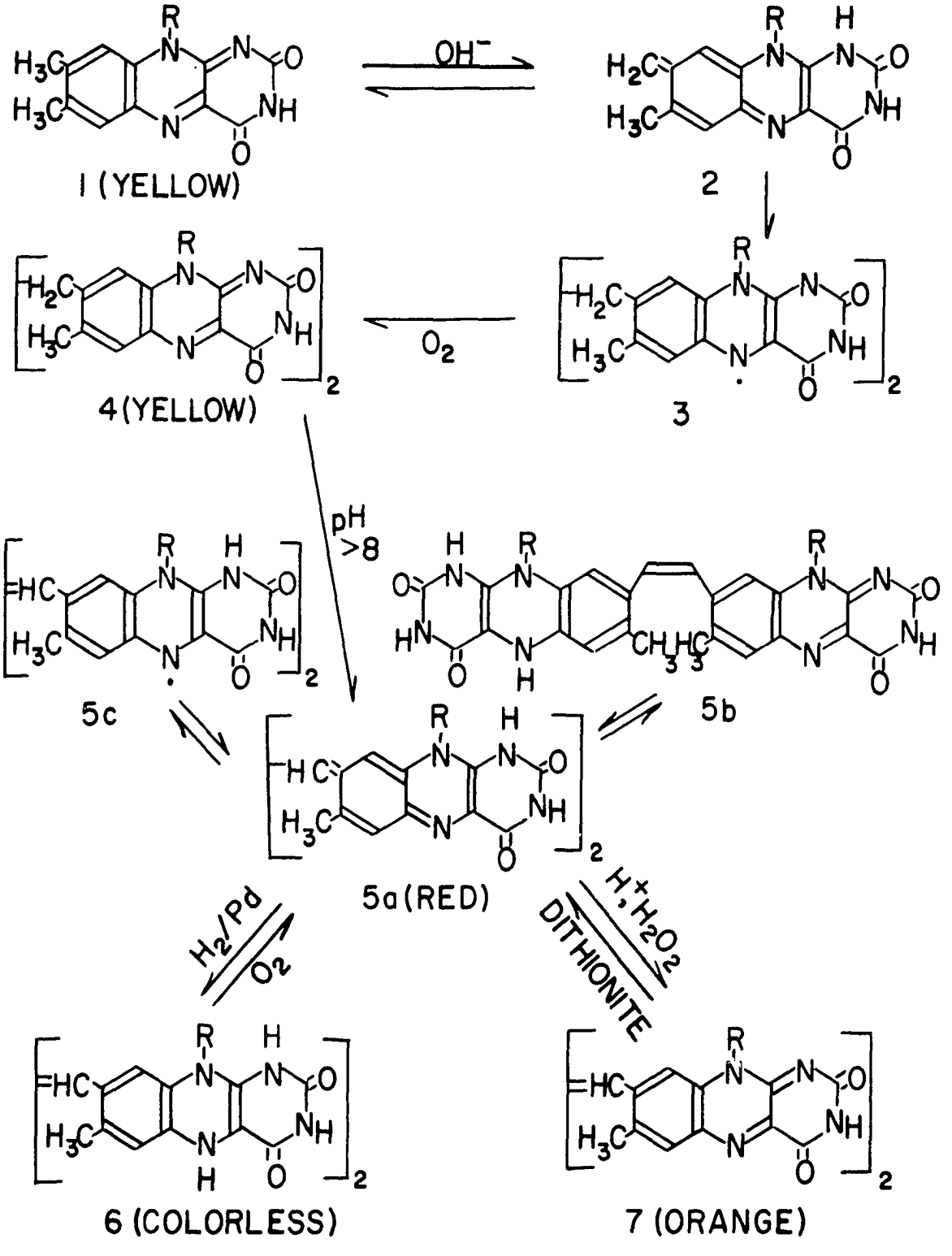
R' = $-H$ OR $-CH_3$

volving the 2 and 4 positions (205, 206), 2- and 4- deoxy compounds (207, 208, 209) which are unstable in aqueous solutions, the akyl, aryl and alkoxy derivatives of the deoxy compounds and 9-acetyl leucoflavin and its derivatives (207, 210) (Figure 4 structure II). The 9-acetyl compound exists in the form shown in structure III. Not only were these compounds synthesized, but their chemical reactivities were determined.

The most exciting developments in the work of Hemmerich et al. have been in the dimerization of riboflavin and lumiflavin in basic solution (211). This dimerization of riboflavin occurs as outlined in the scheme in Figure 5. The compound of greatest interest is the one depicted by structure 5a in the figure. This compound is red and has a spectrum similar to the semiquinone form of the flavins. The author has added structures 5b and 5c to the scheme of Hemmerich et al. to show that structure 5a can be depicted as a pair of flavins, one reduced, one oxidized 5b, a pair of flavin semiquinone radicals 5c or a pair of oxidized flavins as shown in structure 4. It should be pointed out that there are several other dimeric compounds possible as well as other routes to obtain the compounds shown in Figure 5.

The author cannot correlate the observed red color of structure 5a to any of the alternate structures. The structures 5a, b, c, cannot have any intramolecular flavin -

Figure 5. The scheme of Hemmerich *et al.* (211) for the alkaline dimerization of flavins and subsequent reactions



R=-$CH_2(CHOH)_3CH_2OH$ OR-CH_3

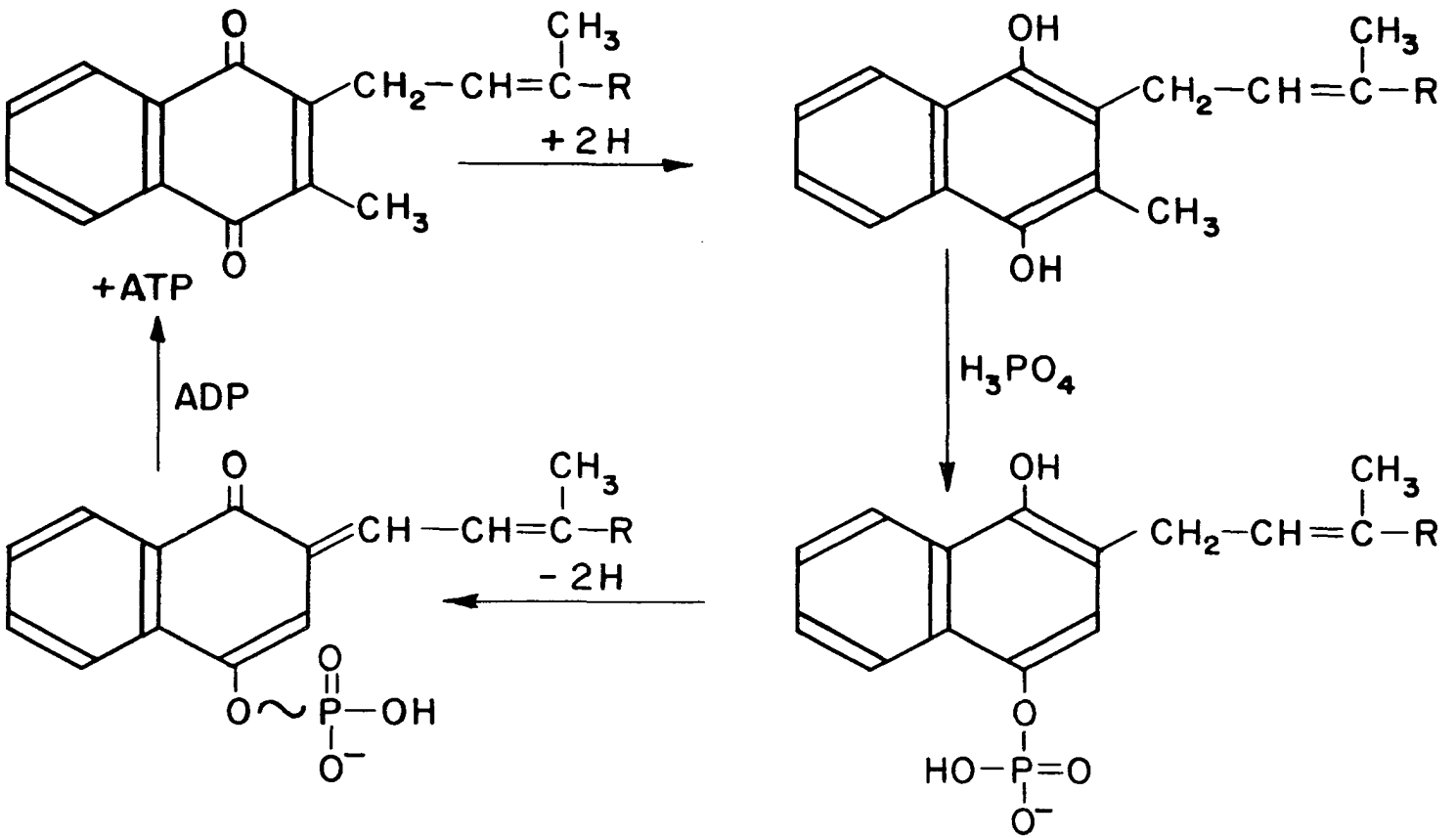
flavin interaction due to the steric hindrance about the ethylene or vinyl linkage and consequently, the red color is probably due to the same phenomenon which occurs in the monoflavin compounds.

Since the color is very similar to the red color observed in many flavin systems, enzyme and chemical, a correct analysis of these structures is considered of great significance.

Because riboflavin fits into the respiratory chain along with the other oxidation-reduction agents and is coupled to oxidative phosphorylation, perhaps a word on the schemes thus far envisioned would be pertinent. All schemes proposed thus far involve p-quinones, of which riboflavin is an analog. Wessels (212) was perhaps the first person to present a mechanism of oxidative phosphorylation. His scheme involved vitamin K, in which the p-hydronaphthoquinone (reduced vitamin K) is esterified with phosphate. When this ester is reoxidized, the phosphorus atom must be transferred as a phosphonium ion, thereby coupling with another phosphate (adenosine diphosphate) to form a pyrophosphate linkage (adenosine triphosphate). This scheme is shown in Figure 6. Similar mechanisms were proposed and demonstrated by Harrison (213), Wieland and Patterman (214), and Clark et al. (215, 216) in Todd's laboratory.

The formation of the phosphate ester of the hydroquinone

Figure 6. Wessel's mechanism of oxidative phosphorylation



$$R = -(CH_2)_3CH(CH_2)_3CH(CH_2)_3CHCH_3$$

is the part of these schemes which is hard to conceive and is, perhaps, the core of oxidative phosphorylation. Grabe et al. (186, 197, 198, 217) attempted to solve this by having the phosphate ester form at the 2 position of riboflavin at the time of a hydride attack at the 10 position (Figures 3 and 7).

Similar schemes may be proposed for coenzyme Q (ubiquinone) and even vitamin E, in which a chroman ring is involved. Coenzyme Q (218) or vitamin K can form a dehydrochroman ring, which when reduced gives the chroman ring. Such a set of compounds would only differ as to reduction potential, i.e., only the side chains differ. Figure 8 shows the possible structures for these compounds.

Recent reviews by Slater (219) and Todd (220) give an extensive and elaborate coverage of the mechanisms proposed for oxidative phosphorylation.

In a study of riboflavin catalyzed redox reactions, the possible substrates one could use may be chosen for a long list. Flavoenzymes are known to catalyze many oxidation-reduction reactions and more systems are being discovered all the time. In order to give an indication of the wide substrate nonspecificity of the flavin moiety, a brief description of the enzyme systems thus far attributed to the flavins will be given. A more comprehensive list is given in a

Figure 7. Grabe's mechanism for oxidative phosphorylation occurring with the flavin-pyridine nucleotide couple

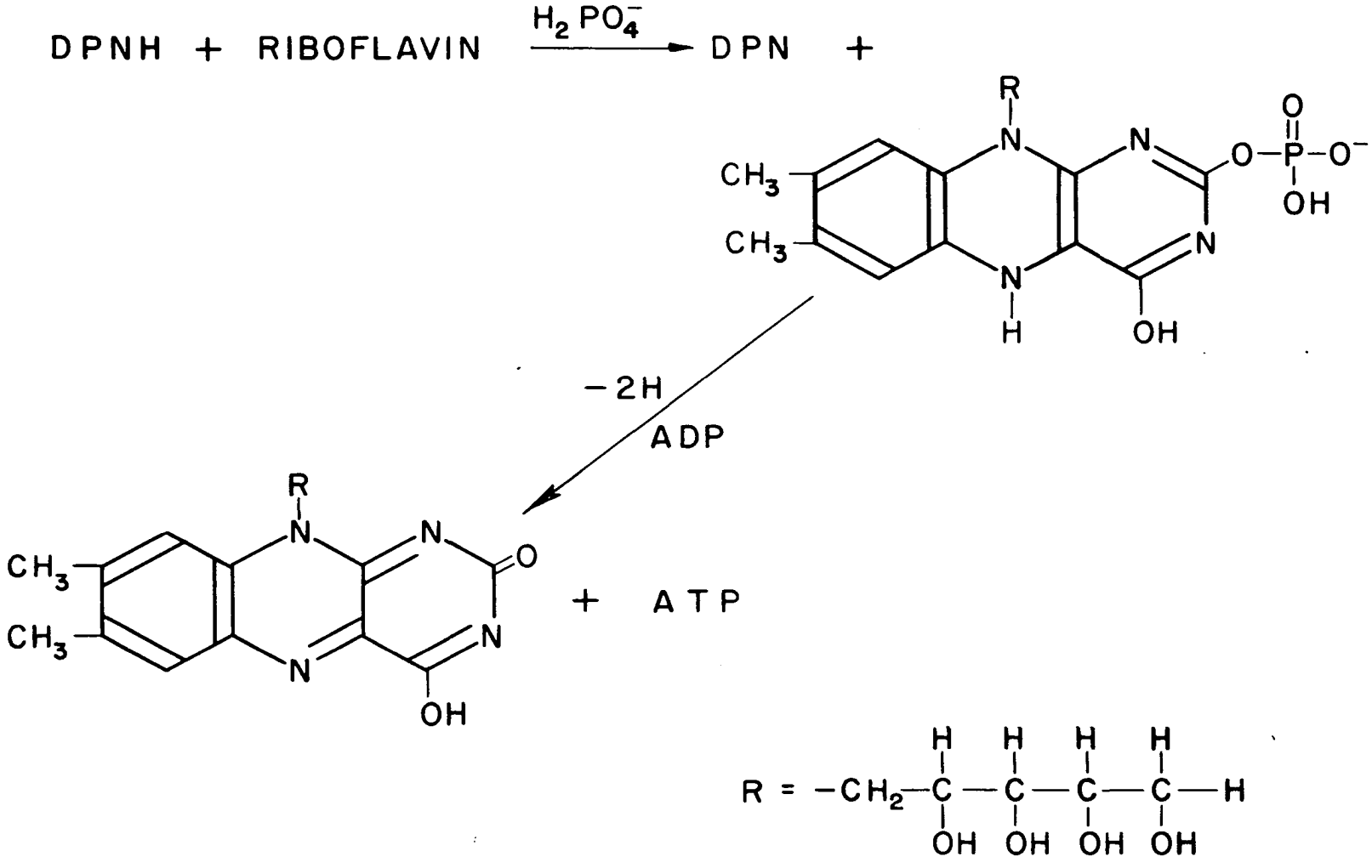
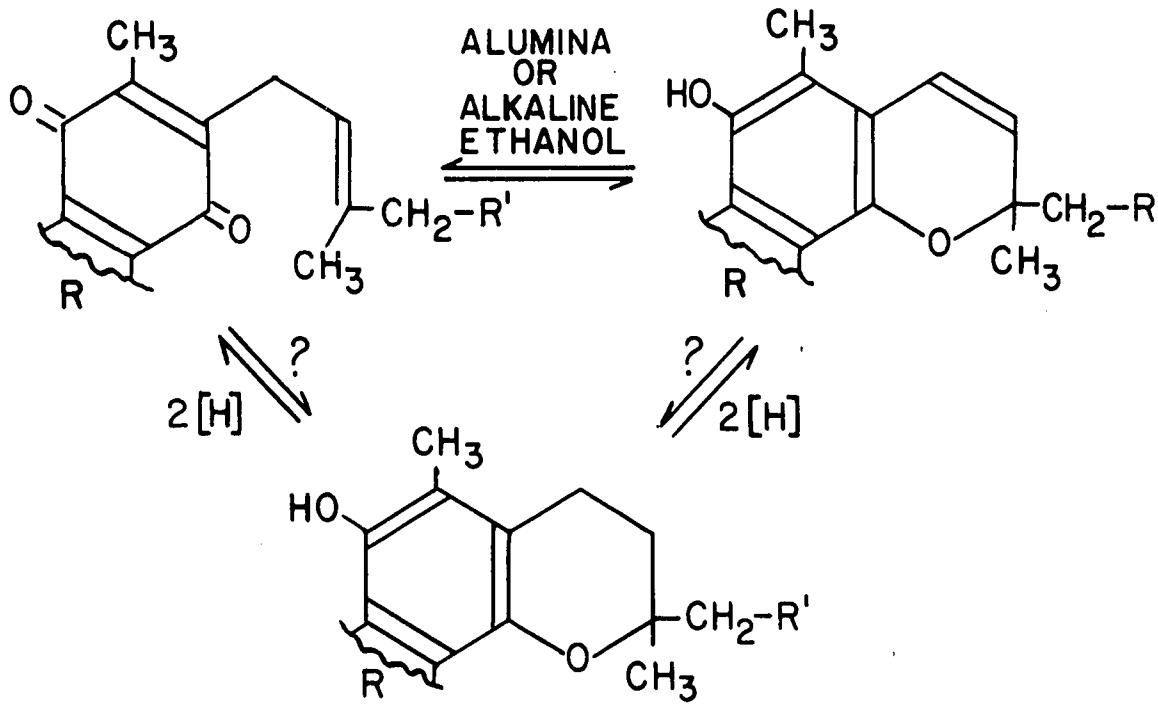

DPNH + RIBOFLAVIN
$H_2PO_4^-$
DPN +
R
N
N
O
P
O⁻
OH
CH_3
CH_3
N
N
H
OH
−2H
ADP
R
N
N
O
CH_3
CH_3
N
N
OH
+ ATP
R = $-CH_2-$
H H H H
C C C C
OH OH OH OH
H

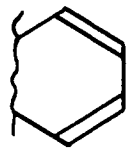
Figure 8. Quinone and chroman structures for vitamin E, vitamin K, and coenzyme Q



$R = \begin{cases} OCH_3 \\ OCH_3 \end{cases}$

$R = -(CH_2-CH=\overset{CH_3}{\overset{|}{C}}-CH_2)_9H$

} COENZYME Q_{10}

$R =$ 

$R' = -(CH_2-CH_2-\overset{CH_3}{\overset{|}{C}}-CH_2)_3H$

} VITAMIN K_1

$R = \begin{cases} CH_3 \\ CH_3 \end{cases}$

$R' = -(CH_2-CH_2-\overset{CH_3}{\overset{|}{C}}-CH_2)_3H$

} VITAMIN E
(α-TOCOPHEROL)

recent review (221). About fifty well-documented flavoproteins are listed.

From the days of the "old yellow enzyme," which oxidized the reduced pyridine nucleotides, things have progressed to a point where many flavoenzymes are known. In rapid succession in the late 1930's and early 1940's, the following enzymes were prepared: D-amino acid oxidase (D-α-amino acids $\rightleftharpoons$ ketoacids), diaphorase (reduced pyridine nucleotides $\rightleftharpoons$ oxidized pyridine nucleotides), cytochrome c reductase (ferricytochrome c $\rightleftharpoons$ ferrocytochrome c), xanthine oxidase (hypoxanthine $\rightleftharpoons$ xanthine $\rightleftharpoons$ uric acid and aldehydes $\rightleftharpoons$ acids), fumaric hydrogenase (dyes, fumarate, maleate, crotonyl alcohol, and geraniol $\rightleftharpoons$ reduced compounds), aldehyde oxidase (only aldehydes $\rightleftharpoons$ acids, α-hydroxy acids $\rightleftharpoons$ α-keto acids), snake venom L-amino acid oxidase (only L-α-amino acids $\rightleftharpoons$ α-keto acids), glycine oxidase (only glycine $\rightleftharpoons$ glyoxalic acid), glucose oxidase (glucose $\rightleftharpoons$ gluconolactone) and histaminase and diamine oxidase (amines $\rightleftharpoons$ aldehydes). More recently the following enzymes have been isolated: glycolic acid oxidase (glycolic acid $\rightleftharpoons$ glyoxalic acid), triphosphopyridine nucleotide - nitrate reductase (transfers hydrogen to various nitrogen compounds in different oxidation states), succinic acid dehydrogenase (succinic acid $\rightleftharpoons$ fumaric acid), fatty acyl coenzyme A dehydrogenases (oxidizes the α-β bond of the fatty acid derivatives to an alkene

linkage), lactic acid oxidative decarboxylase (222) (lactic acid → acetate), oxalic acid oxidase (223), dihydroocotic acid dehydrogenase (224, 225), dihydrolipoyl dehydrogenase (69, 70, 71, 226, 227, 228, 229, 230), Δ^1-, Δ^4-5-α-, Δ^4-5-β-steroid dehydrogenases (231), thyroxine deiodinase (232), and lactose dehydrogenase (233).

From the above impressive but incomplete list, one may conclude that the flavin moiety is non-specific in its oxidation-reduction reactions, whereas, the apoenzyme is, in most cases, quite specific. Since all the above substrates are available to a study of riboflavin oxidation-reduction reactions, the role of riboflavin may well be elucidated through the use of model reaction.

EXPERIMENTAL

Materials

Benzylamine hydrochloride was prepared from the free base and recrystallized from water, m.p. 268-269°C (268°C).

The ∝-phenyl glycine used was recrystallized from material on hand.

The ∝-amino acids used were alanine, glycine, threonine and valine, all of which were commercial preparations.

Methyl hydrocinnamate was prepared by refluxing hydrocinnamic acid (Eastman Organic Chemicals) in an excess of methanol with sulfuric acid as a catalyst. The product was distilled under 3 mm Hg at 90-92°C, b.p. 238°C (239°C).

Methyl cinnamate was used after distillation under 2 mm Hg at 95-96°C, b.p. 262°C (262°C).

Benzylacetone, obtained from K & K Laboratories, was distilled under 13 mm Hg at 86-88°C, b.p. 236-237°C (235°C).

Benzalacetone was distilled from material on hand under 3 mm Hg at 109-111°C, m.p. 41-42°C (42°C), b.p. 260-261°C (260-262°C) with slight darkening.

Methyl hydrogen succinate was prepared from succinic anhydride in a slight excess (1:1) of methanol, refluxing for 45 minutes. The product was recrystallized from water, m.p. 57-58°C (58°C).

Dimethyl succinate was prepared from succinic acid in an excess of methanol with hydrogen chloride as a catalyst. The product was recrystallized from water, m.p. 20° (18.5, 19.5, 20°).

The methyl-hydrogen and dimethyl maleate were prepared in a manner similar to that used to synthesize the corresponding succinates, dimethyl b.p. 202° (205°). The purity of the methyl hydrogen was determined by equivalent weight because the melting point was not reliably reproduced and no value is given in the literature. Eq. wt. 130.5.

Methyl hydrogen fumarate was prepared after Spatz and Stone (234) using thiourea to catalyze the inversion of methyl hydrogen maleate, m.p. 144-145° (142-144°).

Dimethyl fumarate was prepared in a manner similar to that used to synthesize the corresponding succinate, m.p. 101° (102°).

Diethyl ethylmalonate was prepared from the diethyl ester of malonic acid using equal molar amounts of sodium and ethyl iodide in an excess of ethanol. After evaporating off the ethanol, the product was distilled under 15 mm Hg at 94-96°C, b.p. 208° (211°).

Ethyl malonic acid was prepared by saponification of the ester, m.p. 111°C (111.5°C).

Diethyl ethylidene-malonate was prepared as was the diethyl ethylmalonate using acetaldehyde in place of the

ethyl iodide, b.p. 114-116° (115-120°).

The lipoic acid used was a commercial preparation of California Corporation for Biochemical Research.

Reduced ∝-lipoic acid was prepared from ∝-lipoic acid by the method of Gunsalus et al. (235) using sodium borohydride, b.p. 170-172° (169-172°C).

Nicotinamide-1-propochloride was prepared by the method of Holman and Wiegand (236) as described for the methochloride, m.p. 192-194°.

Orotic acid was a preparation from California Corporation for Biochemical Research. Purity was confirmed by titration to a bromcresol purple end point and by its absorption spectrum.

Methyl orotate was prepared from orotic acid using an excess of methanol with hydrogen chloride as a catalyst. The purity was confirmed by this preparation requiring no base to reach a bromcresol purple end point and having the same λ_{max} and a_M as the protonated form of orotic acid. An attempt to produce dihydroorotic acid and its ester by low pressure did not prove fruitful. Only about half of a mole of hydrogen was taken up per mole of acid or ester. l -Dihydroorotic acid was obtained from California Corporation for Biochemical Research and used as such.

β -Napthoic acid was prepared by hydrolysis of the available nitrile and by carbon dioxide addition to the β-

lithium-naphthalene as prepared by the exchange of butyl lithium and β-bromonaphthalene after Gilman and Moore (237) Also, commercially available β-naphthoic acid was obtained from Eastman Organic Chemicals. All the products after recrystallization from water, were identical as to melting point (185-185.5°) and spectrum.

The dihydronaphthoic acids were prepared after Derick and Kamm (238) using a sodium amalgam reduction of naphthoic acid in basic aqueous solution. The Δ^{2-} and Δ^{3-} acids were separated by fractional precipitation and were subsequently recrystallized from water. The Δ^{1-} acid was prepared by heating the Δ^{3} acid with barium hydroxide solution in sealed tubes at 140° for 10 hours. pK_a of Δ^{1-}, Δ^{2-}, Δ^{3-}dihydro-β-naphthoic acids, and β-naphthoic acid are 4.2, 4.7, 4.5, and 4.1 respectively as determined spectrophotometrically. The melting points of the dihydro acids are: Δ^{1}, 117° (118); Δ^{2}, 160° (161°); and Δ^{3}, 101° (101°). The spectra of the compounds agreed both in λ_{max} and a_M with those given by Schrecker, et al. (239).

The ethyl esters of the dihydronaphthoic acids were prepared using an excess of ethanol with hydrogen chloride as a catalyst. An attempt to prepare the ester of the Δ^{3-} acid by the thionyl chloride method gave dark unidentifiable products. The boiling points of all the esters were in the range 265-270° with much darkening. Values of 159-160°,

163°, and 152-3° (under 12 mm Hg) were reported for the Δ^{1-}, Δ^{2-}, and Δ^{3-}, esters, respectively (240). The spectra of these compounds were nearly identical to the spectra of the respective acids in alcohol (Figure 9).

The sodium hydrosulfite (dithionite) used was a technical grade commercial preparation of General Chemical Division of Allied Chemical and Dye Corporation.

The riboflavin (U.S.P.) used was a commercial preparation of Merck and Company. However, most of the work presented in this thesis was conducted using riboflavin (R.S., U.S.P.) from Commercial Solvents. This preparation was dried and stored in the dark. When riboflavin, U.S.P. was used, it will be noted.

Riboflavin-5'-phosphate (FMN) was obtained gratis from Sigma Chemical Company.

Lumiflavin (6,7,9-trimethylisoalloxazine), 2-lumiflavin (2-deoxy-2-iminolumiflavin), and 3-methyllumiflavin were prepared by the method of Hemmerich, et al. (205). The flavin character was confirmed by the characteristic flavin spectrum with λ_{max} 265, 365, and 445 mμ. The 2-lumiflavin was readily converted to a lumazine compound in base as determined spectrophotometrically. The 9-methyl group is apparently lost (205, 241).

1'-Formyllumiflavin was prepared from riboflavin using periodic acid after Fall and Petering (242).

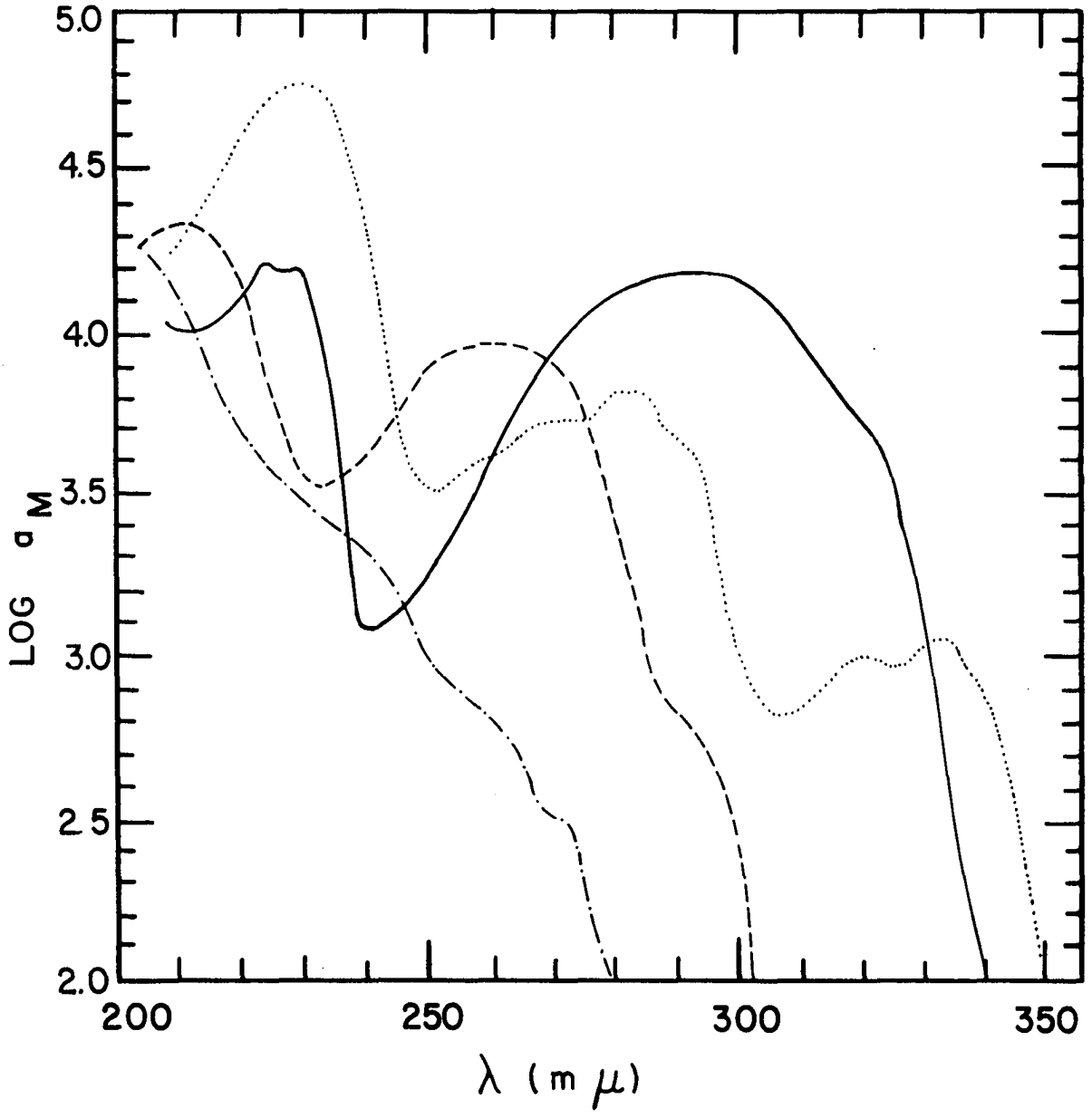
Figure 9. Spectra of the ethyl esters of β-naphthoic acid and the dihydro-β-naphthoic acids

. . . . . β-Naphthoate

——————— Δ^1-Dihydro-β-naphthoate

—·——·— Δ^2-Dihydro-β-naphthoate

– – – – – – Δ^3 Dihydro-β-naphthoate


5.0
4.5
4.0
3.5
3.0
2.5
2.0
LOG a_M
200
250
300
350
λ (mμ)

1'-Hydroxymethyllumiflavin was prepared from the above compound using sodium borohydride (242). As reported (242), these lumiflavin derivatives as well as most flavins, have virtually identical spectra.

Isoriboflavin (5,6-dimethyl-9-(1'-D-ribityl)-isoalloxazine) and flavin adenine dinucleotide (FAD) were obtained from California Corporation for Biochemical Research and were used as such.

Methods

Heat stability

The heat stability of riboflavin and leucoflavin in the presence or absence of other compounds was carried out under conditions similar to those used in the oxidation-reduction reactions discussed below. The samples (10^{-4} M in each reagent) were heated in an oven (dark) at various temperatures for varying lengths of time. The degradation was followed at 445 mμ where only riboflavin absorbs radiation. In the case of leucoflavin, the samples were reoxidized before observing the absorbance.

Light stability

The photoreduction of riboflavin under anaerobic conditions was studied by exposing highly evacuated modified thunberg tubes, containing a riboflavin solution (10^{-4} M),

to a germicidal or fluorescent lamp. Making use of the cuvette portion of the modified thunberg tubes, the decay of absorbance as well as the increase on subsequent reoxidation was observed at 445 mμ.

Lumiflavin was also irradiated in a like manner. The effect of added compounds on lumiflavin photoreduction was used to deduce the mode of riboflavin photoreduction.

Solubility

The riboflavin solubility in various solvents was determined by spectrophotometry. Solutions of riboflavin, in equilibrium with solid riboflavin in a thermostatted bath, were pipetted into cuvettes and the absorbances were observed. The concentrations were calculated from standard solutions using Beer's law.

Metal ion interactions

In an attempt to find a method of determining the stability of riboflavin-metal ion interactions, metal ion titrations and polarography were used. In the metal ion titrations, aliquots of a 1.0 M solution of metal ion were added to a 10^{-3} M riboflavin solution. The change in pH was observed and a maximum stability constant was calculated.

The polarograph used was a Sargeant Model XXI. Various concentrations of riboflavin (10^{-4} to 10^{-3} M) and metal ions (10^{-5} to 10^{-1} M) were used in 1.0 M acetate buffer (ph 5.0)

as the carrier electrolyte. The determination was based on the riboflavin half-wave rather than the customary metal ion half-wave. This reversal was necessary because most of the metal ions used have half-wave potentials more negative than does riboflavin. The determinations and calculations were made as outlined by Souchay and Fauchere (243).

Molecular complexes

Polarography was used to determine the molecular complex stabilities in a manner similar to the metal ion determinations.

The intermolecular interaction of riboflavin-5'-phosphate with itself was observed using the absorbance between 420 and 530 mμ. Riboflavin-5'-phosphate solutions from 2 x 10^{-5} to 5 x 10^{-2} M were used. The absorbances of the more concentrated solutions were determined by use of spacers (reduced pathlength). The more dilute solutions were contained in 10 cm cylindrical cells.

Attempted amino acid oxidations

The attempted oxidations of amines and amino acids were carried out using equal volumes of a solution (10^{-1}M) of the corresponding compcund and a solution of riboflavin-5'-phosphate (10^{-2} M) plus buffer (various ionic strengths). Such mixtures were heated to temperatures up to 95°, at various pH, with and without metal ions present, and for

varying lengths of time up to three hours. In attempting to show a reaction over a longer time, up to 24 hours were used in some cases.

The reaction mixtures were assayed for ammonia by a modified Nessler reagent after Johnson (244). The reaction mixtures were made strongly basic and air was drawn through the mixtures into fresh acid solutions. The acid solutions were then assayed for ammonia.

Fatty acid analog oxidations

The absorbance of unsaturated carbonyl compounds between 260 and 300 mμ was used as the basis for following the α-β oxidation of compounds by riboflavin. The experiments were started by mixing a solution of reduced compound (10^{-4} M) with a solution of riboflavin (10^{-4} M) in equal volumes plus buffer. These reaction mixtures were then heated to various temperatures up to 120°, at various pH, and for varying lengths of time.

The reverse reactions, i.e., the reduction of the oxidized compounds by reduced riboflavin, were attempted in thunberg tubes with attached cuvettes. A solution of dithionite was tipped in from the side arm into a solution of riboflavin and oxidized compound. The absorbance at 260 to 300 mμ was observed as well as the absorbance at 445 mμ.

Dihydro-β-naphthoate oxidations

The reduced β-naphthoates were oxidized and studied in

various ways. The studies of the effects of pH, ionic strength, solvent, and inorganic ions on the light catalyzed reaction were carried out in a pyrex test tube of the dimensions: inside diameter: 1.81 cm, height: 15 cm, and wall thickness: 0.119 cm. This tube was located 12 inches from a 16 inch - 15 watt Sylvania fluorescent tube with a white reflector. To standardize the procedure, the reaction was also conducted in a 1 cm silica cell which was placed in a similar location.

The temperature study of the light catalyzed reaction was conducted in the pyrex test tube immersed in a constant temperature bath at various temperatures. The fluorescent lamp was placed outside the glass bath container.

In the above cases, aliquots were removed from the reaction tube at intervals and the absorbance measured at the appropriate wavelength (Figure 9). In all cases, a riboflavin blank was used as a reference due to the high absorbance of riboflavin in the region employed (Figure 1). The Δ^{3-} dihydro-β-naphthoate oxidation was followed at 260 and 330 mμ with corrections being made for the β-naphthoate formed. The Δ^{2-} and Δ'-dihydro-β-naphthoates were followed at 274 and 300 mμ, respectively, as well as at 330 mμ.

A sample experiment used 8 ml riboflavin solution (1.5×10^{-4} M), 16 ml water, 0.8 ml of 1.0 ionic strength buffer and 0.8 ml of ethyl Δ^{3-} dihydro-β-naphthoate (4×10^{-3} M).

In the various experiments, the total volume remained constant with the amount of water being altered to compensate for the increase of the other components. All the components, except the dihydro-naphthoate, were placed in an aluminum foil covered test tube. The dihydro-naphthoate was placed in a cup suspended from the test tube stopper. After a minimum of five minutes, the test tube was inverted and the components mixed. The test tube was removed from its aluminum foil cover, placed in the light beam and the reaction rate recorded.

The dark experiment was carried out at elevated temperatures in aluminum foil covered sealed screw top vials. The vials were wrapped with plastic tape to stabilize the foil covering. Individual vials were taken out of the covered metal bath and plunged into ice water. The reaction was assayed in a manner similar to that of the light catalyzed reaction.

In all cases, total spectra were obtained at various stages using the Cary Model 14 recording spectrophotometer.

Oxidation-reduction systems of other compounds

The orotic acid oxidation-reduction systems were conducted in a manner similar to that described for the fatty acid analog oxidations. ∝-Lipoic acid systems were also similar with the absorbance at 330 and 445 mμ being observed. The glucose oxidation was followed by acid pro-

duction or by the hydroxamic acid (245) formed from the product, gluconolactone.

The 1-propyl-1,4-dihydronicotinamide reactions studied by Suelter and Metzler (41, 77) were carried out under anaerobic conditions in order to check the anaerobic character of systems developed as well as to evaluate the second order rate constant for this reaction. These experiments were conducted in modified thunberg vessels with a spectrophotometer cuvette attached. The reaction was followed at 360 and 445 mμ using a Varian linear recorder (Model G-10) attached to a Beckman DU spectrophotometer. The cell compartment was thermostatted at 25°. Initial readings were obtained within 10 seconds after mixing.

Because approximately equimolar concentrations of 1-propyl-1,4-dihydronicotinamide (NPrNH) and riboflavin were used (10^{-4} M), the second order rate constant was equal to the reciprocal of the slope of a $2a\sqrt{K}\,t$ versus ln $\frac{1-\frac{x}{a}(1-\sqrt{K})}{1-\frac{x}{a}(1+\sqrt{K})}$ plot, where K is equal to the equilibrium constant of the reaction: leucoflavin + $NPrN^+$ $\rightleftharpoons$ riboflavin + NPrNH + H^+, t is time, a is the initial concentrations and x is the concentration which has reacted at time t. Such a plot is made for a reversible second order reaction. From the equilibrium constant, an approximate reduction potential for the nicotinamide-1-propochloride was calculated.

Leucoflavin

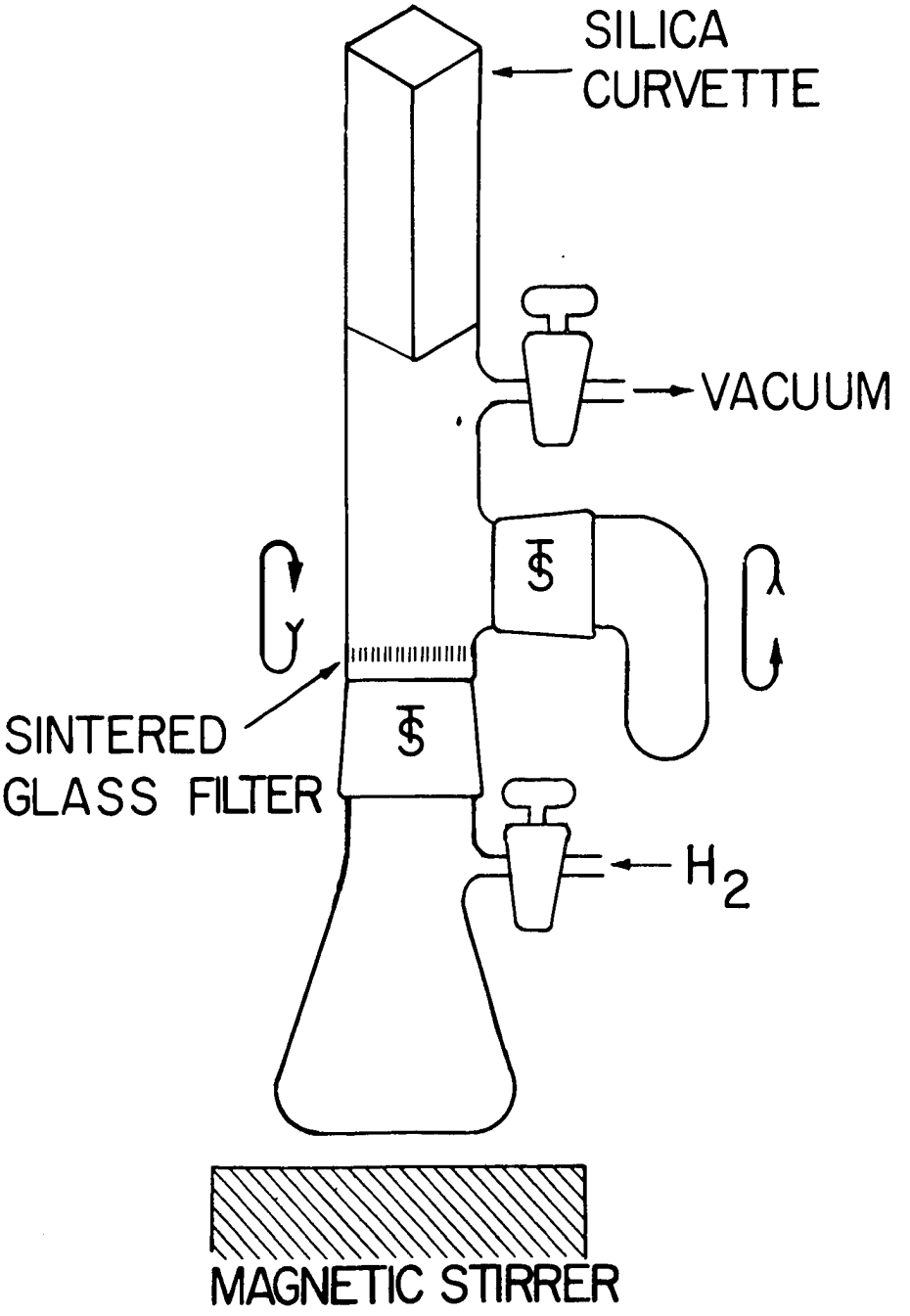
Leucoflavin was prepared and studied in the catalytic hydrogenation apparatus represented by Figure 10. A solution of riboflavin was placed in the flask of the apparatus along with a trace of palladium black and a stirring bar. The system was evacuated and filled with hydrogen several times. The solution was stirred. When completely reduced, the solution of leucoflavin was filtered into the cuvette. If anything was to be added, it was tipped in from the rotating side arm. Any reaction was then followed in a modified spectrophotometer (Beckman DU or Cary Model 14), with a cell compartment cover to accomodate the reduction apparatus.

In the case of substrate reductions, the compound to be reduced was placed in the side arm. The absorbance of the mixture was followed at the wavelength of maximum absorbance of the oxidized compound or reduced compound or at 445 mμ, a peak of riboflavin absorption.

The semiquinone form was prepared by placing equal volumes of a riboflavin solution in both the side arm and the flask. Water was added to the flask to bring the volume in the flask to about 4 ml. Concentrated sulfuric acid was also added to the side arm. The reactions were conducted as for the above solutions.

The semiquinone was also observed by photoreducing a riboflavin solution to the point where only about half of the

Figure 10. Catalytic hydrogenation apparatus for preparation and characterization of leucoflavin


SILICA
CURVETTE
VACUUM
SINTERED
GLASS FILTER
H_2
MAGNETIC STIRRER

flavin remained in the oxidized form. On acidifying, the red semiquinone spectrum appeared and was recorded.

The reoxidation of leucoflavin by oxygen was accomplished by allowing micro drops of water to enter the apparatus. The introduction of water drops was made by use of an addition funnel attached through the side arm of the apparatus depicted in Figure 10. Total spectra were recorded.

RESULTS AND DISCUSSION

Physical Properties of Riboflavin

Heat stability

Knowledge of the effect of heat upon the stability of riboflavin, in the presence or absence of other compounds, was necessary since heat was used as a means of activating possible reactions. As depicted in Tables 1 and 2, the reduced forms of the compounds used stabilized riboflavin and

Table 1. Effects of compounds on riboflavin heat stability at pH 6.15 (phosphate, 0.1 ionic strength) and at 95°C for 12 hours

Compound (7×10^{-5} M)	% Decomposition of riboflavin (7×10^{-5} M)
Methyl hydrocinnamate	7
Methyl cinnamate	37
Benzylacetone	57
Benzalacetone	49
Dihydroorotic acid	0
Methyl orotate	34
Methyl hydrogen succinate	6
Methyl hydrogen fumarate	0
Diethyl malonate	0.2
No addition	37

Table 2. Effects of orotates on leucoflavin heat stability at pH 6.15 (phosphate, 0.1 ionic strength) and at 95° for 12 hours

Compound (7×10^{-5} M)	% Decomposition of leucoflavin (7×10^{-5} M), determined as riboflavin
Dihydroorotic acid	25
Methyl orotate	0
No addition	28

the oxidized forms of the compounds used stabilized leucoflavin. This effect may be ascribed to molecular complex formation between riboflavin and the added compounds. Heat is known to destroy riboflavin much faster in dilute solutions than in more concentrated solutions (11). A possible explanation is that the riboflavin may stabilize itself in higher concentrations due to self-polymerization similar to the intercompound complexes which have been described in the Review Section (p. 24).

Riboflavin solutions were stabilized by increasing the ionic strength as shown in Table 3. Figure 11 describes the time dependence of the decay of riboflavin on heating in the dark. The rate of degradation is first order over many hours.

The spectrum of the heat degradation product obtained after 3 days at 95 to 100°, is presented in Figure 12

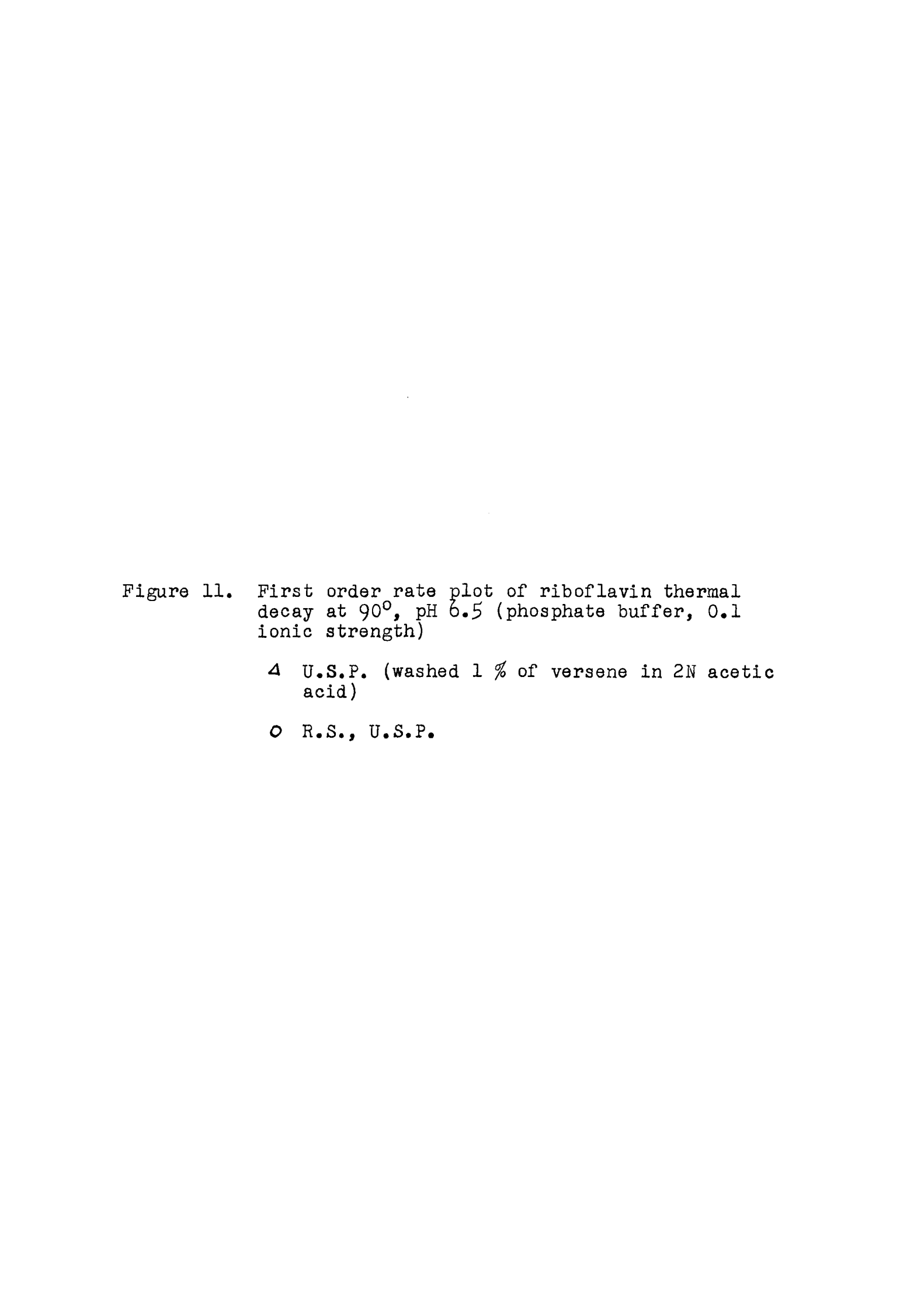


Figure 11. First order rate plot of riboflavin thermal decay at 90°, pH 6.5 (phosphate buffer, 0.1 ionic strength)

△ U.S.P. (washed 1 % of versene in 2N acetic acid)

○ R.S., U.S.P.

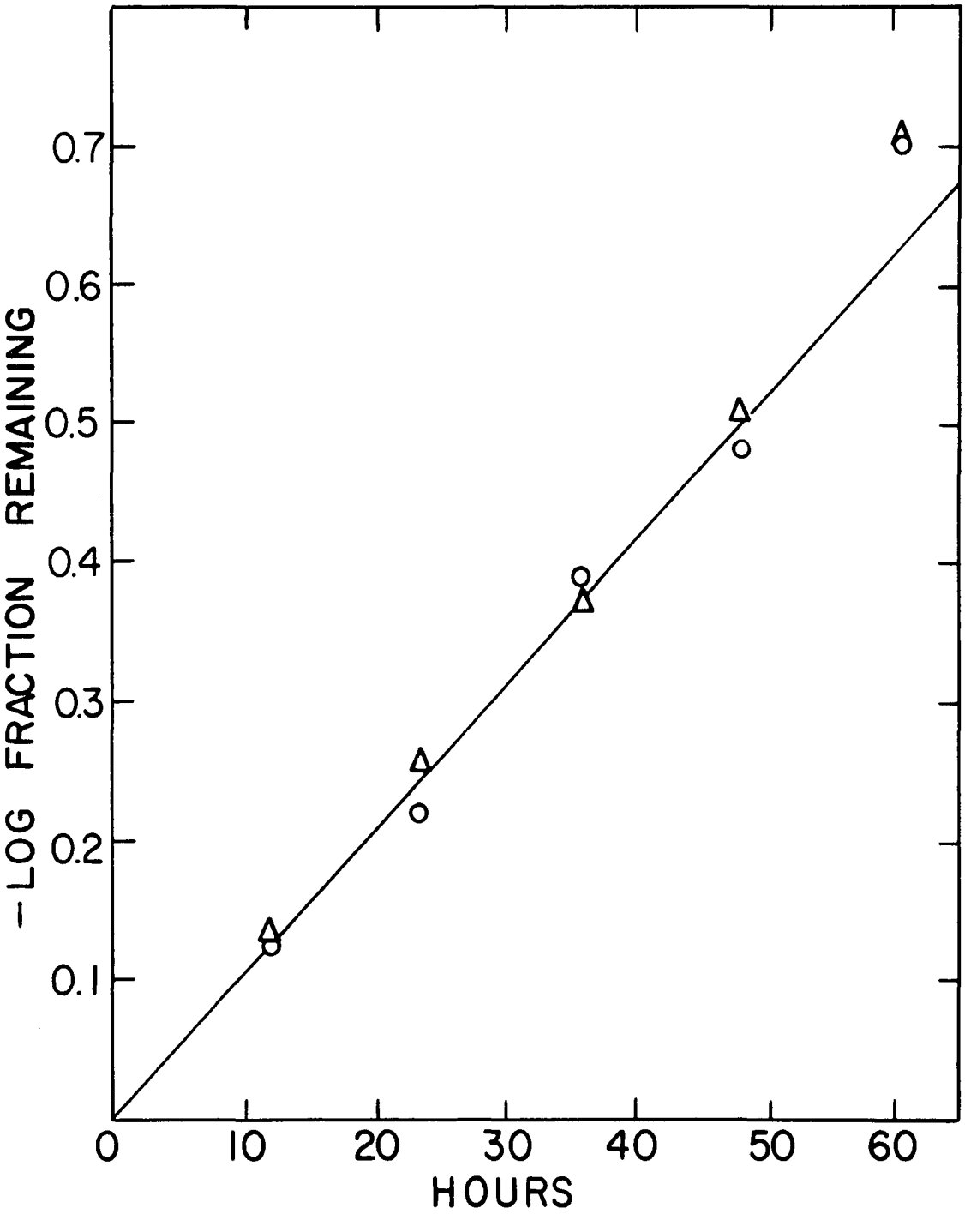

0.7
0.6
0.5
0.4
0.3
0.2
0.1
-LOG FRACTION REMAINING
0
10
20
30
40
50
60
HOURS

Figure 12. Absorption spectra of the riboflavin heat degradation product and the riboflavin alkaline degradation product (insert (246, 247))

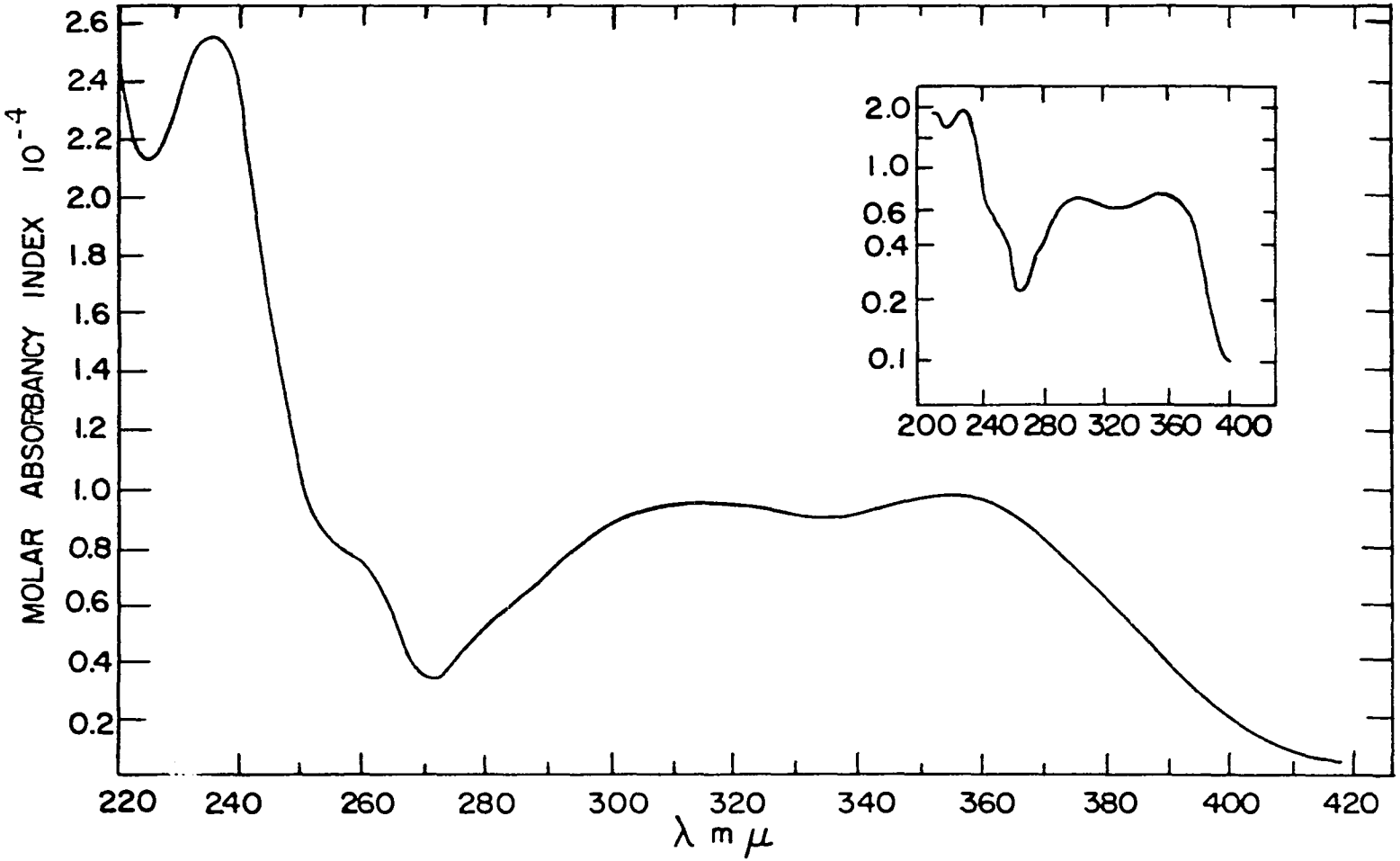
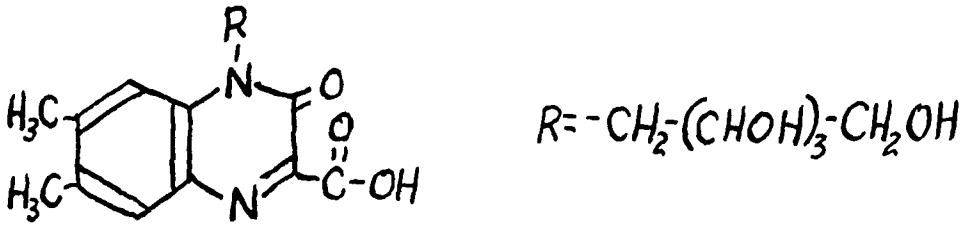

MOLAR ABSORBANCY INDEX 10^{-4}
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
220 240 260 280 300 320 340 360 380 400 420
λ mμ
2.0
1.0
0.6
0.4
0.2
0.1
200 240 280 320 360 400

Table 3. Effect of ionic strength on riboflavin heat stability at pH 6.15 (phosphate) and at 95° for 36 hours

Riboflavin solution (10^{-4} M)	% Decomposition
R.S., U.S.P. (no buffer)	99
R.S., U.S.P. (0.1 ionic strength)	61
R.S., U.S.P. (0.2 ionic strength)	40
U.S.P. (no buffer)	95
U.S.P. (0.1 ionic strength)	59
U.S.P. (0.2 ionic strength)	40

($^{a}M235 = 2.55 \times 10^{4}$, $^{a}M310 = 9.3 \times 10^{3}$, $^{a}M355 = 9.45 \times 10^{3}$). This product is undoubtedly 1,2-dihydro-6,7-dimethyl-2-keto-1-(1'-D-ribityl)-3-quinoxaline-carboxylic acid. This is the



same compound as the one obtained from the alkaline degradation (246, 247). The spectrum of the latter compound is given in the insert in Figure 12.

Light stability

Under strong irradiation, riboflavin is converted, aerobically, into many degradative products with modified side chains on the 9 position. Under anaerobic conditions, the irradiated riboflavin solutions (10^{-4} M) are bleached as observed at 445 mμ. In Table 4, the decay of 445 mμ absorbance is given for an irradiated riboflavin solution. The solution was irradiated by a 15 watt fluorescent bulb at 9 inches. Figure 13 is a plot of the absorbances recorded as the reduced compound is reoxidized by allowing air to diffuse into the solution. Use of a germicidal lamp (General Electric 30 watt) gave similar results, accompanied by more decomposition as determined by failure to attain as high a per cent recovery of absorbance at 445 mμ as with the fluorescent bulb.

Lumiflavin (10^{-4} M) was not reduced under these conditions. However, upon the addition of ethanol (1.0 M) or glycerol (1.0 M), the photoreduction proceeded at a rate comparable to that of the riboflavin photoreduction (Table 5). With the alcohol concentrations at 1.0 M, the rate observed may be dependent upon the light intensity and, subsequently, the concentration of photoexcited flavin molecules. The reoxidation (allowing air to diffuse in) of the photoreduced lumiflavin-alcohol solution was also similar to the reoxidation of the photoreduced riboflavin solution (Figure 13).

Table 4. Anaerobic photoreduction of a riboflavin solution. Initial pH 6.3 (no buffer). Final pH 7.1

Time	Absorbance 445 mμ
0 Min.	1.279
2	1.246
22	1.175
44	1.106
74	1.048
12 Hours	0.567
15	0.452
17	0.395
21	0.285

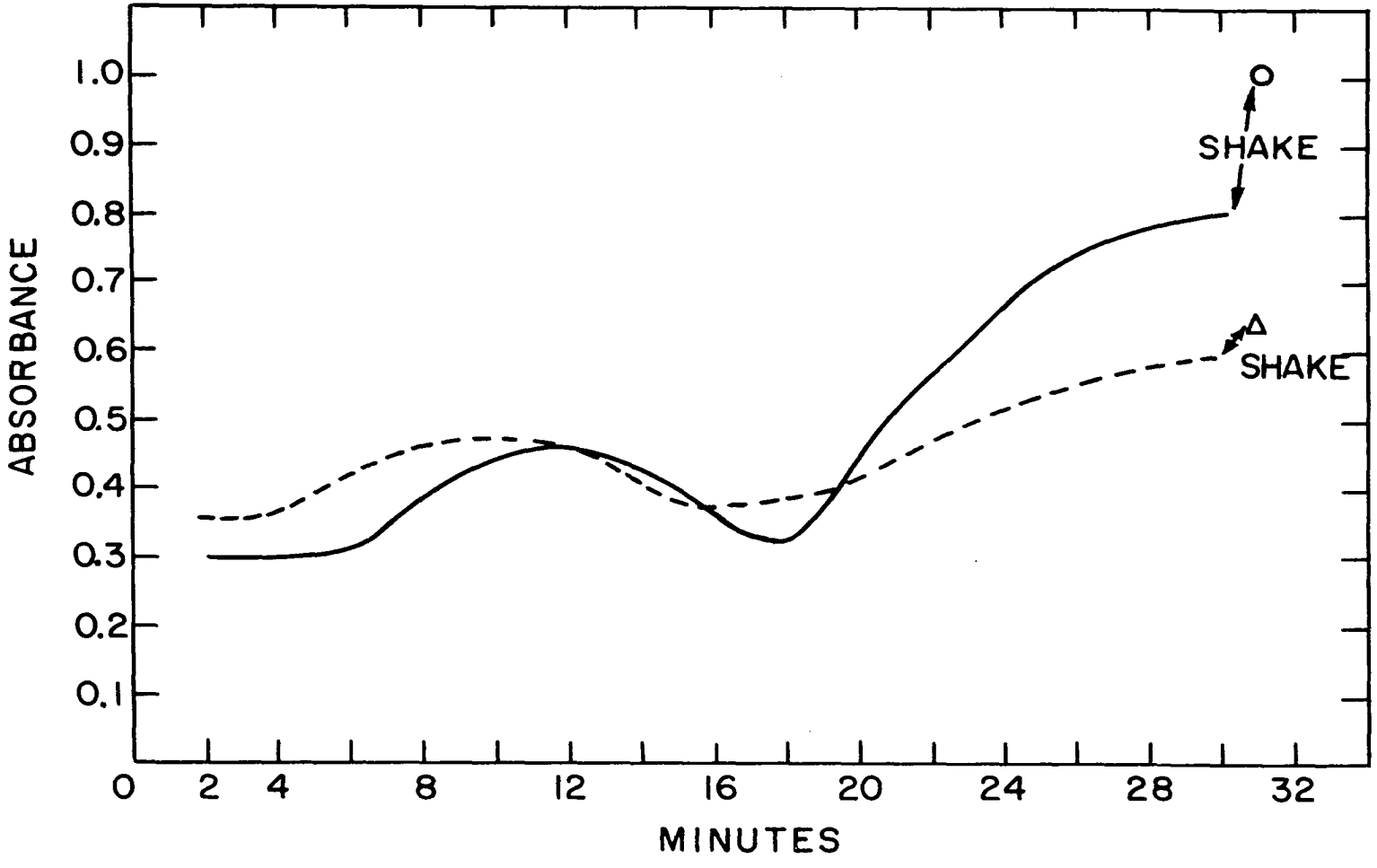
Table 5. Anaerobic photoreduction of lumiflavin solutions in the presence of 1.0 M ethanol or 1.0 M glycerol pH 6.15 (cacodylate, 0.03 ionic strength)

Time	Plus ethanol	Plus glycerol
0 Min.	.750	.745
30	.706	.695
60	.671	.642
2 Hours	.623	.591
4	.559	.530
8	.436	.419
12	.355	.319

Figure 13. Absorbance versus time plot which represents the oxidation of photoreduced flavins by allowing air to diffuse in (refer to Tables 4 and 5)

_____ ,O Riboflavin

- - - -,Δ Lumiflavin ethanol


ABSORBANCE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
8
12
16
20
24
28
32
MINUTES
O
SHAKE
Δ
SHAKE

The conclusions from these experiments are a reiteration of the conclusions for the analogous methylene blue experiment in water and aqueous ethanol solutions (138, 145) (p. 21). A water solution of lumiflavin does not bleach because there is nothing in the solutions to oxidize as there is in an aqueous ethanol solution. In the case of riboflavin, photoreduction occurs in water because the oxidizable substance is present in the flavin itself - the ribityl side chain. The conclusion is that flavins do not photolyze water, but are oxidizing other materials present whether inter- or intra-molecular. The findings of Kuhn et al. (14, 15) substantiate this reasoning. These workers found that when riboflavin is irradiated anaerobically in neutral solution, "deuteroleucoflavin" is formed which can be air oxidized to "deuteroflavin." "Deuteroflavin", when treated with base, gives lumiflavin. Therefore, the bleached material is not leucoflavin but some reduced flavin analog which can be converted to lumiflavin on oxidation and treatment with base.

On reoxidation of the photoreduced material (Figure 13), the variable increase and decrease at 445 mμ may be a reflection of the leucoflavin-oxygen complex of Gutfreund (72) or the semiquinone. This aspect will be discussed in more detail in the section on leucoflavin.

Solubility

No non-aqueous solvents have been reported to dissolve riboflavin to any extent greater than 10^{-5} M. The addition of water to such solvents increases the riboflavin solubility. A solution of riboflavin in 95% ethanol is 10^{-4} M. With the possibility of conducting non-aqueous experiments, the necessity for a solvent was recognized. Dimethyl formamide was found to be a suitable solvent for riboflavin. A saturated solution of riboflavin in dimethyl formamide is 2.8×10^{-4} M at 25°. This is comparable to that of an aqueous solution of ordinary riboflavin, U.S.P. The riboflavin is quite stable in this solvent for several weeks.

Metal Ion Interactions

Heavy metal ions have been reported to have an interaction with riboflavin based on titration studies. The titration work of Albert (40) was thought to be erroneous due to the fact that the constants reported were similar in value to those obtained by neglecting the most important blank -ion hydrolysis. More recently, another laboratory (197) has reported similar interactions of the heavy metal ions, with the exception of ferrous ion. The published curves are too steep for the reactions described and may also represent some ion hydrolysis.

The only metal ions unambiguously known to react with riboflavin are silver ion and cuprous ion. Both of these ions produce a red colored solution when mixed with riboflavin. A solution of the red silver chelate-complex, on standing for days, forms collodial particles or microcrystals which precipitate. When such a mixture if filtered, a red crystalline material is obtained. The solid complex was digested with perchloric acid-nitric acid to destroy the riboflavin and the silver was determined as silver chloride. The compound was found to have a 1:1 composition as verified by the analyses yielding 22.6% silver. The theoretical silver content is 22.4%. A direct silver chloride gravimetric determination is not possible because the precipitated silver chloride absorbs riboflavin. As previously mentioned, this compound has been thoroughly studied by Baarda (200).

Titration of riboflavin solutions (10^{-3} M) with solutions of metal ions (1.0 M) does give the expected pH decrease, assuming a proton displacement as in the case of the silver ion. Cupric ion was the only one of several (Fe^{++}, Fe^{+++}, Zn^{++}) ions to give any pH decrease and this decrease only occurring after an anomalous pH increase. When 10 ml of the riboflavin solution (10^{-2}mM pH 3.2) was titrated with a 1.0 M copper sulfate solution (pH 3.2), the pH increased until 1.4mM of Cu^{++} were added and decreased thereafter. If a calculation is made from the pH decrease (after once started),

the maximum formation constant is about 10^4. This value is less than the one of Albert (40) or Harkins and Freiser (197), but similar to that of Hemmerich and Fallab (198).

In searching for another system which may be more successful in detecting a riboflavin interaction with metal ions, polarography was tried. At pH 5.00 (1 M acetate buffer as carrier electrolyte), riboflavin exhibits a typical simultaneous two electron polarographic wave at -0.370V (versus a saturated calomel electrode, + 0.246V) which is in good agreement with the reported values (56, 60, 61).

Polarography is a useful tool in determining the stability of metal ion-ligand complexes. In these cases, the metal ion is reduced. Riboflavin is reduced prior to most of the metal ions used. In order to avoid fouling of the dropping mercury electrode, reduction of the ligand, i.e., riboflavin, was used. There are no theoretical grounds to vitiate such a determination. None of the metal ions (Cu^{++}, Co^{++}, Ni^{++}, Fe^{++}, Fe^{+++}, Zn^{++}) gave any indication of shifts in the half-wave potential of riboflavin. From consideration of polarographic theory after Souchay and Fauchere (243), metal ion complexes would be expected to shift the reduction potential of riboflavin in a negative direction. A solution composed of 10^{-4} M riboflavin and 10^{-2} M metal ion should have a potential shift of -0.059V if the stability constant were 10^4. Since no shifts were observed, the only conclusion

that can be made is that any interactions are weak or non-existent.

Molecular Complexes

The anticipated molecular complexes of tryptophan, caffeine and 1-propyl-1,4-dihydroncotinamide did not exhibit the usual negative shifts in the riboflavin polarographic half-wave potential. 1-Propyl-1,4-dihydronicotinamide had no effect on the half-wave potential. Caffeine (10^{-4} M to 10^{-1} M) had a positive effect, i.e., the half-wave potential of riboflavin shifted from -0.384V (pH 5.44) to -0.374V in the presence of 10^{-1} M caffeine. This result was considered anomalous since complexes shift the half-wave potential to a more negative value.

In changing the concentration of tryptophan from 10^{-4} M to 10^{-2} M, the riboflavin half-wave potential shifts from -0.360V to -0.373V at pH 5.04. It may be noted that the values are more positive than the riboflavin half-wave potential. On increasing the tryptophan concentration, the potential shifts in a negative direction. Therefore, at no time should the potential be more positive. This observation, also, was considered anomalous. Subsequently, polarography was abandoned due to the belief that the abnormal results obtained for riboflavin and its possible interactants could be questioned on the basis that other phenomena were occurring.

One such phenomenon is the absorption of the compounds on the mercury surface, thereby fouling the electrode and vitiating the data.

Harbury, et al. (175) have used potentiometric titrations to show shifts of -15mV for a riboflavin-5'-phosphate solution (4×10^{-4} M) in the presence of 0.025 M tryptophan and +14mV in the presence of 0.08 M caffeine. These values are similar to the ones obtained by polarography, -13mV and +10mV, respectively. Harbury, et al. explained the positive shift of caffeine by assuming that caffeine complexes more strongly with leucoflavin than with riboflavin. In the polarographic determination, the same explanation can be used, but with the consideration that there is not a true equilibrium situation at the electrode. With the results being similar, the conclusion of a relatively strong caffeine-leucoflavin complex should be further analyzed. Harbury, et al. did not report any anomalous behaviour of tryptophan in the potentiometric titrations. Therefore, the anomaly must be unique to the polarographic techniques.

Riboflavin-5'-phosphate solutions, which can be made more concentrated than riboflavin solutions, have a red-orange coloration. Spectra were determined for various solutions (2×10^{-5} M to 5×10^{-2} M). The spectra are shown in Figure 14. Isenberg and Szent-Györgyi, (174) have attributed the shoulder observed at 485 mμ to a flavin-flavin

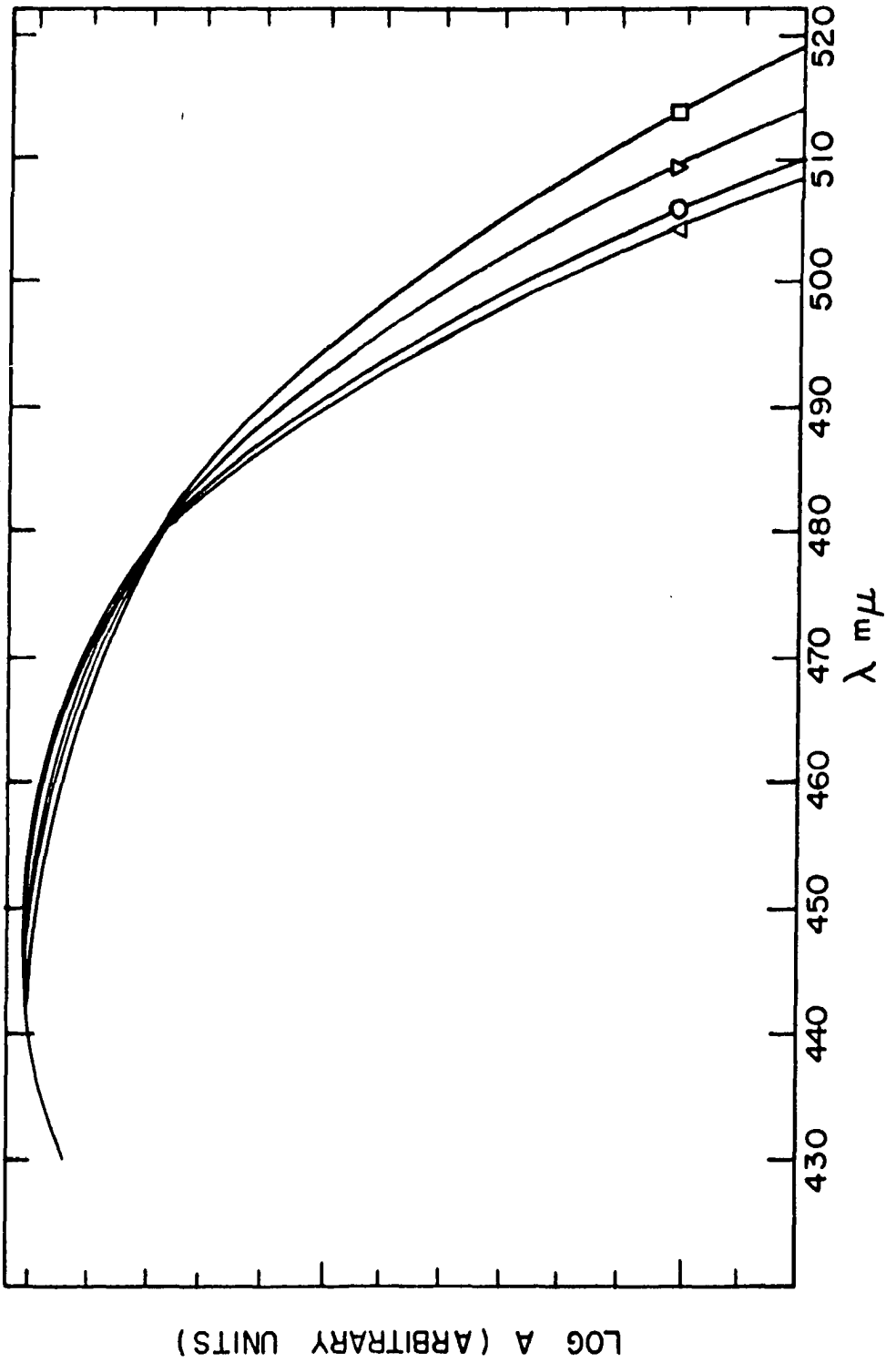
Figure 14. Log absorbance plot for various concentrations of riboflavin-5'-phosphate

△ - 2×10^{-5} M

○ - 1×10^{-4} M

▽ - 5×10^{-3} M

□ - 5×10^{-2} M


LOG A (ARBITRARY UNITS)
λ mμ
430
440
450
460
470
480
490
500
510
520

interaction. The above workers used an integrating sphere to observe this phenomenon at low concentrations. Higher concentrations were used in the results reported here. In the studies represented by Figure 14, there was a considerable difference in absorbance even at low absorbances. The continued high absorbance may be due to a new absorption band of the possible flavin polymers.

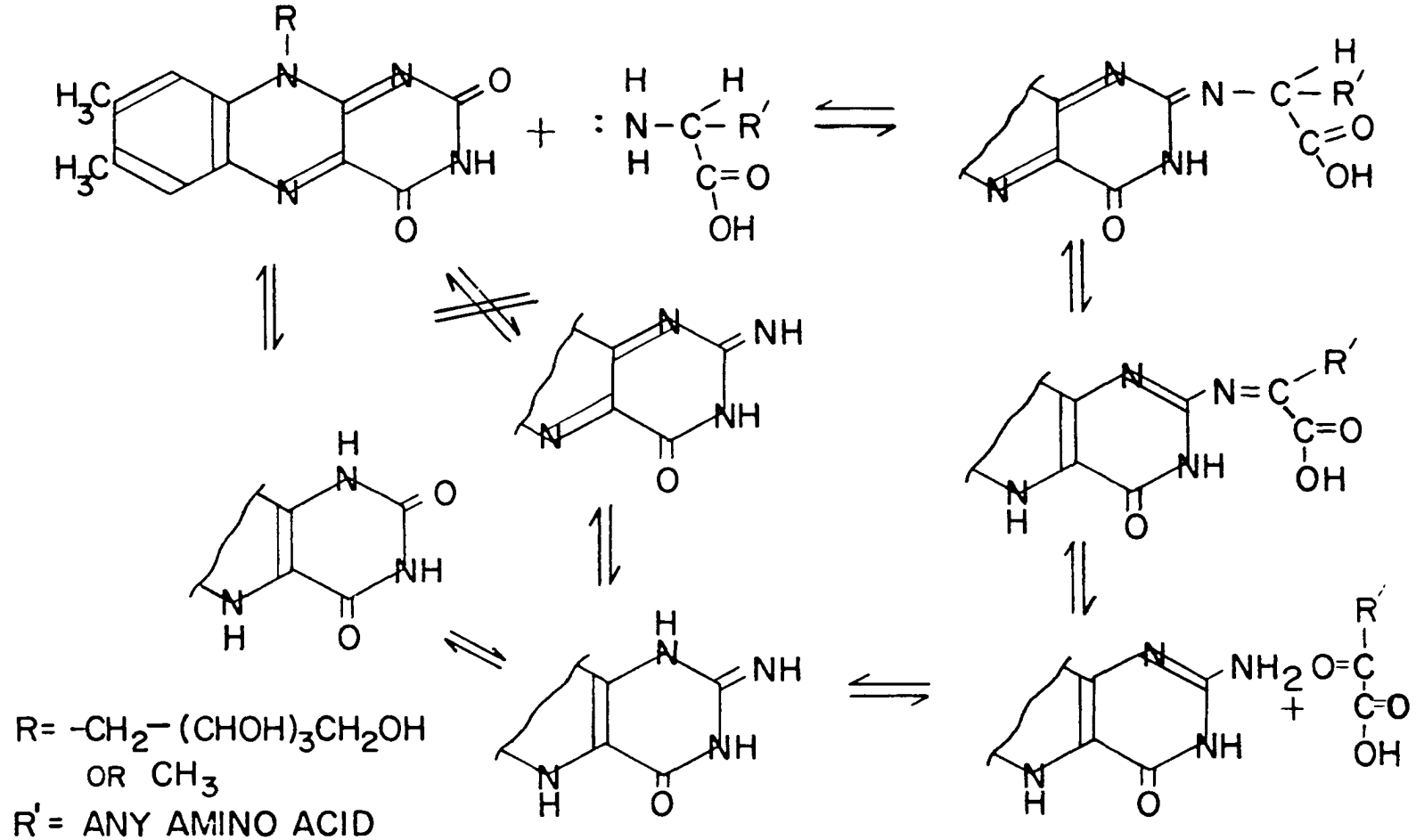
Attempted Amino Acid Oxidation

Using the methods described in an earlier section, very small amounts (ca 1%) of ammonia were detected in the riboflavin-5'-phosphate (10^{-2} M) oxidation of amines and amino acids (10^{-1} M).

Benzylamine and the amino acids, ∝-phenylglycine, alanine, glycine, valine, and threonine, were used. The oxidation attempts were based on the hypothesis that the riboflavin oxidation of amino acids may proceed by the way of a mechanism similar to the one proposed by Braunshtein (248) for pyridoxal. The proposed mechanism is shown in Figure 15, a similar scheme could be constructed for the 4-position of riboflavin.

From studies suggested by the above scheme, several statements can be made about the results. For a one hour reaction at 95°, the yield of ammonia was about 1 to 2% of

Figure 15. Proposed scheme for riboflavin catalyzed oxidation of amino acids


R= -CH2-(CHOH)3CH2OH
OR CH3
R' = ANY AMINO ACID
SIDE CHAIN

the amino acid used. The yields from the amino acids followed the sequence: glycine > alanine > valine > threonine. The rate of ammonia formation was slower at higher ionic strengths. The yield of ammonia exhibited an optimum at pH 6. The nitrogen base buffers, tris-(hydroxymethyl)-methane(Tris) and triethanol amine (TEA) also yielded ammonia under reaction conditions, Tris forming more ammonia than TEA. Metal ions were without effect.

Two reasons were set forth as the most plausible ones for the low and never increasing yields of ammonia. The ammonia could be combined in some unhydrolyzable form. Such a compound could be 2-riboflavimine. The possible existence of this compound was proven by the synthesis of the lumiflavin analog as set forth in the materials section. As depicted in Figure 15, the 2-leucoflavimine is hydrolyzable in base, but 2-riboflavimine is not (206, 241). The 4-lumiflavimine, however, is hydrolyzable (206). In base, 2-lumiflavimine loses the 9-methyl group with the imine remaining (203). The second possible explanation was that the ammonia formation was from another reaction. As mentioned in the review section, Frisell et al. (91) and others have shown that riboflavin catalyzes the photooxidation of many nitrogen containing compounds, including amino acids. The products of these oxidations are many small molecules of a degradative nature. One of these molecules is ammonia.

The conclusion must be made that the yields of ammonia were due to a possible photoxidation and that any ammonia formed by the proposed route was either negligible or combined in an unhydrolyzable compound, e.g., 2-riboflavimine.

Fatty Acid Analog Oxidations

The enzymic α-β oxidation of fatty acyl derivatives suggested another class of compounds on which riboflavin catalyzed oxidations should be attempted. All the fatty acid analogs on pages 47 and 48 were synthesized in order to observe what factors were essential to have an α-β oxidation occur.

Riboflavin-fatty acid analog reaction mixtures were subjected to heat (ca. 100°), at various pH, for varying lengths of time. The spectrum of the reaction mixture was observed in the range where the oxidized compounds would absorb radiation if present. None of these compounds could be made to react. With negative results, it was not possible to decide what factors were most important for a reaction to occur.

Activation by the carbonyl group was anticipated as the most important factor influencing the labilization of the α-hydrogen, whereby the α-carbon bonding electrons would facilitate the removal of a hydride ion from the β-position. Also, stabilization of the transition state by either

electrostatic, covalent or molecular complexes was considered because of a suggestion that other reactions utilize such complexes. Such a case for the latter type is that of reduced diphosphopyridine nucleotide (76) or 1-propyl-1,4-dihydronicotinamide (41, 77) oxidations catalyzed by flavoenzymes or riboflavin, respectively. A driving force such as higher conjugation or aromaticity was also considered very important for success. Finally, an oxidant with a sufficiently positive reduction potential was considered important even though the reactions were irreversible ones as far as electrode reactions are concerned. Only ethyl Δ^3-dihydro-β-naphthoate was a successful substrate for the systems studies and will be discussed in detail below.

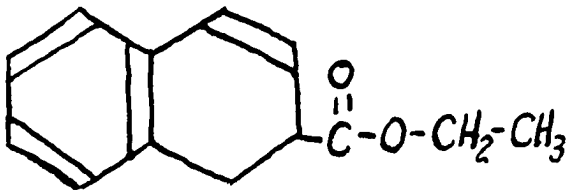
The oxidation of benzylacetone was attempted in non-aqueous solvents. The reaction was carried out by mixing one ml each of equimolar solutions of benzylacetone (in dimethyl-formamide), sodium tertiary butoxide (in tertiary butanol), and 3-methyllumiflavin (in dimethyl formamide). No oxidation was observed in this system.

Dihydro-β-Naphthoate Oxidations

Kinetics

In contrast to all the failures in the preceding section, one fatty acid analog oxidation did occur and this oxidation was unambiguous. The substrate for this non-enzymic oxi-

dation was ethyl Δ^3-dihydro-β-naphthoate, whose structure is:



Although the heat catalyzed reaction proceeded quite rapidly at 95°C, the oxidation was greatly catalyzed by light. Because the light reaction was faster and more convenient to follow than the dark reaction, most of the results are for the light catalyzed reaction. Since photochemical reactions are often accompanied by side reactions, total spectra were recorded for the reaction mixture, at intervals, until the reaction was nearly complete. The results for the oxidation of ethyl Δ^3-dihydro-β-naphthoate are represented by Figure 16.

When the log of the fraction of ester remaining was plotted against time, a straight line was obtained. The slope of the plot being the first order rate constant.

The oxidation of ethyl Δ^3-dihydro-β-naphthoate was also first order with respect to the riboflavin concentration as shown in Figure 17. Since the reaction represented by this plot is dependent upon light energy, any absorption by

Figure 16. Successive spectra of the photo-reaction mixture, riboflavin-ethyl- Δ^3-dihydro-β-naphthoate, at times (in minutes): 0, 5, 10, 20, 30, 45, 60, and 70, plus ethyl naphthoate standard. pH 6.08 (cacodylate, 0.03 ionic strength)

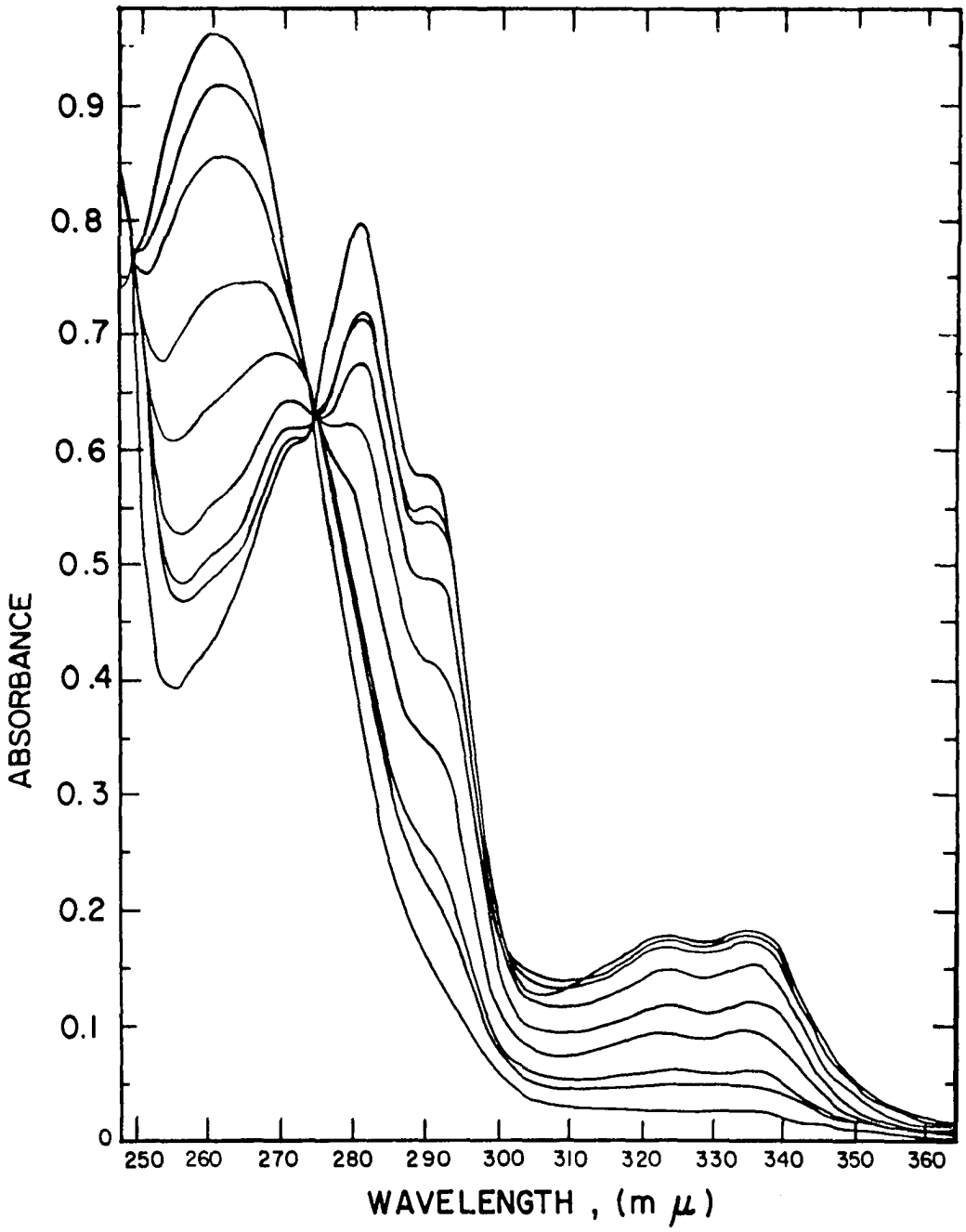
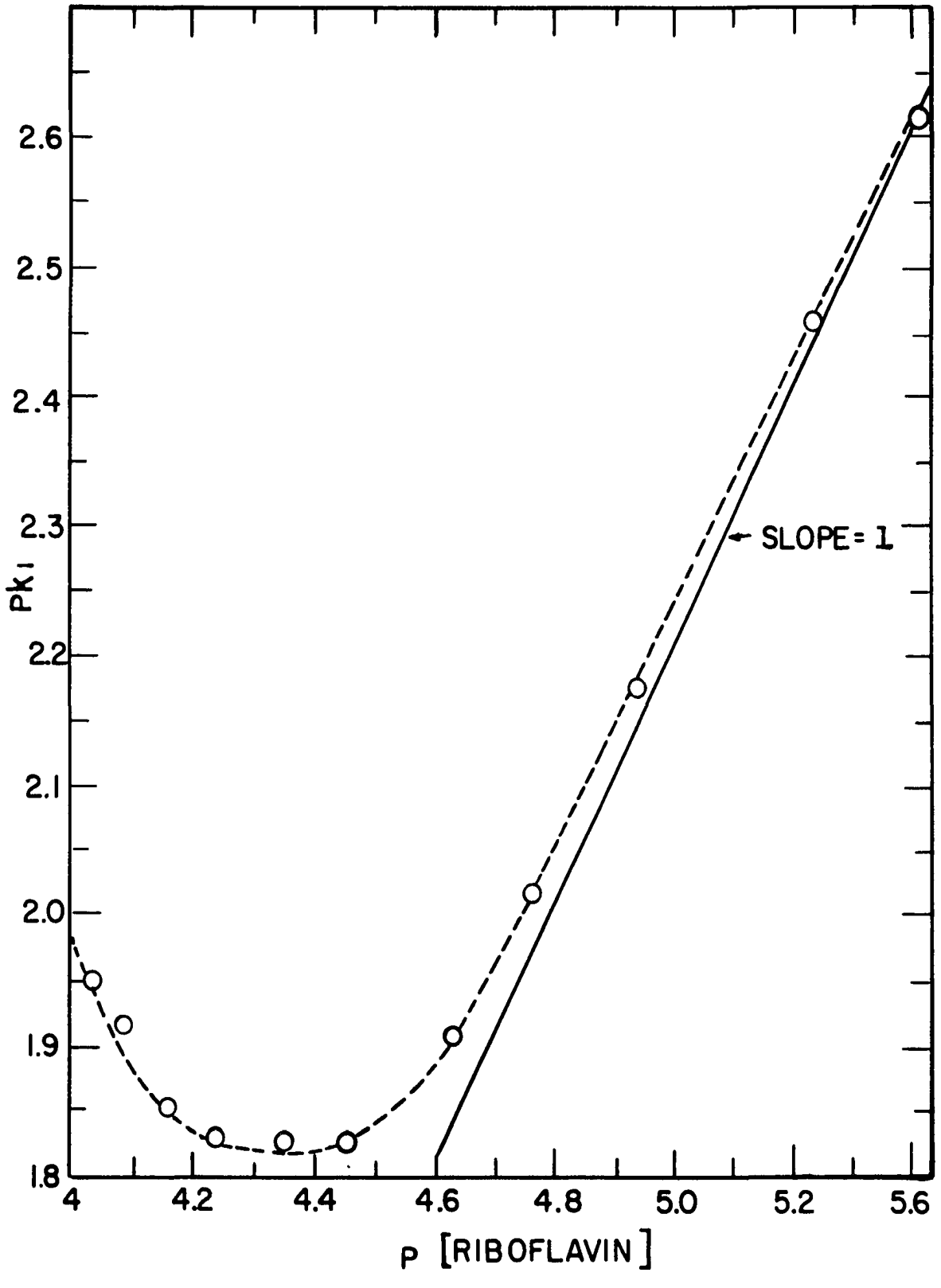

ABSORBANCE
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
250 260 270 280 290 300 310 320 330 340 350 360
WAVELENGTH , (m μ)

Figure 17. Minus log first order rate constant (pk_1) for oxidation of ethyl Δ^3-dihydro-β-naphthoate versus minus log riboflavin concentration (p [Riboflavin]) pH 6.08 (cacodylate, 0.03 ionic strength)


2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
pK1
SLOPE = 1
4
4.2
4.4
4.6
4.8
5.0
5.2
5.6
P [RIBOFLAVIN]

the solution itself would decrease the reaction rate. This effect is shown in Figure 17 where p [Rb] < 4.6, but the plot asymptotically approaches the theoretical line of slope one as expected for a first order effect. Being a first order aerobic riboflavin oxidation, this oxidation is similar in molecularity to that found by Singer and Kearney (76) for the riboflavin oxidation of reduced diphosphopyridine nucleotide and by Suelter and Metzler (41, 77) for the riboflavin oxidation of 1-propyl-1,4-dihydronicotinamide.

The above analysis of the system verifies that the riboflavin remains oxidized due to dissolved oxygen in the solution (2.6×10^{-4} M). Needless to state, the kinetic analyses in this case are simpler than those in an anaerobic system. The first order results were also verified by observation at 445 mμ where no decrease in the absorbance occurred during the reactions. As reported by Beinert (63) and shown in this dissertation, any leucoflavin formed would have reduced the 445 mμ absorbance greatly. Also, oxygenation of the reaction vessel did not enhance the rate, but caused a slight inhibition.

The second order rate constant may be calculated since

$$\frac{-d\,[DHNE]}{dt} = k_2\,[DHNE]\,[Rb]$$

With the riboflavin concentration remaining constant,

$$k_2 = \frac{k_1}{[Rb]}$$

where k_1 = first order rate constant.

k_2 = second order rate constant.

[Rb] = molar concentration of riboflavin.

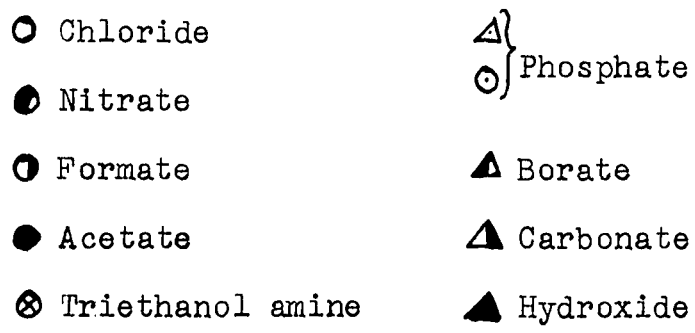
[DHNE] = molar concentration of ethyl Δ^3-dihydro-β-naphthoate.

It should be pointed out that the rate constants in the photochemical oxidations are, in fact, arbitary. The arbitary nature of the rate constants is due to the uniqueness of the system used, i.e., the test tube and light source would not be reproducible by other workers. In order to make the rate constants less arbitary, with only the light source being non-reproducible, the reaction was conducted in a 1 cm silica cuvette at a distance of 12 inches from the 15 watt fluorescent tube. The rates obtained in this manner were 1.5 times those obtained in the test tube photooxidations. The k_2 were 5.38×10^2 l mole^{-1}min^{-1} and 3.62×10^2 l mole^{-1}-min^{-1}, respectively.

Rate of photooxidation versus pH

The effect of pH on the reaction rate is represented by Figure 18 (circles). The lower part of the plot, as completed with a dashed line from pH 2 to 6, is very reminescent of the fluorescence versus pH plot in Figure 19 (249, 250) (solid line). However, as the dashed line indicates, the

Figure 18. First order rate constant of ethyl Δ^3-dihydro-β-naphthoate photooxidation versus pH. Buffers (0.03 ionic strength, except pH~0)



Values at pH 6.06 and 6.21 (phosphate) include cacodylate determinations

[Riboflavin] = 4.7×10^{-5} M (circles)

[3-Methylriboflavin] = 4.6×10^{-5} M (triangles)

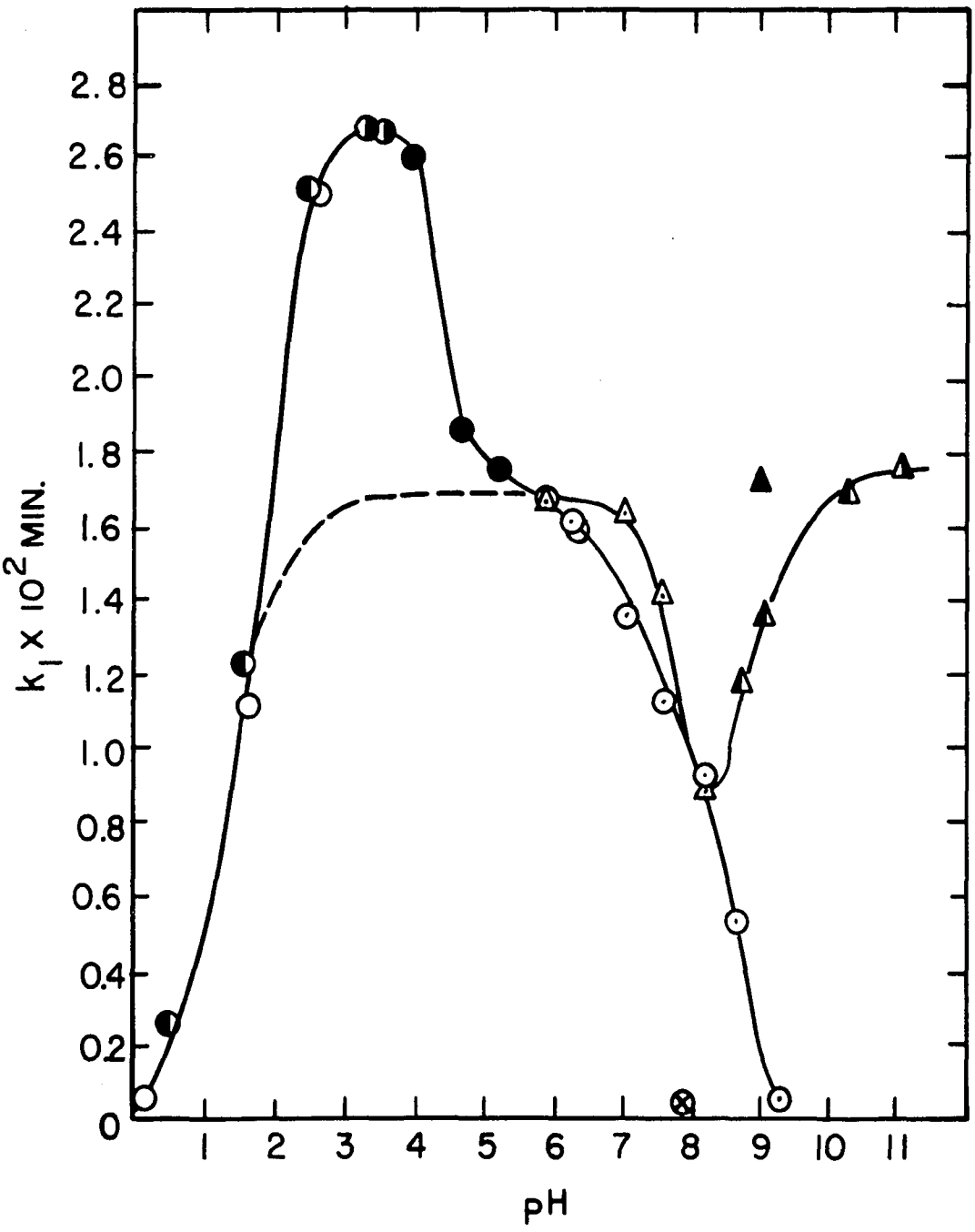
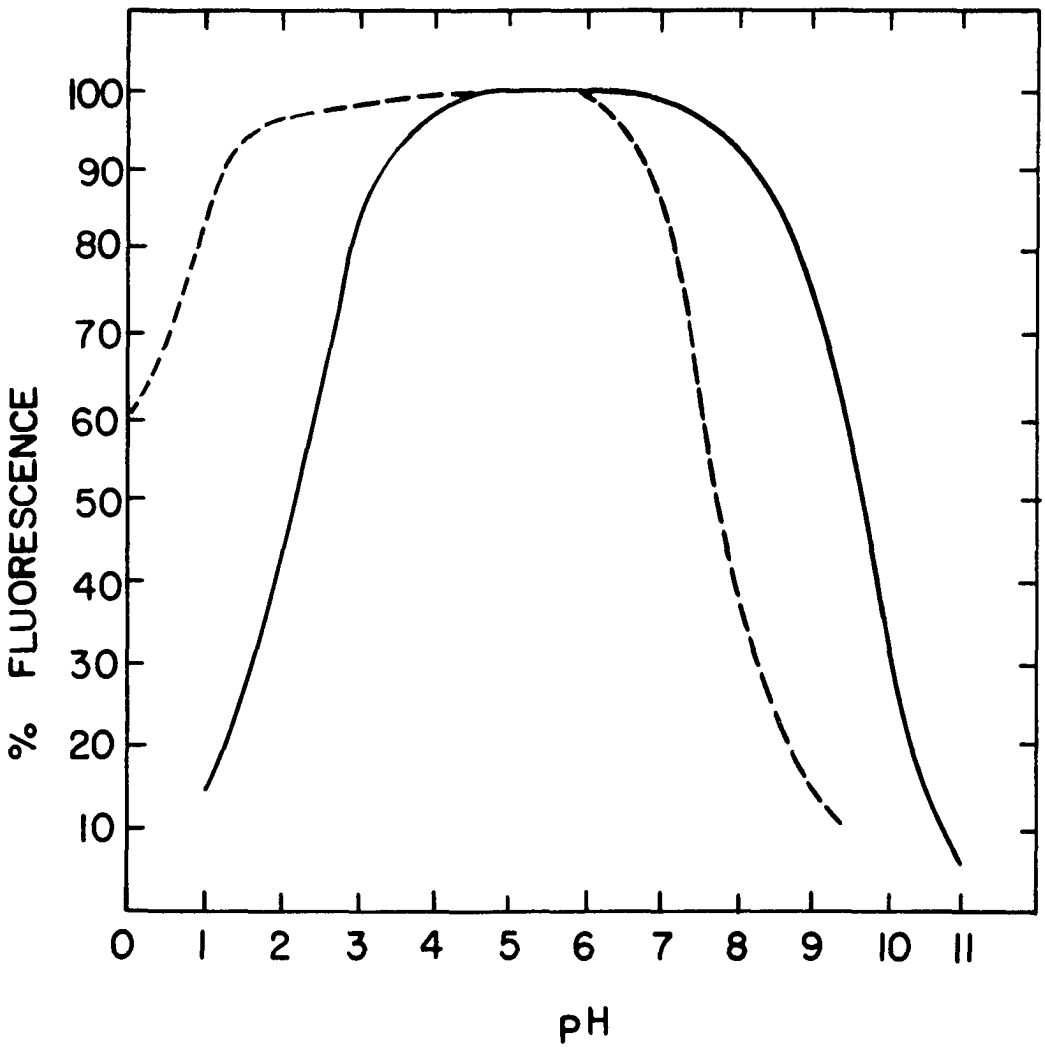

2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
$k_1 \times 10^2$ MIN.
1
2
3
4
5
6
7
8
9
10
11
pH

Figure 19. Per cent riboflavin fluorescence (of maximum) versus pH

- - - - Acetate, tartarate, phosphate, and borate (251)

_____ Hydrochloric acid, sulfuric acid, citrate-sulfuric acid, citrate-phosphate, phosphate-carbonate, carbonate, hydroxide (250)


% FLUORESCENCE
100
90
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
pH

curve may be shifted by the use of different buffers (251). The solid curve is generally considered the more correct chiefly because more workers have reported such. Although in contrast to other results (250), there are reports that monohydrogen phosphate (251) and borate (112) inhibit the fluorescence of riboflavin. If this is true, perhaps the discrepancy between the kinetic data and the fluorescence data at the higher pH may be due to the buffers used. This will be further elaborated on below in respect to 3-methyl-lumiflavin. Amine buffers cannot be used as witnessed by the triethanol amine value at pH 7.88 (Figure 18). The effect of amines probably illustrates the photooxidation of amines by riboflavin (p. 17). Apparently, the amine oxidizes more readily than the naphthoate and inhibits the oxidation of the naphthoate by competition.

The upper part of the plot (pH 3 to 6) is very reminescent to the rapid increase in rate observed by Suelter and Metzler (41, 77) for the 1-propyl-1,4-dihydro-nicotinamide-riboflavin system. This increase, as pH is lowered, may be attributed to the protonation of riboflavin, protonation of a riboflavin-ethyl-Δ^3-dihydro-β-naphthoate complex, or general acid catalysis. The decrease at lower pH made it impossible to study these effects.

With the above data in evidence, it is proposed that the pH dependency of the photooxidation of ethyl Δ^3-dihydro-β-

naphthoate by riboflavin is affected by two consecutive processes (Figure 25). The riboflavin must absorb the light energy and form a reactive complex with ethyl-Δ^3-dihydro-β-naphthoate. More justification will be given for the proposed reactive complex in a later section. Since the energy acceptance or transfer may be very similar to those of fluorescence, the pH dependence should be correspondingly similar. At low and high pH, where the rate is dependent upon the loss in ability of the flavin to accept or transfer energy, there is observed a loss in rate. In the region pH 3 to 6, where energy acceptance or transfer by the flavin is constant, the influence of the second factor, proton activation, may be observed.

Oxidation rate by various flavins

The rate of oxidation by the various flavin analogs available are presented in Table 6. An analysis of the rates was considered a way of learning more about the riboflavin reaction. Since 3-methyllumiflavin has no ionizable hydrogen in the pH range studied, the rate was expected to be constant at all pH above 6. The pH dependence above 6 is given in Figure 18 (triangles). There is a decrease in rate between pH 7 and 8.5, but this effect is not as great as in the case of riboflavin. This difference may be due to a smaller effect of the monohydrogen phosphate ion and boric

Table 6. Relative rates of ethyl Δ^3-dihydro-β-naphthoate photooxidation by various flavins

Flavin	k_1 [a]	k_1 [b]	pK_1	pK_2
Riboflavin	1[c]	0	0.12	9.95
Riboflavin-5'-phosphate	1.2	--	0.05	10.32
Isoriboflavin	0.5	0	-1	10.0
Lumiflavin	0.9	0.3	--	9.95
2-Lumiflavimine	1.1	--	--	--
1'-Hydroxymethyl-lumiflavin	0.9	--	--	--
1'-Formyllumiflavin	0.5	--	3.50	--
3-Methyllumiflavin	0.8	0.8	0.18	--
Flavin adenine dinucleotide	0.2	--	--	--

[a] pH 6.06, Phosphate (0.03 ionic strength).

[b] pH 9.14, Borate (0.003 ionic strength).

[c] Riboflavin is assigned the relative rate of 1.

acid in the absence of a polyhydric side chain. Also, the protonated form of borate appears to be the one inhibiting the reaction and the ionized species is less effective or does not inhibit. This conclusion is evidenced by the increase around the pK of borate. The value at pH 9.3 (no buffer) substantiates these conclusions about buffer effects. There is no decrease in the rate at high pH because 3-methyllumiflavin does not have an ionizable hydrogen in this range.

The polyhydric side chain as an influencing factor in the monohydrogen phosphate ion and boric acid inhibition may be suggested by the fact that lumiflavin (no hydroxyl groups) at pH 9.14 (borate buffer) has a rate of 0.3 that at pH 6, whereas riboflavin has no measurable rate.

The other rates listed may be explained as merely differences in the fluorescent properties and consequently, energy acceptance and transfer properties. This is particularly evident in the case of flavin adenine dinucleotide. With internal complexation by the adenine moeity, the flavin fluorescence is greatly quenched (42). Also, some of the decrease in rate of oxidation may be due to the assumption that the ethyl Δ^3-dihydro-β-naphthoate, due to steric hindrance, cannot get within the proximity of the flavin to be oxidized.

In the case of the 1'-formyllumiflavin, the visual fluorescence is similar to that of riboflavin and one could not explain the decrease in rate on this basis. In fact, the rate was expected to increase on the basis of the interaction shown in Figure 20. The interaction is based upon the relatively high pK of 3.5 (41). Also, the infrared spectrum of the free base shows a peak at 1105 cm^{-1} (aldehyde) which is absent in the hydrochloride spectrum. The hydrochloride has a peak at 1047 cm^{-1} (alcohol), absent in the spectrum of the free base.

Isoriboflavin, which has a rate equal to that of 1'-formyllumiflavin, has only a very slight fluorescence which is more yellow than the yellowish-green riboflavin fluorescence. In this case, a decrease would be expected on the basis of fluorescence and a decrease was observed.

Effect of solvent

The rate of the oxidation diminished quite rapidly on increasing the per cent ethanol in the reaction solution (Figure 21). At 62% ethanol by volume, the rate had decreased so much that it was not measurable. There are several things which this decrease could represent. One explanation would be that the possible hydrophobic bonding needed between riboflavin and the ethyl Δ^3-dihydro-β-naphthoate was weakened or destroyed. Another explanation is that the

Figure 20. 1'-Formyllumiflavin A. Neutral form B. Suggested structure for the reduced compound C. Suggested structure for the cation

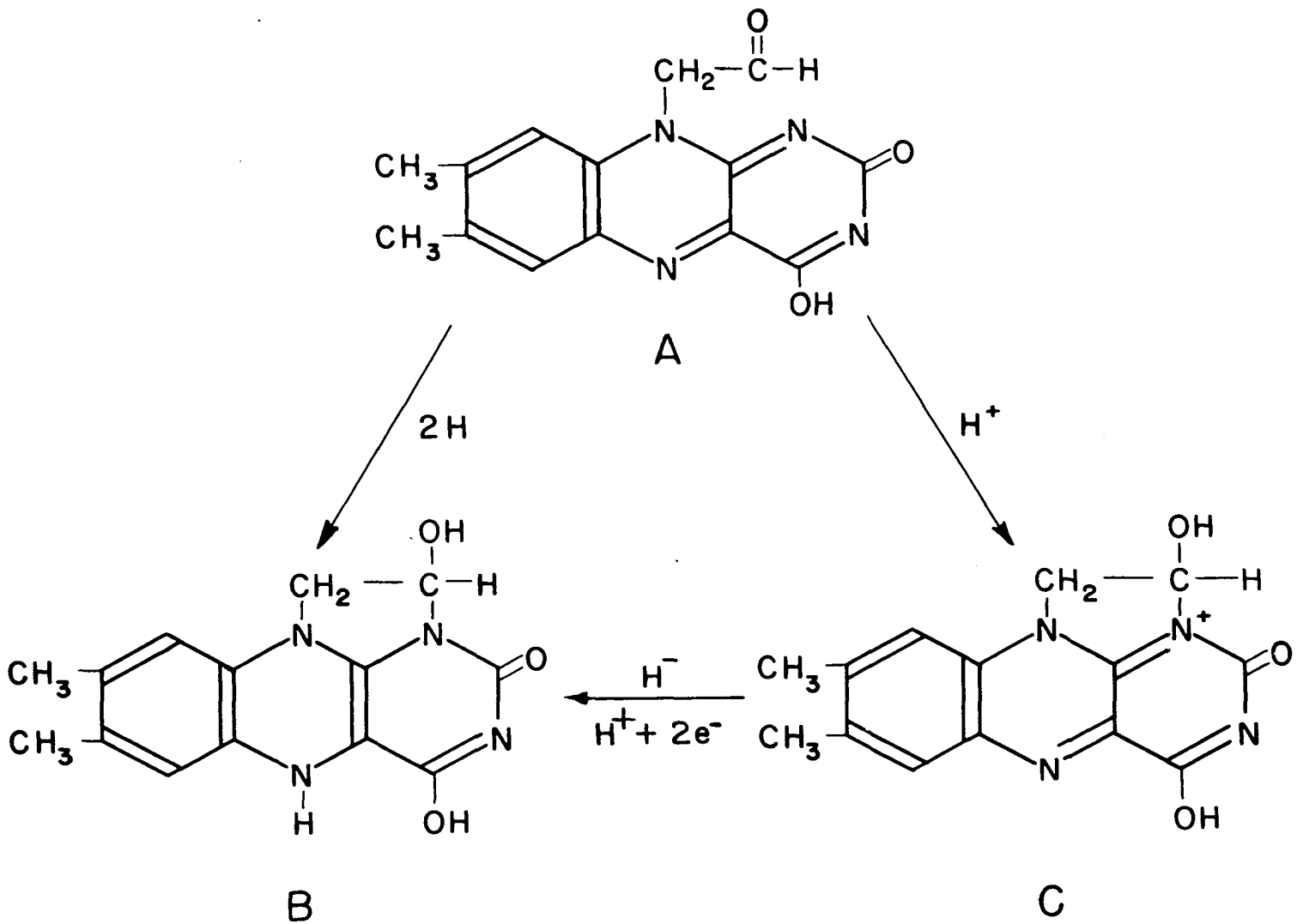
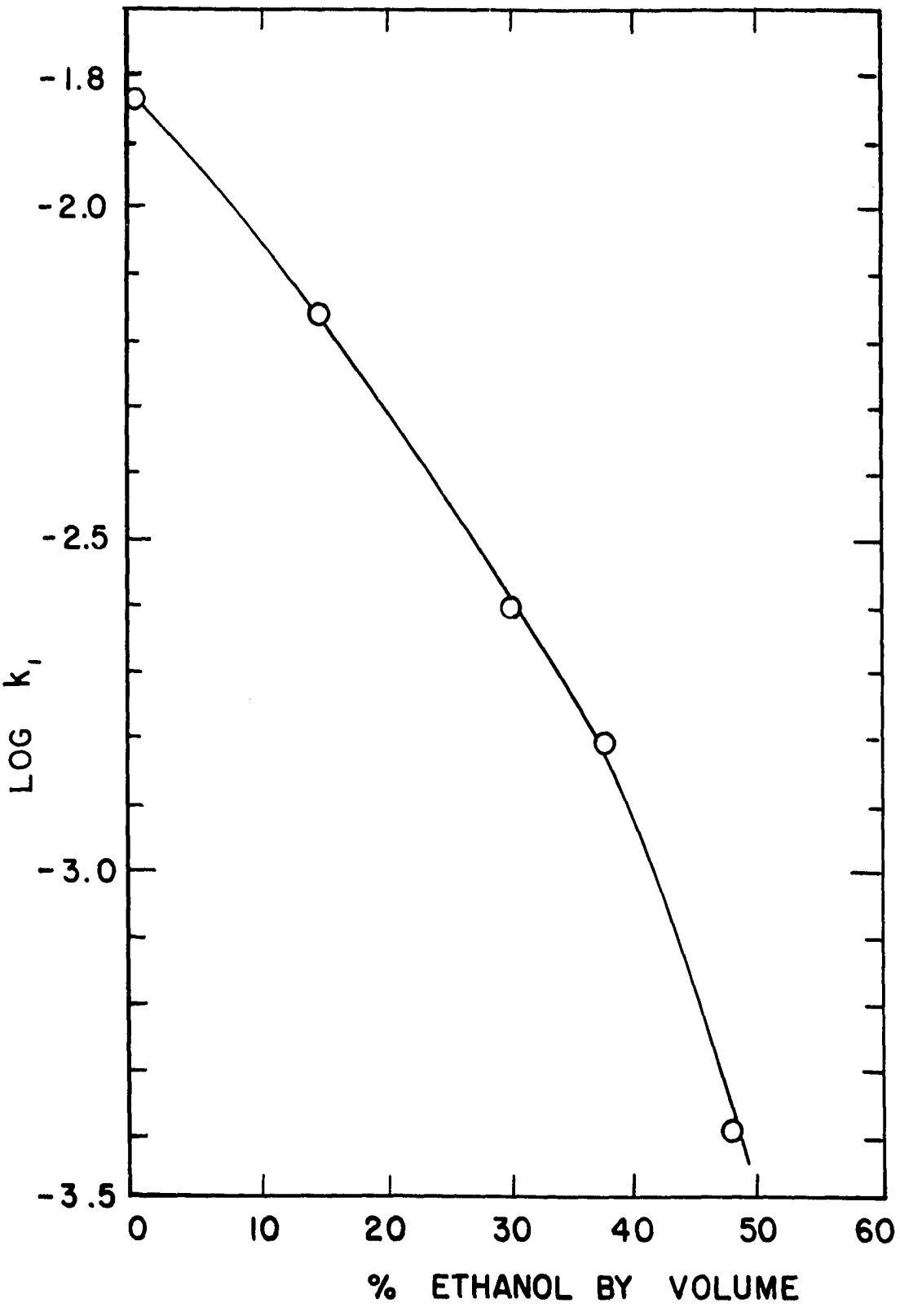

A
2H
H+
B
C
H−
H+ + 2e−

Figure 21. Log first order rate constant versus per cent ethanol in reaction mixture. [Riboflavin] = 4.7×10^{-5} M pH 6.08 (cacodylate, 0.03 ionic strength)


-1.8
-2.0
-2.5
-3.0
-3.5
LOG k_1
0
10
20
30
40
50
60
% ETHANOL BY VOLUME

photoexcited flavin has a shorter half-life in the less polar solution. Just like amines, the alcohol may be a competing substrate for the photoexcited flavin. The explanation that the less polar solvent effects polar intermediates appears not to be valid as evidenced in the next section.

Effect of ionic strength

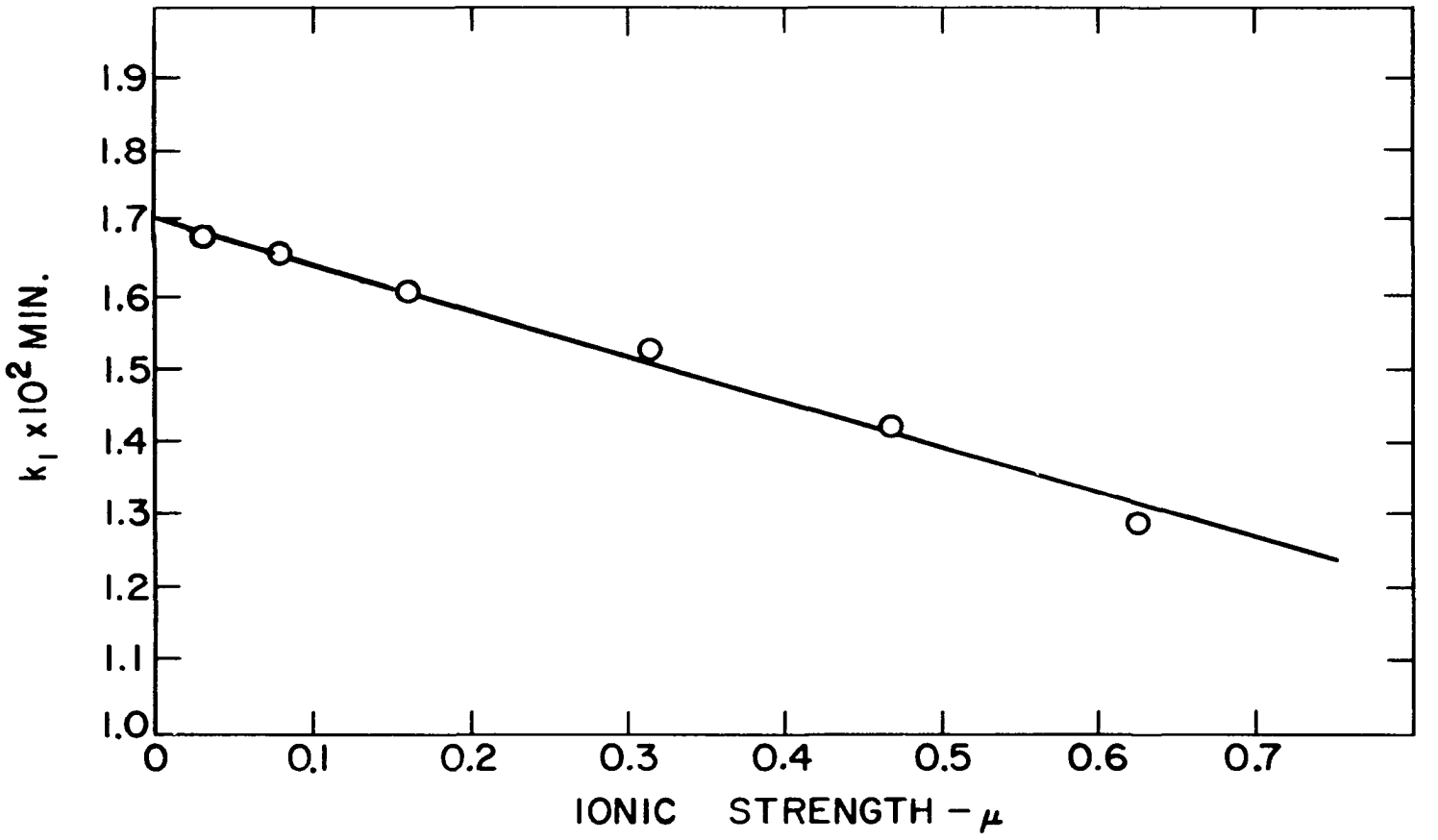
The rate of the oxidation was only slightly dependent upon ionic strength (pH 6.08, cacodylate buffer) as depicted in Figure 22. Increasing the ionic strength decreased the rate. This is expected if one assumes that any intermediate formed was less polar than the reactants. Since both reactants are very much non-polar, no ionic intermediate is likely. The reasoning suggests some non-polar mechanism such as a complex or a radical mechanism.

Effect of inorganic ions

Inorganic ions (10^{-4} M) had varying effects on the reaction rate (pH 6.08, cacodylate buffer). Fe^{+++}, Zn^{++}, Mg^{++}, Ca^{++}, Br^{-}, and Cl^{-} had no effect on the rate. Fe^{++}, Cu^{++}, Co^{++}, Ni^{++}, Ag^{+}, $S_2O_3^{=}$, $SO_3^{=}$, and I^{-} all exhibited about 30% inhibition. From these results, a decision about whether the reaction involves a free radical or not becomes difficult.

Weiss (252) has set down a rule which is undoubtedly

Figure 22. First order rate constant versus ionic strength (μ) [riboflavin] = 4.7×10^{-5} M pH 6.08 (cacodylate)


1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
$k_1 \times 10^2$ MIN.
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
IONIC STRENGTH – μ

valid here. Unless one knows exactly what is going on in the intermediate stages, it would be impossible to decide what the step-wise reactions are in a photochemical oxidation-reduction system. Since the photoexcited dye is willing to accept electrons, any oxidizable material (of the correct nature) present can contribute electrons to the excited state of the dye and is, thereby, oxidized. Whether one wishes to call this couple a reactive complex or not, the two moeities have changed oxidation states.

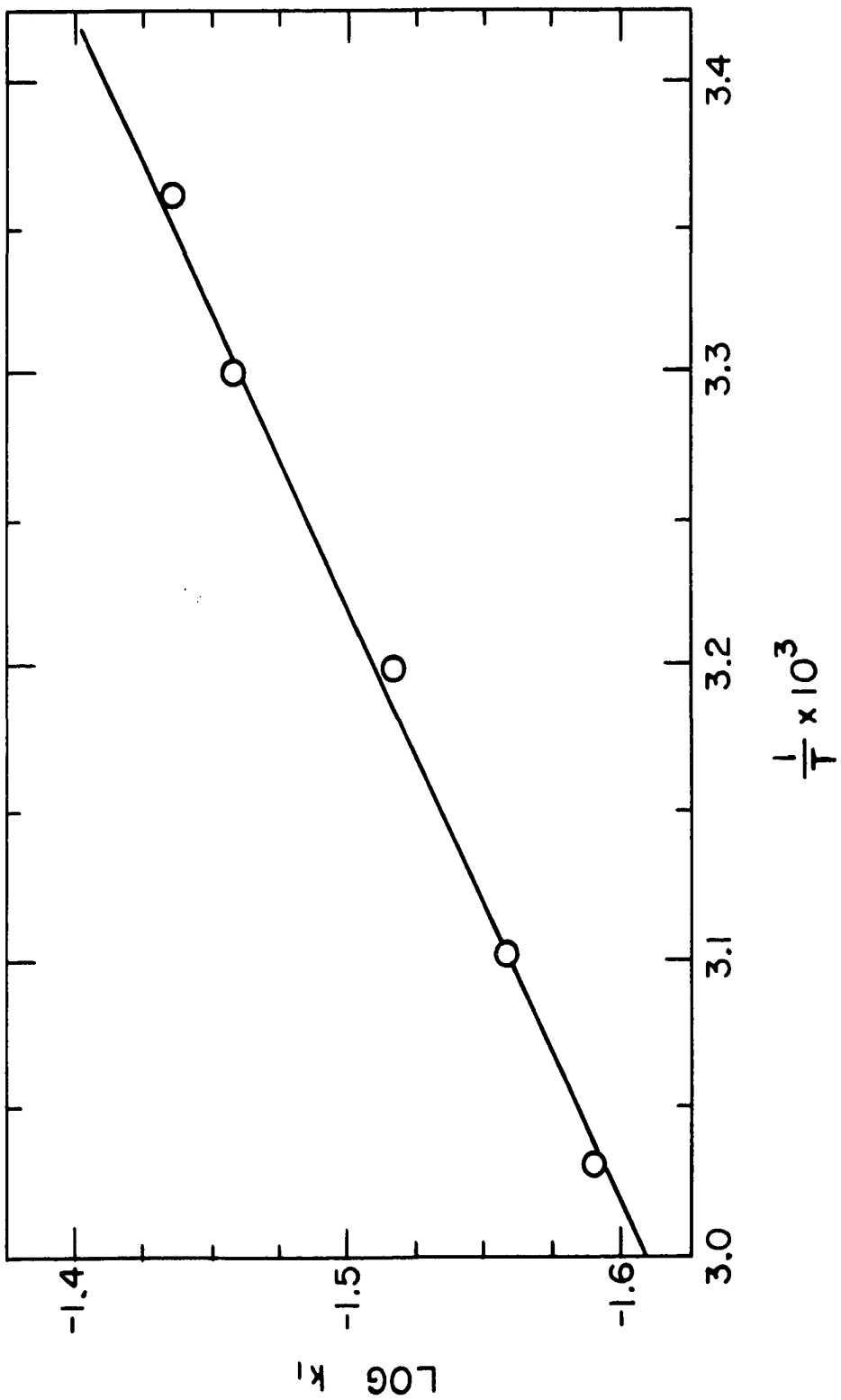
Also, Weiss (253) has concluded that all reactions can be described as one electron reactions. However, one cannot tell if a radical is formed since this depends on the primarily formed one electron transfer complex. If the radical leaves the complex, a free radical could be detected.

The effect of the ions used seems to be more in line with fluorescence quenching than anything else. There are two exceptions to this. Silver ion is not a good fluorescence quencher in other systems but is in the case of riboflavin. This result may be a reflection of a silver-riboflavin interaction. Br^- is known to be a good fluorescence quencher in other systems but is without effect in the present system.

Effect of temperature on the photooxidation

The photoreaction was carried out at various temperatures with the results shown in Figure 23. The figure is an

Figure 23. Log first order rate constant of ethyl-Δ^3-dihydro-β-naphthoate photooxidation versus the reciprocal of the absolute temperature (Arrhenius plot) [riboflavin] = 4.7×10^{-5} M pH 6.08 (cacodylate buffer, 0.03 ionic strength)


LOG k_1
-1.4
-1.5
-1.6
3.0
3.1
3.2
3.3
3.4
$\frac{1}{T} \times 10^3$

Arrhenius plot whose slope is indicative of the heat of activation. In this case, the heat of activation is - 2.25 kcal $mole^{-1}$. A negative heat of activation is expressed by a decrease in the rate on increasing the temperature. This is a highly unusual result which has been used to substantiate the intervention of a complex in the reaction. A temperature increase usually increases certain intermediate reactions. This increase would be overshadowed by the failure to form the prerequisite complex of the reactants in the case of the photochemical reactions where the intermediate reactions are nearly heat independent.

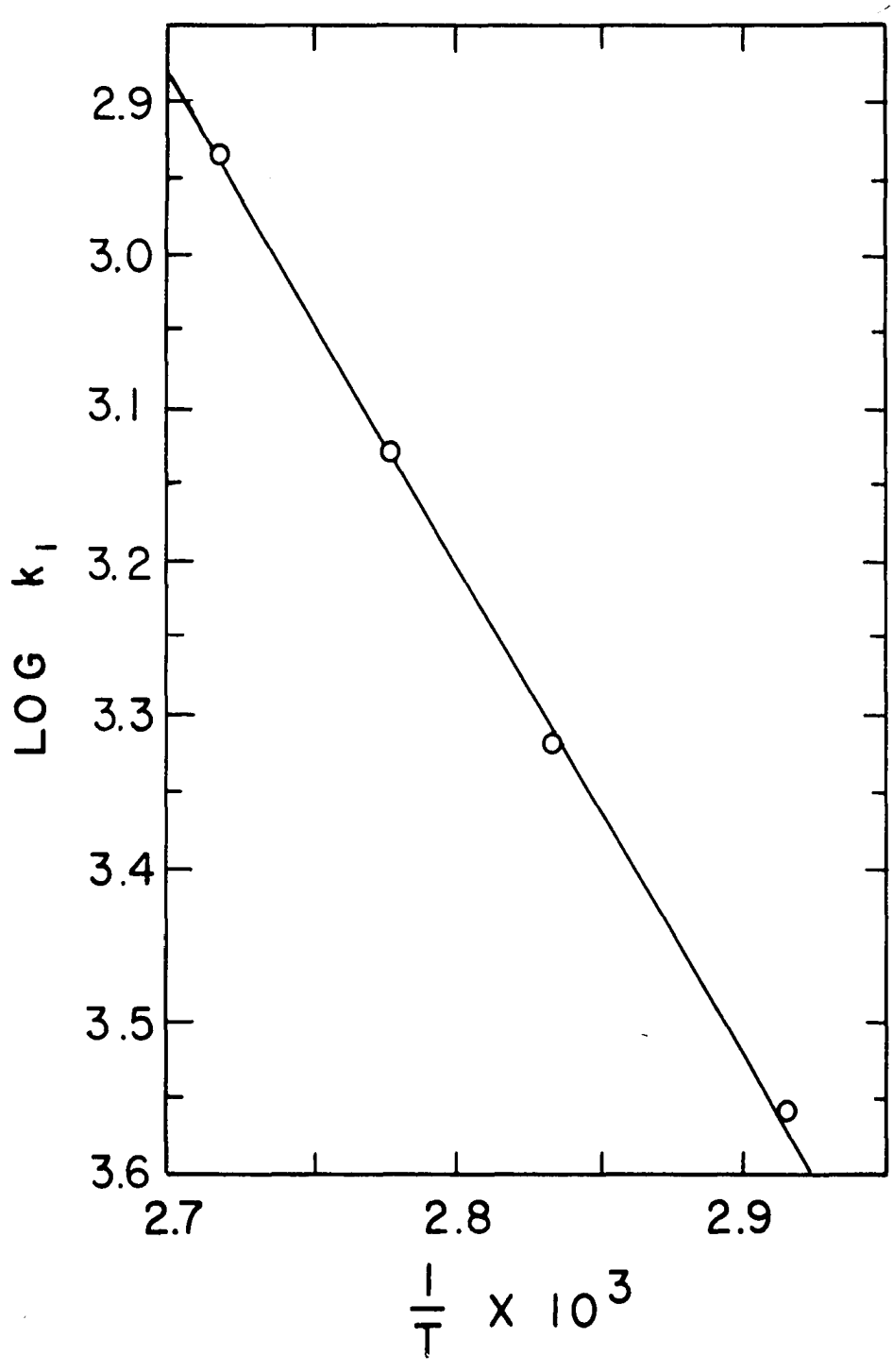
Effect of temperature on the dark reaction

The dark reaction was not studied too thoroughly because complications such as light catalysis and sample evaporation were not easy to overcome. Also, the reaction is much slower at 95° than at 25° with light. The reaction at 95° (pH 6.08) has a half-life of about 4 hours whereas the light reaction has a half-life of about 18 minutes. In the dark reaction (pH 6.08, cacodylate buffer, 0.03 ionic strength), the heat of activation is +14.6 kcal $mole^{-1}$ as calculated from the data presented in Figure 24. The product of the dark reaction is also exclusively β-naphthoate as determined at intervals for 5 hours. The k_2 of the reaction at 95° is 25 $l mole^{-1} min^{-1}$.

Figure 24. Log first order rate constant of ethyl Δ^3-dihydro-β-naphthoate heat oxidation versus the reciprocal of the absolute temperature (Arrhenius plot)

$[\text{Riboflavin}] = 4.7 \times 10^{-5}$ M

pH 6.08 (cacodylate, 0.03 ionic strength)


2.9
3.0
3.1
3.2
3.3
3.4
3.5
3.6
LOG k_1
2.7
2.8
2.9
$\frac{1}{T} \times 10^3$

The explanation which was given in the preceding section can now be more thoroughly justified. In the dark experiments, the only external source of energy was heat. Consequently, the intermediate reactions are not temperature independent as in the photoreaction and the rate increases with temperature as expected.

Oxidation of the Δ^1- and Δ^2-ethyldihydro-β-naphthoates and the dihydro-β-naphthoic acids

The isomeric esters of the Δ^3 acid (Δ^1 and Δ^2) did not undergo photooxidations in the systems described above. No change was observed in the total spectra of the reaction mixtures during irradiation. These results are in contrast to the benzoquinone and o- and p-naphthoquinone thermal oxidations of the dihydronaphthalenes (160, 161). In these systems, 1,4-dihydronaphthalene was oxidized about 15 times as rapidly as 1,2-dihydronaphthalene. The presence of the carboxyl group and its influence may be a reason for the difference in these two systems. Also, the difference in the mode of activation may be another contribution to the differing results.

The dihydro-β-naphthoic acids did not give the unique photooxidation to β-naphthoic acid. The Δ^3-acid had another reaction proceeding, at all pH, simultaneously with the oxidation as revealed by total spectral analyses during

the reaction interval. The formation of the internal lactone is suggested as a possible concurrent reaction. The reaction

is analogous to the reaction of the acid with iodine to yield the iodolactone (239). The Δ^1-acid did not oxidize but isomerized to something with an unidentifiable spectrum similar to those of alkyl substituted benzenoid compounds. This acid was studied at pH 3 and 6. The spectra during the photoreaction of the Δ^2-acid showed the appearance of some β-naphthoic acid at pH 3 but not at pH 6. Because of its low absorbancy above 250 mμ (Figure 9), the appearance of an isomerized compound was not evident. However, the absorbances at 290 and 330 mμ did not correspond, in proportion, to those for β-naphthoic acid. Another reaction was suggested by these results.

Other systems

Nicotinamide-1-propochloride did not oxidize the dihydro-β-naphthoates under any of the conditions used. Also, methyl hydrocinnamate was not photooxidized by riboflavin under the conditions above.

Proposed scheme of oxidation

A brief resume of the findings will lay a basis for the proposed scheme of oxidation. The riboflavin oxidation of ethyl Δ^3-dihydro-β-naphthoate is greatly catalyzed by light. Both the photoproduct and the thermal product are ethyl-β-naphthoate. The reaction is a bimolecular one, first order in both ester and riboflavin. The reaction is faster with higher light intensities. The energy transfer of the photoreaction is characterized by the same effects as is fluorescence. The pH plots of each is similar to the other. Ionic fluorescence quenchers reduce the rate of reaction. With an exception, the rate of reaction by the various flavins is indicated by the amount of fluorescence.

From solvent and temperature effects, the probable intervention of a reactive complex was deduced. The failure of methyl hydrocinnamate to be photooxidized also suggests a specific interaction or the need for formation of an aromatic system.

In studies of the acid versus the ester, no results could be obtained as to α-hydrogen activation because the acid undergoes another simultaneous reaction. The studies on the isomeric esters revealed that hydrogen activation was not sufficient, but that the hydrogens must be α-β. The reaction does not proceed through any polar intermediates as evidenced by a decrease in rate as ionic strength is increased.

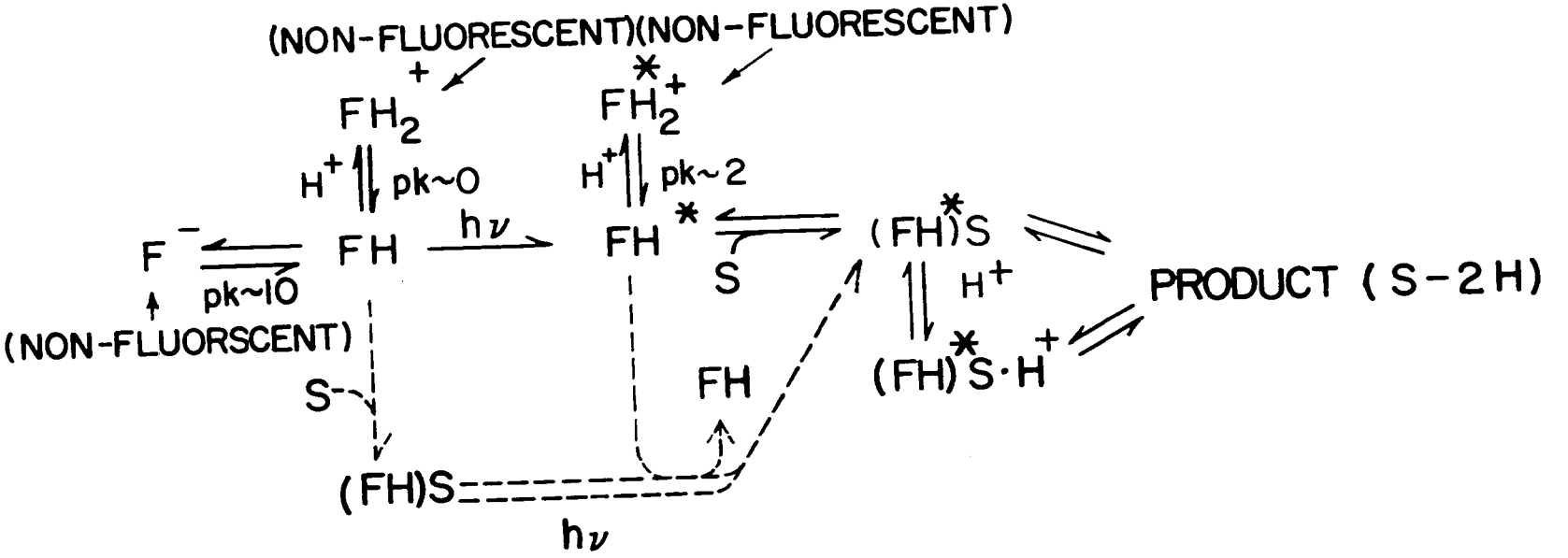
The following is the proposed scheme for the photooxidation (Figure 25). The ethyl Δ^3-dihydro-β-naphthoate (S) forms a reactive complex with photoexcited riboflavin (FH^*). The oxidation-reduction reactions occur by means of the photoexcitation energy. The oxidized substrate and leucoflavin separate and the flavin is regenerated by oxygen, forcing the reaction to completion. For the dark reaction, where heat furnishes the energy for the oxidation-reduction reactions, no such scheme can be constructed due to lack of experimental data.

Of the alternate schemes depicted in Figure 25 (dotted lines), the energy transfer scheme may be eliminated since the reaction is first order in flavin. The other alternate scheme is just as valid as the proposed scheme if one assumes the flavin-substrate complex has energy acceptance and transfer properties similar to free flavin.

Posthuma and Berends (109) have suggested a flavin-(triplet)polyene(triplet) energy transfer for the riboflavin or lumichrome photooxidation of pimaricin (a polyene antibiotic). This conclusion was based on the inhibition by oxygen or other paramagnetic compounds.

The manner in which the photooxidation-reduction reactions occur could not be ascertained. Hydride ion transfer may be proposed on the basis of proton activation, but it is known that flavin semiquinone radicals are stabilized

Figure 25. Proposed mechanism for the riboflavin photo-oxidation of ethyl Δ^3-dihydro-β-naphthoate


(NON-FLUORESCENT)(NON-FLUORESCENT)
FH2+
FH2*+
H+ pk~0
H+ pk~2
F−
pk~10
FH
hν
FH*
S
(FH*)S
H+
PRODUCT (S-2H)
(NON-FLUORSCENT)
S-
FH
(FH*)S·H+
(FH)S
hν

by protonation and a one electron transfer may equally be justified on the basis of proton activation. Justification for a one electron transfer also comes from the fact that the reaction was faster in solutions of lower ionic strength and the fact that the reaction was greatly catalyzed by light which is known to activate radical reactions.

Since only one product was formed and the reaction was quite specific for one isomer and for a bicylic system, it must be concluded that if the reaction occurs by one electron transfer, the transfer must be quite specific, suggesting a prerequisite complex.

Oxidation-reduction Systems of Other Compounds

Orotic Acid

Similar to the fatty acids and their analogs, particularly ethyl Δ^3-dihydro-β-naphthoate, is a compound now known to be a substrate for a flavoenzyme. Dihydroorotic acid was thought to possess the necessary requirements for a flavin catalyzed oxidation. This oxidation was previously thought to be a diphosphopyridine nucleotide catalyzed oxidation. The flavin non-enzymic reaction was attempted at the time the enzyme was isolated and shown to be a flavoenzyme.

Contrary to the enzymic studies, the non-enzymic reactions did not exhibit an oxidation reaction with riboflavin.

A photooxidation system, similar to the one for the dihydro-β-naphthoate, did not result in an oxidation. Since the enzymic reaction equilibrium favors the reduced orotate, the reverse reaction was thought to be more promising.

Studies with leucoflavin, prepared by dithionite reduction, appeared to give a reduction of methyl orotate, although the results were very inconsistent (Table 7). The results are similar to the catalytic hydrogenation of methyl orotate (about 50% conversion). Also, with the possibility of the reduction occurring by means of a riboflavin-dithionite complex, the decision was made to wait for a more elaborate system, not using dithionite, to be developed. Such a system is described in the leucoflavin section.

Lipoic acid

The reduced α-lipoic acid anaerobic oxidation was similar in results to the orotates. The rate of reaction was not rapid enough to do a quantitative study (Table 8). Although the results indicate an oxidation, the systems were not as anaerobic as the system developed for the leucoflavin experiments. The slowness of the reaction may have been a result of oxygen remaining in the thunberg vessels.

Glucose

The attempted oxidation of glucose was not successful. No gluconolactone could be detected by titration or by

Table 7. Reduction of orotate by riboflavin-dithionite. The reaction mixtures contain 2 ml each of riboflavin (1.41×10^{-4} M and methyl orotate (1.35×10^{-4} M) solutions, 1 ml pH 7 phosphate buffer (0.3 ionic strength), and 0.2 mg dithionite

Reaction mixture	Absorbance - 280 mμ		
	I[a]	II[b]	III[c]
Methyl orotate, riboflavin, and dithionite	.280	.291	.281
Methyl orotate and dithionite	.520	.495	.554

[a]I. Reacted 4 hours - allowed to stand 4 hours to reoxidize and clarify.

[b]II. Reacted 4 hours - reoxidized and clarified immediately with oxygen.

[c]III. Reacted 10 hours - allowed to stand 4 hours to reoxidize and clarify.

formation of the hydroxamate.

1-Propyl-1,4-dihydronicotinamide

Studies on the riboflavin oxidation of 1-propyl-1,4-dihydronicotinamide have been reported by Suelter and Metzler (41, 77). Because this reaction was one free of ambiguity, it offered a fine system to check the anaerobic systems which were developed.

Table 8. Reduced ∝-lipoic acid oxidation by riboflavin. The reaction mixture contains 2 nl each of reduced ∝-lipoic acid (10^{-3} M) and riboflavin (10^{-4} M) at pH 6 (no buffer)

Hours	Absorbance - 445 mμ
0	1.510
12	1.060
36	.742
36 (open to air)	1.200

When a 10^{-4} M riboflavin solution was reduced by an equimolar amount of 1-propyl-1,4-dihydronicotinamide in an evacuated thunberg vessel, the 445 mμ absorbance decayed until less than 2% of the initial riboflavin absorption remained.

With equimolar concentrations, the second order rate constant (k_2), calculated from the slope $\frac{1}{k_2}$ of a $2a\sqrt{K'}\,t$ versus

$$\ln \frac{1 - \frac{x}{a}(1-\sqrt{K'})}{1 - \frac{x}{a}(1+\sqrt{K'})}$$

plot, had a value of 105 $\text{lmole}^{-1}\text{sec}^{-1}$. Such a plot is made to evaluate the rate constant of a reversible second order reaction. This value is in agreement with the one found by Suelter and Metzler (41, 77) using the first order rate

constant divided by the riboflavin concentration. The equilibrium of this reaction lies at a value of about 98% reduction of the flavin. This equilibrium would assign the nicotinamide-1-propochloride-1-propyl-1,4-dihydronicotinamide couple a reduction potential of about -0.3V (pH 7, 20°). The reduction potential of nicotinamide-1-propobromide as obtained from cyanide addition data is reported to be -0.387V (pH 7, 20°) (254). The diphosphopyridine nucleotide couple has a reduction potential of -0.282V (pH 7, 30°).

Leucoflavin

pK Of leucoflavin

The pK of leucoflavin was calculated by the spectrophotometric method to be 6.1. This value agrees with the value of Michealis (39), who used potentiometric titrations in determining the pK. The spectra of the acid and base forms of leucoflavin for this pK are shown in Figure 26. However, leucoflavin is protonated at a lower pH as shown in Figure 27.

The assignment of pK 6.1 to leucoflavin was made from the absorbancy index of a leucoflavin solution at pH 6.15 and the absorbancy indexes of leucoflavin solutions at pH 3.3 and 9.18. The absorbancy index of a pH 12.04 solution was identical to that of the pH 9.18 solution. The absorbancy index of leucoflavin solutions from pH 5.03 to 2.87

Figure 26. Spectra of leucoflavin at pH 9.18 and 12.04 (- - -) and at pH 3.30 and 2.87 (——)

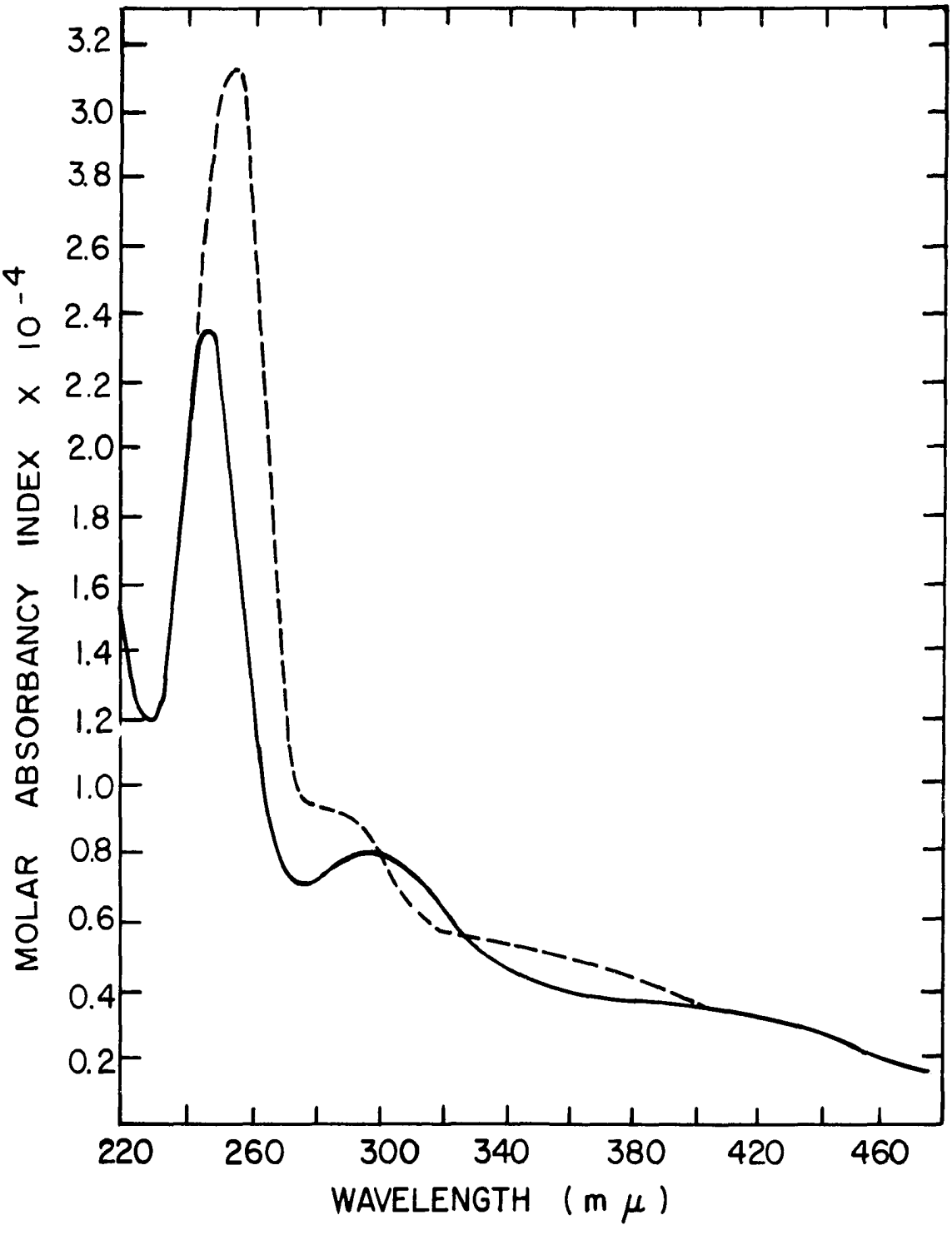
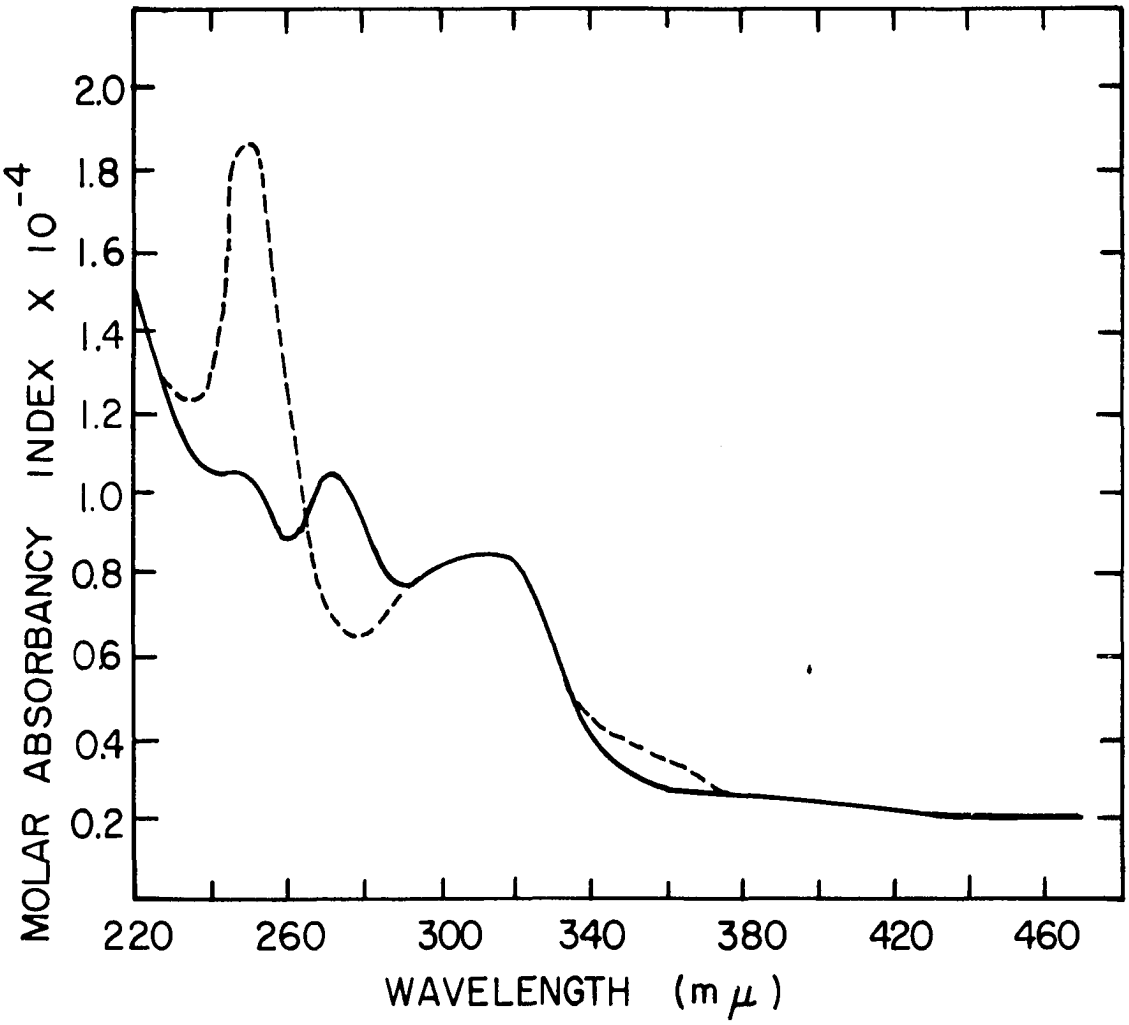

MOLAR ABSORBANCY INDEX X 10^{-4}
3.2
3.0
3.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
220
260
300
340
380
420
460
WAVELENGTH (m μ)

Figure 27. Spectra of leucoflavin in acid solutions of pH 0.53 (- - -) and of 2 N solfuric acid (——)


MOLAR ABSORBANCY INDEX X 10^{-4}
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
220
260
300
340
380
420
460
WAVELENGTH (mμ)

were essentially identical. All solutions were approximately 2×10^{-5} M. The absorbency indexes for leucoflavin are: basic form, $a_{M250} = 2.81 \times 10^4$ and $a_{M260} = 3.12 \times 10^4$; acid form, $a_{M250} = 2.36 \times 10^4$ and $a_{M300} = 8.03 \times 10^3$. There is an isosbestic point at 300 mμ, $a_M = 8.03 \times 10^3$.

Air oxidation of leucoflavin

Leucoflavin, prepared by catalytic hydrogenation, re-oxidizes in a manner similar to that of dithionite reduced riboflavin-5'-phosphate (Figure 28) (63).

Since the solutions used in this study were very dilute (2×10^{-5} M), evidence of other phenomenon such as a semiquinone, a semiquinone dimer or a leucoflavin-oxygen complex could not be detected. It should be pointed out that the micro drops of water containing oxygen used to reoxidize the leucoflavin did not significantly change the volume. This is evidenced by the final spectrum being unchanged after each of three additions.

Formation of the semiquinone

The semiquinone form was prepared by adding a riboflavin solution to a leucoflavin solution prepared by catalytic hydrogenation using the apparatus depicted in Figure 10. The addition of concentrated sulfuric acid to such a mixture yields the spectrum shown in Figure 29. Both flavins had

Figure 28. Spectra showing the conversion of leucoflavin (lower curve above 320 mμ) to riboflavin by water drops containing oxygen

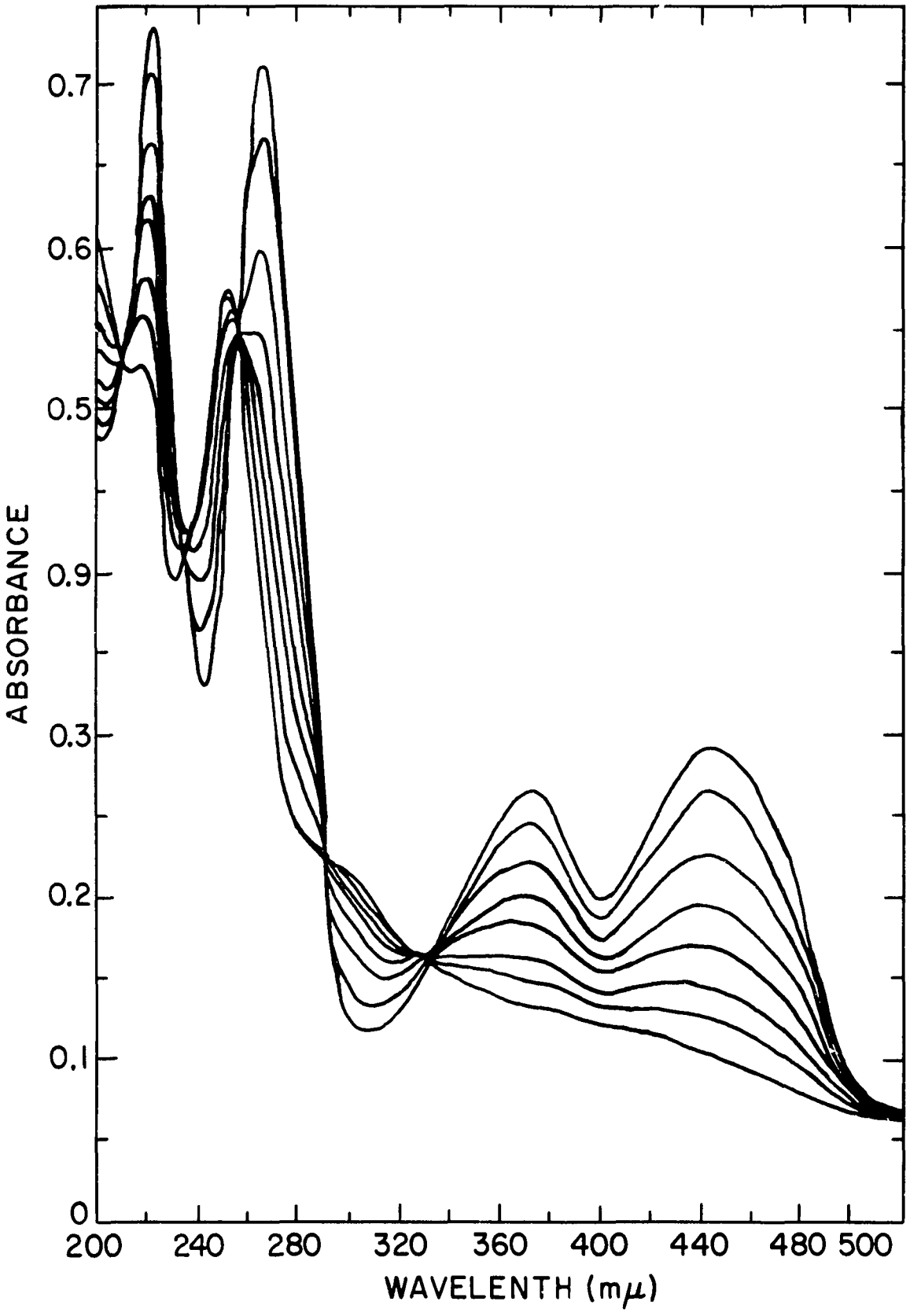
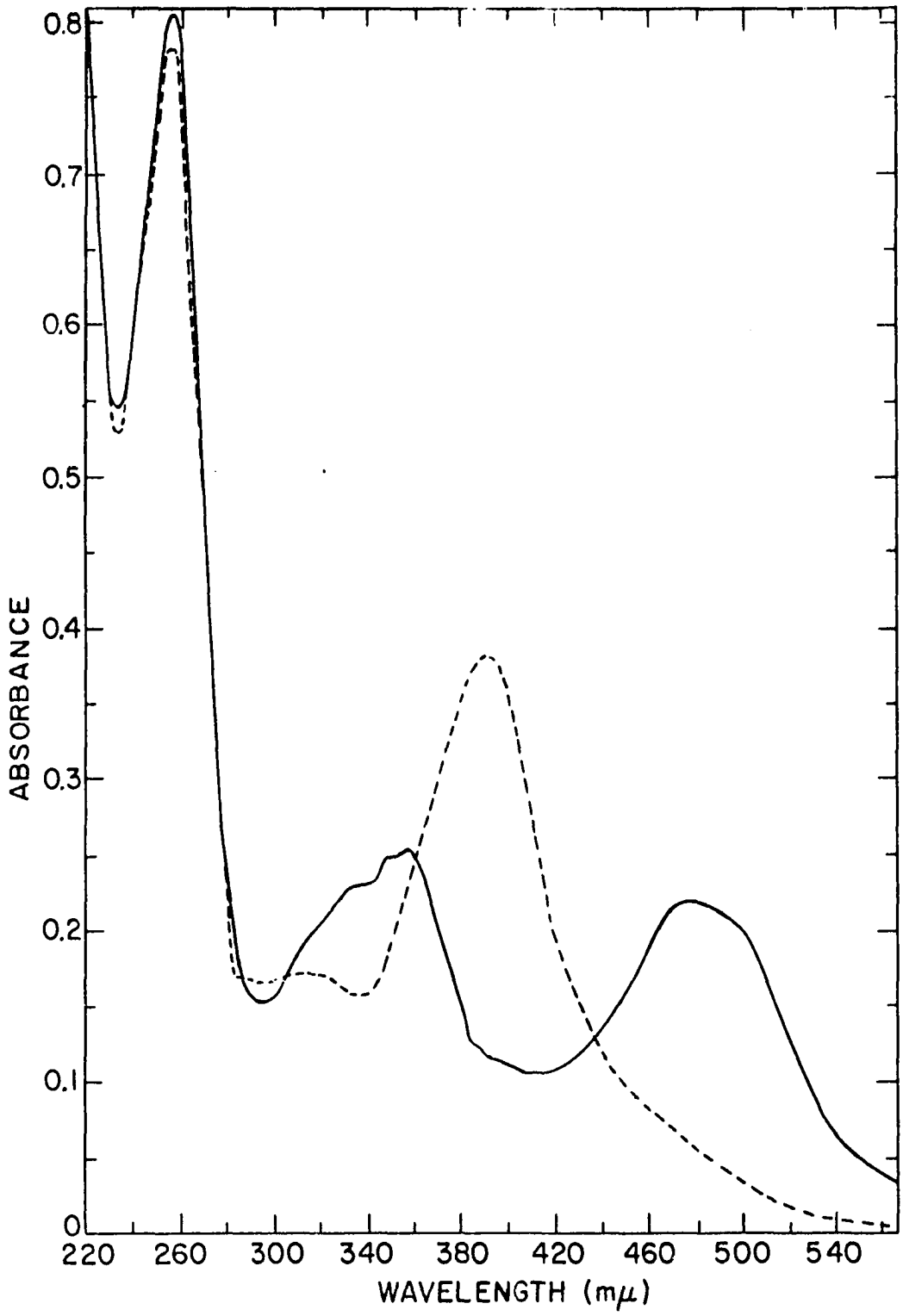

ABSORBANCE
0.7
0.6
0.5
0.9
0.3
0.2
0.1
0
200
240
280
320
360
400
440
480
500
WAVELENTH (mμ)

Figure 29. The spectrum of equimolar amounts of leucoflavin and riboflavin (semiquinone solution) in 2 N sulfuric acid (____) and calculated spectrum of an equimolar mixture assuming no interaction (- - -). Total flavin concentration = 4×10^{-5} M


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
220
260
300
340
380
420
460
500
540
WAVELENGTH (mμ)

the concentration of 1.8×10^{-5} M. The dotted spectrum in Figure 29 is the calculated spectrum of a riboflavin-leucoflavin mixture at the same acidity, assuming no interaction.

The semiquinone form was also prepared by photochemically reducing a riboflavin solution so that the oxidized flavin was at one-half the initial concentration. This solution was then acidified by tipping in concentrated sulfuric acid from the side arm of the modified thunberg tube. The total spectra were recorded. Figure 30 shows the spectrum of such solutions in 2 N sulfuric acid. This is similar to the semiquinone spectrum in Figure 29 where chemically pure leucoflavin and riboflavin were mixed.

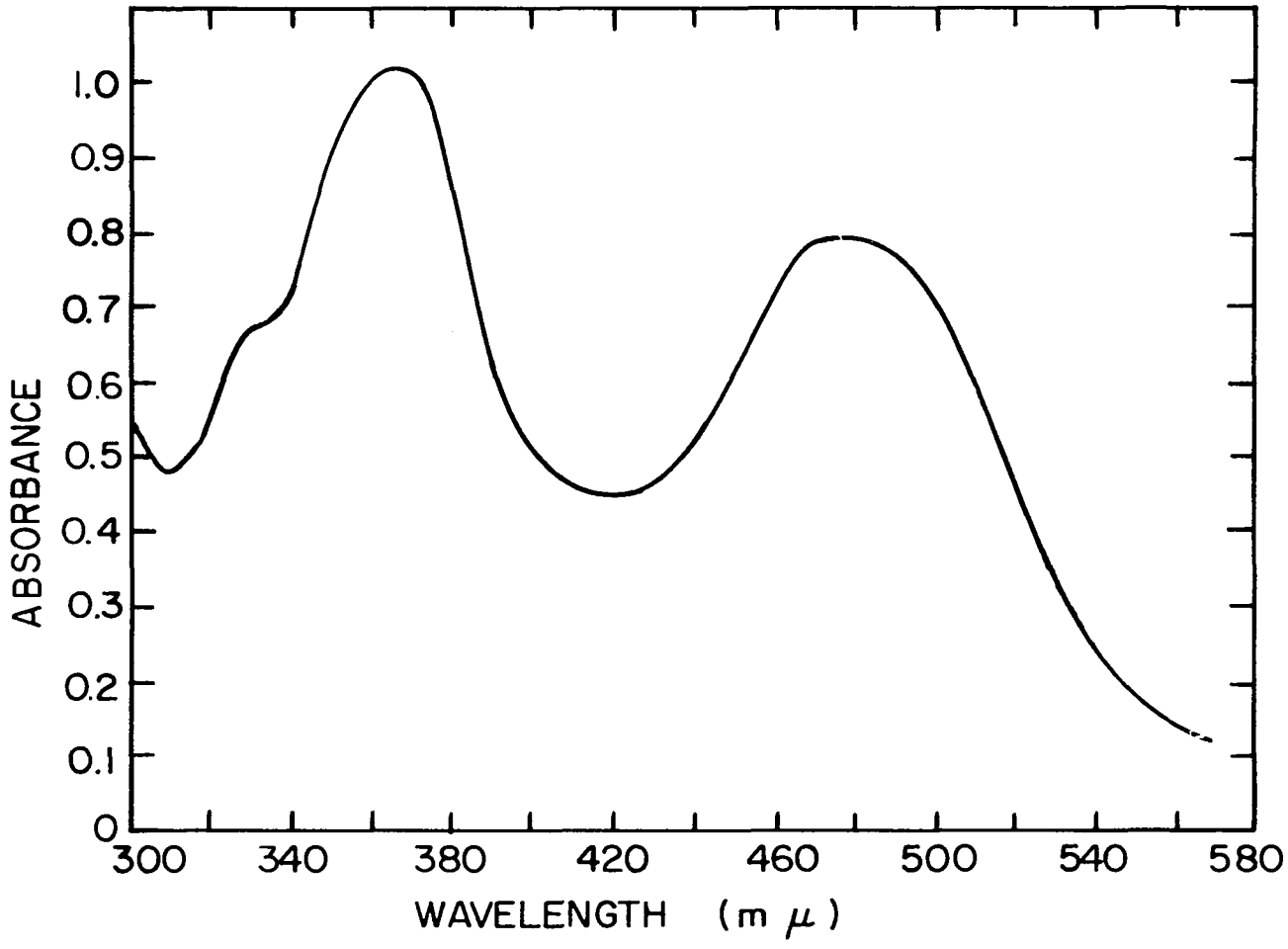
The spectrum of the semiquinone could not be calculated from the spectra obtained due to the spectra being concentration dependent. This dependence may be due to a rate effect or may be a true concentration effect. At the time of this writing, the problem has not been solved.

Oxidation-reduction reactions of leucoflavin

The oxidation of leucoflavin by oxidized compounds was not successful for β-naphthoate, cinnamate, orotate or α-lipoic acid.

These results, in conjunction with the substrate oxidation studies reported on an earlier section, show that many oxidation-reduction reactions of riboflavin are not suitable for non-enzymic studies.

Figure 30. Absorption spectrum of a photolyzed riboflavin solution in 2 N H_2SO_4. Concentration of riboflavin remaining = 7.45×10^{-5} M. Concentration of reduced flavin = 8.07×10^{-5} M


ABSORBANCE
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
300
340
380
420
460
500
540
580
WAVELENGTH (m μ)

SUMMARY

A quite thorough review has been made on flavin chemistry and related topics in order that the results of this dissertation and other current results can be put into a correct perspective.

The physical properties of riboflavin, viz., heat stability, light stability, and solubility have been reviewed. Riboflavin undergoes a first order heat decay in the dark to 1,2-dihydro-6,7-dimethyl-2-keto-1-(1'-D-ribityl)-3-quinoxaline carboxylic acid. This product is identical to the alkaline degradation product in which a molecule of urea is cleaved from the pyrimidine ring of riboflavin.

Under anaerobic conditions, riboflavin is photoreduced. This photoreduction is apparently due to riboflavin oxidizing its own polyhydric side chain and not due to photolysis of water. Lumiflavin (no hydroxy side chain) does not photoreduce but in the presence of alcohols can be made to photoreduce.

Dimethyl formamide has been found to be a suitable non-aqueous solvent for riboflavin. The interactions of metal ions or organic molecules with riboflavin have been studied. No evidence could be found for metal ion interactions except in the case of silver ion and cuprous ion. Polarography was used to attempt detection of possible metal ion or molec-

ular interactions. With the positive results being few and questionable, polarography was considered not to be useful for such experiments.

Attempted riboflavin, non-enzymic oxidations of amino acids were not successful. Small yields of ammonia were attributed to photoreactions.

The oxidation of fatty acid analogs by riboflavin was successful in only one case. The oxidation of ethyl Δ^3-dihydro-β-naphthoate by riboflavin gave the exclusive product, ethyl β-naphthoate. This is the first, riboflavin catalyzed, single product, non-enzymic oxidation reported.

The oxidation of ethyl Δ^3-dihydro-β-naphthoate was greatly catalyzed by light. Both the light and dark reactions yielded β-naphthoate. The photooxidation is a bimolecular one, first order in both ester and flavin.

The pH dependence of the photooxidation suggests that the reaction is influenced by the same properties which influence riboflavin fluorescence and by proton activation. At high and low pH the rate decreases similar to flavin fluorescence. At intermediate pH, the rate increases as pH is decreased, suggesting some form of proton activation.

The photooxidation is inhibited by various fluorescence quenchers and other compounds capable of being photooxidized, e.g., amines and alcohols. The rate of oxidation is slower in solutions of increasing ionic strength.

The various flavins carry out the photooxidation of the dihydronaphthoate at a rate in proportion to their fluorescence.

The photoreaction has a heat of activation of -2.25 kcal $mole^{-1}$, whereas the dark reaction has a value of +14.6 kcal $mole^{-1}$.

The isomeric esters, Δ^{1-} and Δ^{2-}, as well as the dihydroacids did not give the unique oxidation. Methyl hydrocinnamate was not photooxidized.

The proposed scheme of oxidation suggests that a photoexcited flavin-substrate complex rearranges to reduced flavin and oxidized substrate with the reduced flavin being subsequently oxidized by oxygen. The manner in which the oxidation occurs could not be ascertained. However, the mode of oxidation must be quite specific because the isomeric compounds and the monocyclic compound were not oxidized. The inverse effect of ionic strength and the effect of light suggest a possible radical reaction.

Other flavoenzyme substrates such as dihydroorotic acid, reduced α-lipoic acid, and glucose showed no oxidation or questionable oxidation. Dihydroorotic acid exhibited no photoreaction with riboflavin. The reverse reactions of the oxidized compounds, i.e., the leucoflavin reduction of the oxidized compounds showed no reaction either.

The 1-propyl-1,4-dihydronicotinamide-riboflavin system of Suelter and Metzler (41, 77) was examined under an-

aerobic conditions. The second order rate constant of the reaction yielding leucoflavin is 105 l $mole^{-1}sec^{-1}$. An equilibrium at a value of about 98% reduction of riboflavin assigns a reduction potential of about -0.3V (20°, pH 7) to nicotinamide-1-propochloride.

Leucoflavin was found to have a pK_a of 6.1 by spectrophotometric determinations. The spectra of the two acid-base forms for this pK have been reported for the first time. The spectra of leucoflavin in solutions of higher acidity have also been reported.

The air oxidation of leucoflavin was found to be similar to the air oxidation of dithionite prepared reduced riboflavin-5'-phosphate which had been reported previously.

The semiquinone form of riboflavin was prepared by two methods. The spectrum of the semiquinone form of riboflavin cannot be reported at this time since it was observed that the spectrum is concentration dependent even at 10^{-5} M.

ACKNOWLEDGMENTS

The author wishes to express his most sincere gratitude to Dr. David E. Metzler for suggesting the problems discussed in this dissertation and for his guidance and support throughout the course of this work. Also, the helpful discussions with Dr. Ernest Wenkert are acknowledged.

The author wishes to recognize the financial contrbution of The Ethyl Corporation in his final year of study.

Last, but not least, the author expresses the most heartfelt appreciation to his friends, relatives, and especially his mother for inspirational and materialistic assistance in this accomplishment.

BIBLIOGRAPHY

1. Blyth, W. A., J. Chem. Soc., 35, 530 (1879).

2. Kuhn, R., György, P., and Wagner-Jauregg, T., Ber., 66, 317 (1933).

3. Karrer, P., Schöpp, K., and Benz, F., Helv. Chem. Acta, 18, 426 (1935).

4. Kuhn, R., Reinemund, K., Weygand, F., and Ströbele, R., Ber., 68, 1765 (1935).

5. Beinert, H., Flavin Coenzymes, in Boyer, P.D., Lardy, H., and Myrbäck, K., eds., The Enzymes, Vol. 2, pp. 339-416, London, Academic Press. 1960.

6. Warburg, O. and Christian, W., Naturwissen., 20, 980 (1932).

7. _____ and _____, Biochem. Z., 257, 492 (1933).

8. Sebrell, W. H., Jr. and Harris, R. S., Eds., The Vitamins, Vol. 3, New York, Academic Press. 1954.

9. The Association of Vitamin Chemists, ed., Methods of Vitamin Assay, New York, Interscience Publishers, 1951.

10. Wagner-Jauregg, T., Riboflavin, Chemical Properties. In Sebrell, W. H., Jr. and Harris, R. S., eds. The Vitamins, Vol. 3. p. 307. New York, Academic Press. 1954.

11. Farrer, K. T. H., and MacEwan, J. L., Aus. J. Biol. Sci., 7, 73 (1954).

12. Shimizu, S., J. Fermentation Technol. (Japan), 28, 399 (1950). [Abstracted in Chem. Abst., 47, 1755e (1953).]

13. Warburg, O. and Christian, W., Biochem. Z., 266, 377 (1933).

14. Kuhn, R., György, P., and Wagner-Jauregg, T., Ber., 66, 1034 (1933).

15. Kuhn, R., Rudy, H., and Wagner-Jauregg, T., Ber., 66, 1950 (1933).

16. Karrer, P., Salomon, H., Schöpp, K., Schlittler, E., and Fritzsche, H., Helv. Chim. Acta, 17, 1010 (1934).

17. Kuhn, R. and Rudy, H., Ber., 67, 1936 (1934).

18. Karrer, P., Köbner, T., and Zehender, F., Helv. Chim. Acta, 19, 261 (1936).

19. Kuhn, R. and Rudy, H., Ber., 68, 300 (1935).

20. Fukamaiki, C. and Sakurai, Y., J. Vitaminology, 1, 217 (1955).

21. _____ and _____, Vitamins (Kyoto), 7, 939 (1954). [Abstracted in Chem. Abst., 51, 18026g (1957).]

22. _____ and _____, ibid. 7, 1014 (1954). [Abstracted in Chem. Abst., 51, 18026g (1957).]

23. Halwer, M., J. Am. Chem. Soc., 73, 4870 (1951).

24. Shimizu, S., J. Vitaminology, 1, No 2, 39 (1955).

25. Sakai, K., Nagoya J. Med. Sci., 18, 232 (1956). [Abstracted in Chem. Abst., 50, 11829b (1956).]

26. Yagi, K., Igaku to Seibutsugaku, 28, 54 (1953). [Abstracted in Chem. Abst., 48, 2141f (1954).]

27. Brdicka, R., Collection Czech. Chem. Communs., 14, 130 (1949).

28. Commoner, B. and Lippincott, B. B., Proc. Natl. Acad. Sci., 44, 1110 (1958).

29. Fridovich, I. and Handler P., J. Biol. Chem., 235, 1835 (1960).

30. Warburg, O. and Christian, W., Biochem. Z., 298, 150 (1938).

31. Stern, K. G. and Holiday, E. R., Ber., 67, 1104 (1934).

32. Daglish, C., Baxter, N., and Wokes, F., Quart. J. Pharm. Pharmacol., 21, 344 (1948).

33. Singer, T. P. and Kearney, E. B., Arch. Biochem. and Biophys., 27, 348 (1950).

34. Whitby, L. G., Biochem. J., 50, 433 (1952).

35. _____, ibid. 54, 437 (1953).

36. Yagi, K., Bull. Soc. Chim. France, 1543 (1957).

37. Kuhn, R. and Moruzzi, G., Ber., 67, 888 (1934).

38. Karrer, P. and Fritzsche, H., Helv. Chim. Acta, 19, 481 (1936).

39. Michealis, L., Schubert, M. P., and Smythe, C. V., J. Biol. Chem., 116, 587 (1936).

40. Albert, A., Biochem. J., 54, 646 (1953).

41. Suelter, C. H. and Metzler, D. E., Biochim. et Biophys. Acta, (in press).

42. Weber, G., Biochem. J., 47, 114 (1950).

43. Leonhardt, H. and Weller, A., Naturwissen., 47, 58 (1960).

44. Pringsheim, P., Fluorescence and Phosphorescence, New York, Interscience Publishers, Inc. 1949.

45. O'Malley, C. M. and Sievert, C. W., Ind. Eng. Chem., 34, 1117 (1942).

46. Karrer, P. and Ostwald, R., Rec. Trav. Chim., 57, 500 (1938).

47. Kuhn, R. and Wagner-Jauregg, T., Ber. 67, 361 (1934).

48. Michealis, L. and Hill, E. S., J. Am. Chem. Soc., 55, 1481 (1933).

49. Kuhn, R. and Ströbele, R., Ber., 70, 753 (1937).

50. Michealis, L. and Schwarzenbach, G., J. Biol. Chem., 123, 527 (1938).

51. Shimizu, S., J. Fermentation Technol. (Japan), 28, 139 (1950). [Abstracted in Chem. Abst., 47, 1755e (1953).]

52. Kuhn, R. and Moruzzi, G., Ber., 67, 1220 (1934).

53. Barron, E. S. G. and Hastings, A. B., J. Biol. Chem., 105, vii (1934).

54. Stare, F. J., J. Biol. Chem., 112, 223 (1935).

55. Kuhn, R. and Boulanger, P., Ber., 69, 1557 (1936).

56. Tachi, I., J. Agr. Chem. Soc. (Japan), 20, 401 (1944). Abstracted in Chem. Abst., 46, 1892d (1952).

57. Valentinuzzi, M., Cotino, L. E., and Portnoy, M., Anales soc. cient. argentina, 147, 45 (1949). Abstracted in Chem. Abst., 43, 7355g (1949).

58. Stern, K. G., Biochem. J., 28, 949 (1934).

59. Lowe, H. J. and Clark, W. M., J. Biol. Chem., 221, 983 (1956).

60. Lingane, J. J. and Davis, O. L., J. Biol. Chem., 137, 567 (1941).

61. Kaye, R. C. and Stonehill, H. I., J. Chem. Soc., 3244 (1952).

62. Ke, B., Arch. Biochem. and Biophys., 68, 330 (1957).

63. Beinert, H., J. Am. Chem. Soc. 78, 5323 (1956).

64. Burn, G. P. and O'Brien, J. R. P., Biochim. et Biophys. Acta, 31, 328 (1959).

65. Beinert, H., Hauge, J. G., and Page, E., Federation Proc., 15, 217 (1956).

66. Beinert, H., Biochim. et Biophys. Acta, 20, 588 (1956).

67. _____, J. Biol. Chem., 225, 465 (1957).

68. Haas, E., Biochem. Z., 290, 291 (1937).

69. Searls, R. L. and Sanadi, D. R., Biochem. Biophys. Res. Commun., 2, 189 (1960).

70. Searls, R. L., Federation Proc., 19, 36 (1960).

71. Massey, V., Biochem. J., 69, 58p (1958).

72. Gutfreund, H., Biochem. J., 74, 17p (1960).

73. Wellner, D. and Meister, A., J. Biol. Chem., 235, PC12 (1960).

74. Ehrenberg, A., Acta Chem. Scand., 14, 766 (1960).

75. Karrer, P., Schwarzenbach, G., Buchi, J., Fritzsche, H., and Solmssen, U., Helv. Chim. Acta, 20, 72 (1937).

76. Singer, T. P. and Kearney, E. B., J. Biol. Chem., 183, 409 (1950).

77. Suelter, C. H. and Metzler, D. E., Federation Proc., 18, 334 (1959).

78. Vernon, L. P., Biochim. et Biophys. Acta, 36, 177 (1959).

79. Frisell, W. R. and Mackenzie, C. G., Federation Proc., 17, 224 (1958).

80. _____ and _____, Proc. Natl. Acad. Sci., 45, 1568 (1959).

81. Dolin, M. I., Arch. Biochem. and Biophys., 60, 499 (1956).

82. Dolin, M. I., J. Biol. Chem., 225, 557 (1957).

83. Isaka, S. and Kato, J. J. Coll. Arts Sci. Chiba Univ., 1, 4350 (1932). [Abstracted in Chem. Abst., 49, 4737c (1955).]

84. Isaka, S. Ishida, S., J. Coll. Arts Sci. Chiba Univ., 1, 90 (1953). [Abstracted in Chem. Abst., 49, 9699a (1955).]

85. Shugar, D., Acta Biochim. Polon., 1, 3 (1954). [Abstracted in Chem. Abst., 49, 4052i (1955).]

86. Sakurai, Y. and Kuroki, M., Vitamins (Kyoto), 11, 473 (1956). [Abstracted in Chem. Abst., 51, 18026g (1957).]

87. Brauner, L., Naturwissen., 40, 23 (1953).

88. Brauner, L., Z. Botan., 41, 291 (1953). [Abstracted in Chem. Abst., 48, 5296a (1954).]

89. Brauner, L. and Brauner, M., Z. Botan., 42, 83 (1954). [Abstracted in Chem. Abst., 48, 6842h (1954).]

90. Mackenzie, C. G. and Frisell, W. R., J. Biol. Chem., 232, 417 (1958).

91. Frisell, W. R., Chung, C. W., and Mackenzie, C. G., J. Biol. Chem., 234, 1297 (1959).

92. Merkel, J. R. and Nickerson, W. J., Proc. Natl. Acad. Sci., 39, 1008 (1953).

93. _____ and _____, Biochim. et Biophys. Acta, 14, 303 (1954).

94. Galston, A. W., Proc. Natl. Acad. Sci., 35, 10 (1949).

95. _____ and Baker, R. S., Science, 109, 485 (1949).

96. _____, Science, 111, 619 (1950).

97. Meĭsel, M. N. and Dikanskaya, E. M., Doklady Akad. Nauk S. S. S. R., 85, 1377 (1952). [Abstracted in Chem. Abst., 47, 2258e (1953).]

98. Giri, K. V., Kalyankar, G. D., and Vaidyanathan, G. S., Naturwissen., 41, 88 (1954).

99. Krasnovskiĭ, A. A. and Gavrilova, V. A., Doklady Akad. Nauk S. S. S. R., 81, 1105 (1951). [Abstracted in Chem. Abst., 46, 4590b (1952).]

100. Martini, E., Boll. soc. ital. biol. sper., 9, 1235 (1934). [Abstracted in Chem. Abst., 29, 2580^2 (1935).]

101. Hand, D. B., Guthrie, E. S., and Sharp, P. F., Science, 87, 439 (1938).

102. Hopkins, F. G., Compt. rend. trav. lab. Carlsberg, Sér. chim. 22, 226 (1938). [Abstracted in Chem. Abst. 32, 62654 (1938).]

103. Numota, I., Science (Japan), 12, 218 (1942). [Abstracted in Chem. Abst., 45, 10329i (1951).]

104. Sakai, K., Nagoya J. Med. Sci., 18, 245 (1956). [Abstracted in Chem. Abst., 50, 11828b (1956).]

105. Hendriks, B. and Berends, W., Rec. Trav. Chim., 77, 145 (1958).

106. Fukumachi, C., Kuroki, M., and Sakurai, Y., Vitamins (Kyoto), 9, 221 (1955). [Abstracted in Chem. Abst., 51, 18026g (1957).]

107. Nyc, J. F., Friedgood, H. B., Garst, J. B., and Makon, D. M., J. Am. Chem. Soc., 75, 907 (1953).

108. Zondag, E., Posthuma, J., and Berends, W., Biochim. et Biophys. Acta, 39, 178 (1960).

109. Posthuma, J. and Berends, W., Biochim. et Biophys. Acta, 41, 538 (1960).

110. Hioki, H. and Sakurai, Y., Vitamins (Kyoto), 8, 380 (1955). [Abstracted in Chem. Abst., 51, 18026g (1957).]

111. Kolesnikov, P. A., Biochemistry, U. S. S. R., 23, 404 (1958).

112. Vernon, L. P. and Ihnen, E. D., Biochim. et Biophys. Acta, 24, 115 (1957).

113. Oster, G., Bellin, J. S., Kimball, R. W., and Schrader, M. E., J. Am. Chem. Soc., 81, 5095 (1959).

114. Sakai, K., Nagoya J. Med. Sci., 18, 222 (1956). [Abstracted in Chem. Abst., 50, 11828i (1956).]

115. Rutter, W. J., Acta Chem. Scand., 12, 438 (1958).

116. Weber, M. M., Lenhoff, H. M., and Kaplan, N. O., J. Biol. Chem., 220, 93, (1956).

117. Andreae, W. A., Arch. Biochem. and Biophys. 55, 584 (1955).

118. Blum., H. T., Physiol. Revs., 12, 23 (1932).

119. Arnow, L. E., ibid., 16, 671 (1936).

120. Schomer, H. A., The Effects of Radiation on Enzymes. In Duggar, B. M., ed. Biological Effects of Radiation, Vol. 2, pp. 1151-1165, New York, McGraw-Hill Book Company, Inc. 1936.

121. Gaffron, H., Biochem. Z., 184, 453 (1927).

122. Harris, D. T., Biochem. J., 20, 288 (1926).

123. Carter, C. W., ibid., 22, 575 (1928).

124. Harden, A. and Norris, R. V., ibid., 9, 330 (1915).

125. Dixon, M. and Tunnicliffe, H. E., Proc. Roy. Soc. Lond., B, 94, 266 (1923).

126. Lieben, F., Biochem. Z., 184, 453 (1927).

127. Windaus, A. and Brunken, J., Ann. Chem., 460, 225 (1928).

128. _____ and Borgeaud, P., ibid., 460, 235 (1928).

129. Weber, K., Z. Phys. Chem., B, 15, 18 (1931).

130. Martini, E., Biochim. terap. sper., 20, 505 (1933). [Abstracted in Chem. Abst., 28, 34567 (1934).]

131. Weiss, J., Nature, 136, 794 (1935).

132. _____, Naturwissen., 23, 610 (1935).

133. Rabinowitch, E., J. Chem. Phys., 8, 551 (1940).

134. _____, ibid., 560.

135. Ainsworth, S. and Rabinowitch, E., Science, 131, 303 (1960).

136. Weil, L., Science, 107, 426 (1948).

137. _____ and Maher, J., Arch. Biochem. and Biophys., 29, 241 (1950).

138. _____ and _____, Federation Proc., 9, 244 (1950).

139. _____, Gordon, W. G., and Buchert, A. R., Arch. Biochem. and Biophys., 33, 90 (1951).

140. Ferri, M. G., Nature, 168, 334 (1951).

141. _____, Arch. Biochem. and Biophys., 31, 127 (1951).

142. Kutsaya, B. F. and Dain, B. Ya., Ukrain. Khim. Zhur., 17, 820 (1951). [Abstracted in Chem. Abst., 49, 13789c (1955).]

143. Imamura, M. and Koizumi, M., Bull. Chem. Soc. Japan, 28, 117 (1955).

144. Schenck, G. O., Naturwissen., 40, 205 (1953).

145. Koizumi, M. and Obata, H., Bull. Chem. Soc. Japan, 30, 136 (1957).

146. Obata, H. and Koizumi, M., ibid., 142.

147. Oster, G. and Wotherspoon, N., J. Am. Chem. Soc., 79, 4836 (1957).

148. Oster, G. K. and Oster, G., J. Am. Chem. Soc. 81, 5543 (1959).

149. Pestemer, M., Z. Electrochem., 58, 121 (1959).

150. Millich, F. and Oster, G., J. Am. Chem. Soc., 81, 1357 (1959).

151. Mauzerall, D., J. Am. Chem. Soc., 82, 1832 (1960).

152. Broyde, B. and Oster, G., J. Am. Chem. Soc. 81, 5099 (1959).

153. Buckley, D., Dunstan, S., and Henbest, H. B., J. Chem. Soc., 4880 (1957).

154. Horner, L. and Spietschka, W., Ann. Chem., 591, 1 (1955).

155. Treibs, W., Steinert, R., and Kirchhof, W., Ann. Chem., 581, 54 (1953).

156. Barclay, B. M. and Campbell, N., J. Chem. Soc., 530 (1945).

157. Dimroth, O., Angew. Chem., 46, 571 (1933).

158. Dost, N. and van Nes, K., Rec. trav., chim., 70, 403 (1951).

159. Dost, N., ibid., 71, 857 (1952).

160. Braude, E. A. Jackman, L. M. and Linstead, R. P., J. Chem. Soc., 3548 (1954).

161. _____, _____, and _____, ibid., 3564.

162. Braude, E. A., Brook, A. G., and Linstead, R. P., J. Chem. Soc., 3569 (1954).

163. Kenner, J., Tetrahedron, 3, 78 (1958).

164. Braude, E. A., Brook, A. G., and Linstead, R. P., J. Chem. Soc., 3574 (1954).

165. Uehara, K., Muramatsu, A., and Makita, M., J. Vitaminology, 2, 44 (1955).

166. Weil-Malherbe, H., Biochem. J., 40, 363 (1946).

167. Weber, G., Trans. Far. Soc. 44, 185 (1948).

168. Yagi, K. and Matsuoka, Y., Kagaku, 23, 216 (1953). [Abstracted in Chem. Abst., 49, 11368i (1955).]

169. _____ and _____, ibid., 258. [Abstracted in Chem. Abst., 49, 11368i (1955).]

170. _____ and _____, Biochem. Z., 328, 138 (1956).

171. Yagi, K., Okuda, J., Ozawa, T., and Okada, K., Biochem. Z., 328, 492 (1957).

172. Yagi, K., Biochim. et Biophys. Acta, 35, 102 (1959).

173. Dietrich, L. S. and Harland, B. F., J. Biol. Chem., 219, 383 (1956).

174. Harbury, H. A. and Foley, K. A., Proc. Natl. Acad. Sci., 44, 662 (1958).

175. Harbury, H. A., LaNoue, K. F., Loach, P. A., and Amick, P. M., ibid., 45, 1708 (1959).

176. Isenberg, I. and Szent-Györgyi, ibid., 44, 857 (1958).

177. _____ and _____, ibid., 45, 1229 (1959).

178. Karreman, G., Isenberg, I., and Szent-Györgyi, A., Science, 130, 1192 (1959).

179. Searls, R. L., Bethesda, Maryland. Results of Experiments. Private Communication. 1960.

180. Commoner, B., Heise, J. J., Lippincott, B. B., Norberg, R. E., Passonneau, J. V., and Townsend, J., Science, 126, 57 (1957).

181. Commoner, B., Lippincott, B. B., and Passonneau, J. V. Proc. Natl. Acad. Sci., 44, 1099 (1958).

182. Commoner, B. and Hollocher, T. C., Jr., Proc. Natl. Acad. Sci., 46, 405 (1960).

183. Hollocher, T. C., Jr. and Commoner, B., ibid., 46, 416 (1960).

184. _____ and _____, Federation Proc. 19, 28 (1960).

185. Ehrenberg, A. and Ludwig, G. D., Science, 127, 1177 (1958).

186. Grabe, B., Expt. Cell Res., 13, 588 (1957).

187. Grabe, B., Biochim. et Biophys. Acta, 30, 560 (1958).

188. Grabe, B., Arkiv Physiks, 17, 97 (1960).

189. Pullman, B. and Pullman, A., Proc. Natl. Acad. Sci., 44, 1197 (1958).

190. _____ and _____, ibid., 45, 136 (1959).

191. _____ and _____, Compt. rend. soc. chim. biol., 248, 149 (1959).

192. Frost, D. V., J. Am. Chem. Soc., 69, 1064 (1947).

193. Wagner-Jauregg, T., Riboflavin, Chemical Properties. In Sebrell, W. H., Jr. and Harris, R. S., eds. The Vitamins, Vol 3. p. 306. New York, Academic Press. 1954.

194. Sakai, K., Nagoya J. Med. Sci., 18, 237 (1956). [Abstracted in Chem. Abst., 50, 12399a (1956).]

195. Albert, A. and Brown, D. J., Intern. Congr. Biochem., Abstrs. of Commun. 1st Congr., Cambridge, Engl. 1949, 240. [Abstracted in Chem. Abst., 47, 11209b (1953).]

196. Albert, A., Biochem. J., 47, xxvii (1950).

197. Harkins, T. R. and Freiser, H., J. Phys. Chem., 63, 309 (1959).

198. Hemmerich, P. and Fallab, S., Helv. Chim. Acta, 41, 498 (1958).

199. Kuhn, R. and Bor, F., Ber., 67, 898 (1934).

200. Baarda, I. F., Silver Complexes of Riboflavin and Related Compounds. Unpublished M.S. Thesis. Ames, Iowa, Iowa State University of Science and Technology Library. 1959.

201. Foye, W. O. and Lange, W. E., J. Am. Chem. Soc., 76, 2199 (1954).

202. Mahler, H. R., Fairhurst, A. S. and Mackler, B., J. Am. Chem. Soc., 77, 1514 (1955).

203. Walaas, E., Acta. Chem. Scand., 11, 1082 (1957).

204. _____, ibid., 12, 528 (1958).

205. Hemmerich, P., Fall, S., and Erlenmeyer, H., Helv. Chim. Acta, 39, 1242 (1956).

206. Hemmerich, P., Prijs, B., and Erlenmeyer, H., Helv. Chim. Acta, 42, 1604 (1959).

207. Hemmerich, P. and Erlenmeyer, H., Helv. Chim. Acta, 40, 180 (1957).

208. Hemmerich, P., Helv. Chim. Acta, 41, 514 (1958).

209. Bamberg, P., Hemmerich, P., and Erlenmeyer, H., Helv. Chim. Acta, 43, 395 (1960).

210. Hemmerich, P., Prijs, B., and Erlenmeyer, H., Helv. Chim. Acta, 43, 372 (1960).

211. _____, _____, and _____, ibid., 372.

212. Wessels, J. S. C., Rec. trav. chim., 73, 529 (1954).

213. Harrison, K., Nature, 181, 1131 (1958).

214. Wieland, T. and Patterman, F., Angew. Chem., 70, 313 (1958).

215. Clark, V. M., Kirby, G. W., and Todd, A., Nature, 181, 1650 (1958).

216. Clark, V. M., Hutchinson, D. W., and Todd, A., Nature 187, 59 (1960).

217. Lindberg, O., Grabe, B., Löw, H., Siekevitz, P., and Ernster, L., Acta Chem. Scand., 12, 598 (1959).

218. Links, J., Biochim. et Biophys. Acta, 38, 193 (1960).

219. Slater, E. C., Rev. Pure and Applied Chem., 8, 221 (1958).

220. Todd, A., Gazz. chim. ital., 89, 126 (1959).

221. Huennekens, F. M. and Mahler, H. R., Biological Oxidations, New York, Academic Press, in Press.

222. Sutton, W. B., J. Biol. Chem., 226, 395 (1957).

223. Datta, P. K. and Meeuse, B. J. D., Biochim. et Biophys. Acta, 17, 602 (1955).

224. Friedman, H. C. and Vennesland, B., Federation Proc. 17, 224 (1958).

225. _____ and _____, J. Biol. Chem., 235, 1526 (1960).

226. Massey, V., Biochem. J., 69, 58p (1958).

227. _____, Biochim. et Biophys. Acta, 30, 205 (1958).

228. _____, Biochem. J., 72, 7p (1959).

229. Searls, R. L. and Sanadi, D. R., Proc. Natl. Acad. Sci., 45, 698 (1959).

230. Massey, V., Biochim. et Biophys. Acta, 37, 314 (1960).

231. Levy, H. R. and Talalay, P., J. Am. Chem. Soc., 79, 2658 (1957).

232. Tata, J. R., Biochim. et Biophys. Acta, 35, 567 (1959).

233. Nishizuka, Y, Kuno, S., and Hayaishi, O., J. Biol. Chem., 235, PC 13 (1960).

234. Spatz, S. M. and Stone, H., J. Org. Chem., 23, 1559 (1958).

235. Gunsalus, I. C., Barton, L. S., Gruber, W., J. Am. Chem. Soc., 78, 1763, (1956).

236. Holman, W. I. M. and Wiegand, C., Biochem. J., 43, 423 (1948).

237. Gilman, H. and Moore, F. W., J. Am. Chem. Soc., 62, 1843 (1940).

238. Derick, C. G. and Kamm, O., J. Am. Chem. Soc., 38, 400 (1916).

239. Schrecker, A. W., Greenberg, G. Y., and Hartwell, J. L., J. Am. Chem. Soc., 74, 5669 (1952).

240. Auwers, K. and Müller, K., J. Prakt, Chem., 2, 109, 124 (1925).

241. Cresswell, R. M., Hill, A. C., and Wood, A. C. S., J. Chem. Soc., 698 (1959).

242. Fall, H. H. and Petering, H. G., J. Am. Chem. Soc., 78, 377 (1956).

243. Souchay, P. and Fauchere, J., Bull. Soc. Chim. France 1947, 529 (1947).

244. Johnson, M. J., J. Biol. Chem., 137, 575 (1941).

245. Hestrin, S., J. Biol. Chem. 180, 249 (1949).

246. Kuhn, R. and Rudy, H., Ber., 67, 894 (1934).

247. Surrey, A. R. and Nachod, F. C., J. Am. Chem. Soc., 73, 2336 (1951).

248. Braunshtein, A. E., Adv. in Prot. Chem., 3, 1 (1947).

249. Kuhn R. and Moruzzi, G., Ber., 67, 888 (1934).

250. Kavanagh, F. and Goodwin, R. H. Arch. Biochem., 20, 315 (1949).

251. Ellinger, P. and Holden, M., Biochem. J., 38, 147 (1944).

252. Weiss, J., Trans. Faraday, Soc., 32, 1331 (1936).

253. _____, Nature 181, 825 (1958).

254. Wallenfels, K. and Diekmann, H., Ann. 621, 166 (1959).