STUDIES IN THE CHEMISTRY OF FLAVINS AND FLAVOPROTEINS

A thesis submitted for the degree of

Doctor of philosophy by

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ABSTRACT

This thesis is concerned with properties of the excited states of flavins. They may be involved in some photobiological processes and are models for flavoprotein catalysis. Understanding the spectral properties of flavins is important for interpreting the nature of intermolecular interactions in flavoproteins.

The absorption spectra of many flavin derivatives are similar, but the two long wavelength bands are sensitive to both solvent and substituents. Two independent methods for finding the angle between these absorption oscillators are described. The first, measurement of the fluorescence polarisation of a random but rigid array of the chromophore, is a well known technique. The second is novel, and requires the preparation of a partially ordered solution of flavin by selective bleaching with plane polarised light of wavelengths absorbed by only one of the absorption bands. The theory of this technique is developed, and it shows that the angle between the absorption oscillators can be calculated from the dichroic ratios of the extinctions of the two bands after bleaching. This angle does not vary much for different flavins, and the same value (around 30°) is found from both fluorescence polarisation and dichroic bleaching. The

directions of the oscillators relative to the axes of the molecule cannot be measured by either technique, but the effects of substituents on absorption spectra suggest that the visible band is polarised approximately parallel to the long axis of the flavin.

The first excited singlet of a flavin loses some of its energy by emission as fluorescence, but it also participates in singlet-to-singlet energy transfer. This is demonstrated by fluorescence for two flavin derivatives, using a methyl cellulose film as the supporting matrix. Optical observations are possible under these conditions at higher flavin concentrations than are easily studied in liquid solutions.

Excited singlets may also lose energy by intersystem crossing to the lowest triplet state, but no delayed light emission can be detected from aqueous flavin solutions. In a rigid polyacrylamide matrix, however, there <u>is</u> delayed emission, even at room temperature. This is identified as E-type delayed fluorescence on the basis of its spectrum, its temperature and its exciting light intensity dependence. At lower temperatures the delayed fluorescence decreases, and phosphorescence emission dominates.

The intensities and lifetimes of the delayed emissions

of 12 flavins have been measured in the range 77°-315°K. On plotting the logarithm of intensity or of the reciprocal of half-life (decay is exponential) against the reciprocal of temperature biphasic Arrhenius plots are obtained. These can be interpreted in terms of the activation energies of the various rate processes associated with triplet production and inactivation. The activation energies from delayed fluorescence intensities and half-lives (5.9 and 2.6 kcal/mole respectively for FMN) add to a value close to the energy separating the spectroscopic fluorescence and phosphorescence maxima. A kinetic scheme is proposed to account for this. The activation energy for non-radiative decay of the triplet to the ground state can be calculated from the temperature dependence of phosphorescence, and values of about 100 cal/mole are found.

Photochemical reactions of the flavin triplet both in the presence and absence of oxygen have been studied. Anaerobically flavins photo-oxidise many amino acids and amines in their high pH forms, with production of dihydroflavin. When DL-phenylglycine is the reducing agent equimolar amounts of benzaldehyde, carbon dioxide and dihydroflavin are produced. The dependence of reaction rate on reducing agent concentration shows saturation behaviour at high concentrations, which is consistent with a steady state kinetic treatment for

the reaction. The sensitivity of rates to substituents in the DL-phenylglycine can be described by a Hammett ρ-value of-1.1. On the basis of their photoreactivities towards a series of flavin derivatives it is suggested that DL-phenylglycine and benzylamine react with a similar rate determining step (probably hydrogen abstraction). This is different from the photoaddition of phenylacetate.

Anaerobic photoreductions are quenched by some aromatic compounds. From the effects of temperature and by comparison with quenching by potassium iodide it is concluded that inhibition is not simply a collisional process. FAD reacts more slowly in photoreduction than FMN, but in concentrated urea solutions the difference is reduced. Inhibition by aromatic compounds is also reduced under these conditions. This suggests that the forces between flavin triplet and quencher are at least partially hydrophobic.

Aerobic flavin photochemistry is followed by measuring oxygen uptake. Two general types of behaviour are found for oxygen concentrations from 2 μM upwards. Either the reaction rate increases as oxygen is consumed (type I) or it falls (type II). The type I reaction pathway is slow photoreduction followed by rapid reoxidation of dihydroflavin. Evidence for this comes from the dependence of reaction rate on oxygen concentration and pH, and from the Hammett ρ-value for

substituted phenylglycines which is similar to that of anaerobic photoreduction. Oxygen is also a triplet quencher.

Rate constants for the reduction and quenching of FMN triplets by EDTA, diethanolamine and DL-phenylglycine (all type I substrates) are deduced from the dependence of reaction rates on oxygen concentration. In each case quenching is over 10 times as efficient as reaction. The rate constants for all three substrates are similar, increasing in the order DL-phenylglycine < EDTA < diethanolamine. The values for EDTA are $1.38.10^7$ M^{-1} sec^{-1} for reaction and $1.6.10^8$ $M^{-1}sec^{-1}$ for quenching. These rate constants can be used to predict a concentration dependence of anaerobic photoreduction which agrees well with experiment. The most likely mechanism for quenching is a rapidly reversed electron transfer.

Whereas type I substrates are reducing agents for flavin triplets, type II substrates are typically aromatic molecules which quench anaerobic photoreduction. Tryptophan, for example, has type II kinetics and reacts by a complex mechanism in which product inhibition of reaction occurs.

The potential of flavin photosensitized destruction of amino acids as a method for selective chemical modification of proteins is investigated. Tryptophan, tyrosine, histidine, methionine, cysteine and phenylalanine all react but none of

them has the same pH-profile of reactivity. Preliminary experiments on two proteins, L-glutamate dehydrogenase and D-amino acid oxidase show that the method has promise.

An investigation of the behaviour of flavins when they act as Hill oxidants with isolated spinach chloroplasts has also been made. In this reaction light is absorbed by the chloroplast and flavin reacts in its ground state. The rates of reduction of 10 derivatives have been compared. The order of reactivity is different from that found for some model systems: dark reduction by NADH or dihydrolipoic acid, the redox potentials, and photoreduction by DL-phenylglycine. The rates are independent of flavin concentration, but vary with the concentration of chloroplasts and light intensity. It is suggested that the reactivity/to reduction by chloroplasts depends on binding between chloroplasts and flavin as well as on the electronic character of the flavin. The partition coefficients of flavins between water and butan-1-ol lend some support to this hypothesis.

NON STANDARD ABBREVIATIONS AND NOMENCLATURE

APS	ammonium persulphate
BIS	N,N'-methylenebisacrylamide
CMU	3-(p-chlorophenyl)-1,1-dimethylurea
DCMU	3-(2,4-dichlorophenyl)-1,1-dimethylurea
GDH	L-glutamate dehydrogenase
isoriboflavin	5,6-dimethyl,9-ribitylisoalloxazine
lumichrome	6,7-dimethylalloxazine
lumiflavin	6,7,9-trimethylisoalloxazine
TEMED	N,N,N',N'-tetramethylethylenediamine

CHAPTER ONE

General Introduction

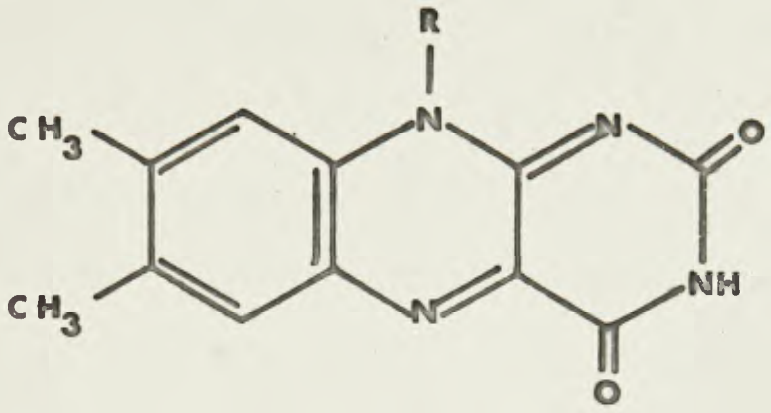
Discovery and Identification of Riboflavin

Pure riboflavin was first identified as a vitamin and isolated (from whey) by Kuhn, Gyorgyand Wagner-Jauregg in 1933. Its structure was deduced from its elemental composition and chemical reactivity, and was finally confirmed when first lumiflavin (Kuhn, Reinemund and Weygand, 1934) and then riboflavin (Karrer, Schöpp and Benz, 1935) were synthesised. The structures of riboflavin, FMN, FAD, and lumiflavin are shown in figure 1-1. It was quickly realised that riboflavin could be obtained from many other natural sources (egg white, urine, etc.) and that a partial purification had been reported as early as 1879 (Blyth, 1879).

At the same time as the first purification of riboflavin Warburg and Christian were isolating from yeast a yellow enzyme with oxidising properties (Warburg and Christian, 1932). The product of alkaline photodegredation of this 'old yellow enzyme' was identical to the product of photodegredation of riboflavin. This compound was subsequently found to have the formula of lumiflavin.

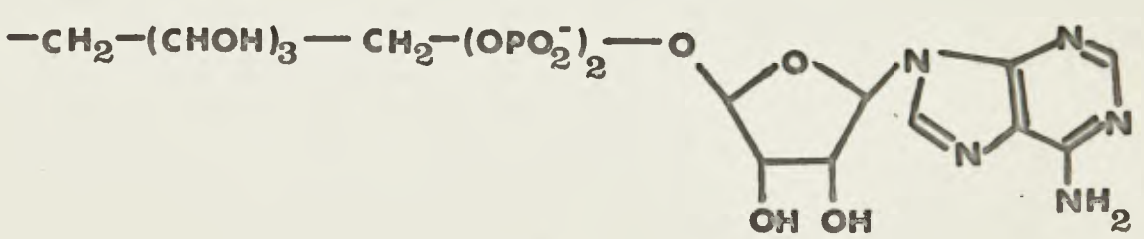
It is now realised that riboflavin, although it occurs free in whey, urine and the retina, is generally present as either FMN or FAD inside living cells (Wagner-Jauregg, 1954).

Fig. 1-1 ; Structures of the most common flavins



R is: $-CH_2-(CHOH)_3-CH_2OH$ (D-ribityl) for riboflavin

$-CH_2-(CHOH)_3-CH_2-OPO_3^{2-}$ for FMN



$-CH_3$ for lumiflavin

These two nucleotides are the prosthetic groups of the many flavoproteins which have now been identified.

Biological Function

Riboflavin (vitamin B_2) is found in the water soluble, heat stable fraction of the vitamin B complex. Its natural action is closely associated with the function of flavin nucleotides in biochemical oxidations. FMN and (more often) FAD function as coenzymes in the oxidation of amino acids, amines, carboxylic acids, reduced nicotinamide nucleotides, thiol groups, etc. Riboflavin deficiency has a very widespread effect on all mammals, greatly inhibiting growth (Horwitt, 1954). This is consistent with the coenzymes being vital for efficient functioning of the mitochondrial electron transport chain. In addition flavins play a part in bacterial dehydrogenation and in nitrate assimilation by fungi and plants, and they are implicated in other systems like that of thyroid iodination (Beinert, 1960).

Flavins can also influence several photobiological processes, though not necessarily through one of their own photochemical reactions. Thus when FMN acts as a cofactor for cyclic photophosphorylation by isolated chloroplasts (Whatley, Allen and Arnon, 1959) it probably acts in its ground state as an electron carrier. FMN enhances bacterial bioluminescence (McElroy and Seliger, 1963), and

may be a photoreceptor in phototropism of plants (Thimann and Curry, 1960). Crystalline riboflavin is present in the eyes of some fish and mammals, but its function is not understood (Pirie, 1960).

Chemistry

The chemical studies on flavins fall into 4 groups; A) spectral properties, B) photochemistry, C) formation of intra- and intermolecular complexes, and D) other chemical and physical properties. A) is covered in the introductions to chapters 2 and 3; B) is covered in the introductions to chapters 4 and 5. C) and D) will be briefly considered here.

D) Chemical and physical properties. Strictly riboflavin is not a nucleoside, nor are FMN and FAD nucleotides, because the sugar side-chain is not linked to the rest of the molecule by a glycosidic bond. The side-chain at N_9 is thus stable to acid hydrolysis, and riboflavin can be crystallised from acid solution as fine yellow needles. These decompose when heated to about 280° (Wagner-Jauregg, 1954). Riboflavin and its nucleotides are water soluble, and dissolve to a lesser extent in alcohols and other organic solvents. Some derivatives with less hydrophylic substituents at N_9 are more soluble in organic solvents than in water.

The electronic absorption spectrum of riboflavin has

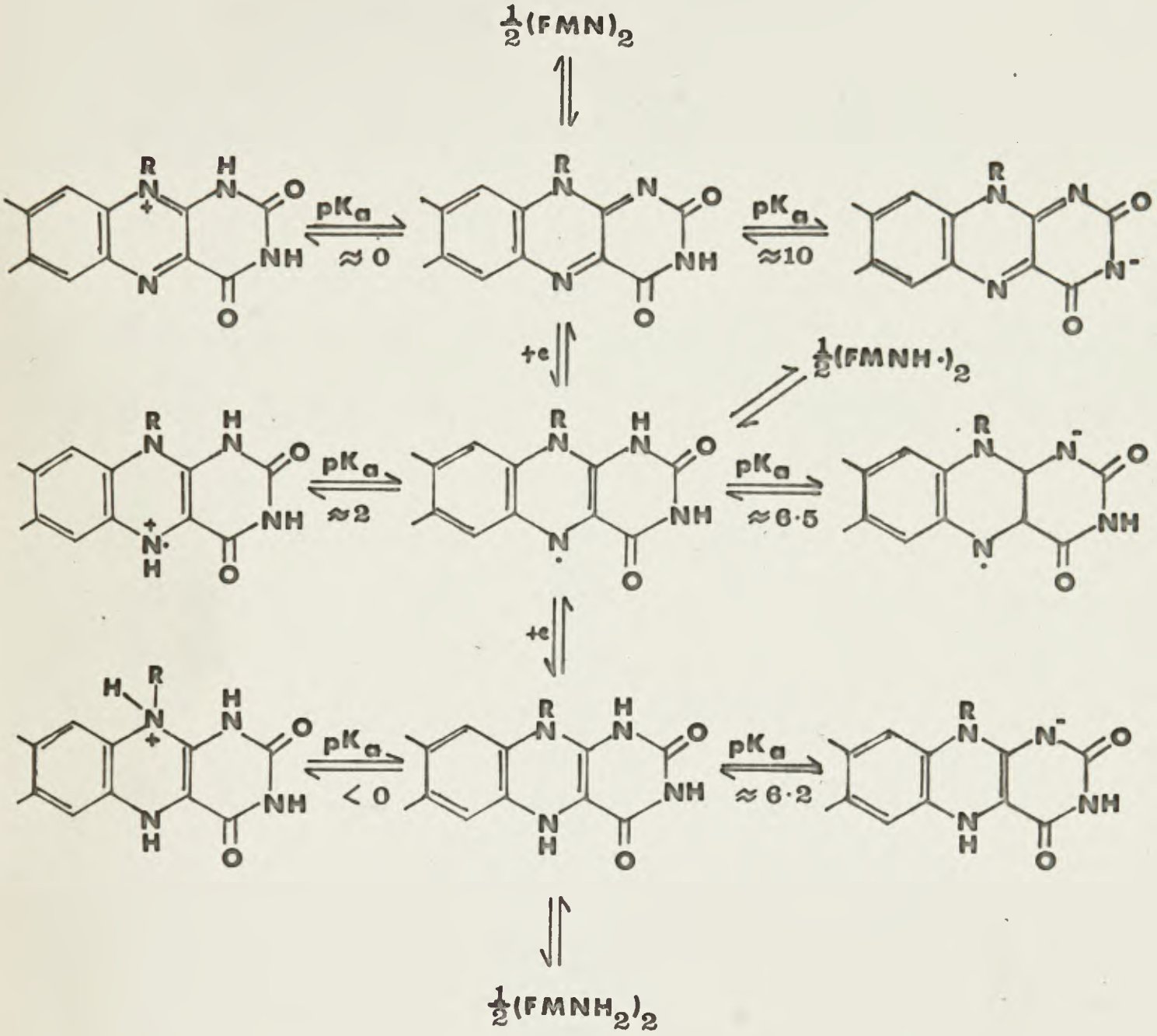
maxima at 220, 265, 375, and 447mμ (see chapter 2). Flavins fluoresce strongly at room temperature (with a maximum at 530mμ for riboflavin) and phosphoresce in glasses at low temperatures (with a maximum at 600-610mμ for riboflavin - see chapter 3). Riboflavin has little optical rotation in the visible region of the spectrum except under alkaline conditions (Kuhn, Rudy and Weygand, 1935). The optical rotatory dispersion curves and the circular dichroism spectra of flavins are the subject of disagreement, at least partly because of artefacts obtained using one instrument (see appendix 1). It seems most probable that there is no Cotton effect associated with the 447mμ absorption, but that there is one at 375mμ.

In solution riboflavin is photosensitive so care must be taken that its properties are studied in the absence of light. In the dark it is stable to air, acids, and most oxidising agents (chromic acid, permanganate, and potassium persulphate are exceptions), but strong alkali decomposes it (Wagner-Jauregg, 1954). Riboflavin is reduced to colourless dihydroriboflavin by a variety of reducing agents, including sodium dithionite, catalytic hydrogenation, zinc and HCl. In the presence of oxygen the reduced compound is rapidly reoxidised to riboflavin and H_2O_2 (Rutter, 1958).

On the basis of potentiometric titrations and magnetic

measurements several authors concluded that reduction of riboflavin occurs in two distinct one-electron steps through the semiquinone intermediate (see fig.1-2) (Beinert, 1960). Two absorption bands are observed during reduction at 570 and 900mμ (Beinert, 1956), and electron spin resonance shows the presence of radicals in solutions of partially reduced FMN (Beinert and Sands, 1961). The extinction at 570mμ is related to the resonance signal, and is due to absorption by the free radical (Gibson, Massey and Atherton, 1962). On the basis of its temperature and concentration dependence the broad absorption band at 900mμ has been attributed to a complex of semiquinones with each other or with other components of the solution (Beinert, 1956). Gibson, Massey and Atherton, however, think it is a charge-transfer absorption of an FMN-$FMNH_2$ complex. They found that the radical concentration was greatest at about 70% reduction of FMN, which can be explained if there is a complex between the semiquinone and reduced flavin. Some of the redox and dimerisation equilibria which are present in aqueous solutions of flavins are summarised in figure 1-2. The rates of several of the processes shown have been measured by fast reaction techniques, flash photolysis (Holmström, 1964), stopped flow (Gibson and Hastings, 1962),

Fig. 1-2 ; Redox and acid-base equilibria of flavins



$$FMN + FMNH_2 \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \underset{\text{transfer}}{\overset{\text{charge}}{FMN - FMNH_2}} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} 2\,FMNH\cdot$$

$$FMNH_2 + FMNH\cdot \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} (FMNH_2.FMNH\cdot)$$

and temperature-jump relaxation (Swineheart, 1965).

The different acid-base forms of the three redox states of flavins have been studied by their absorption spectra, and (for the radical) by electron spin resonance (Ehrenberg, 1964; Ehrenberg, Müller and Hemmerich, 1967). The best resolved resonance spectrum is that of the anion radical, and by use of isotopic substitution it has been shown that most spin density in the lumiflavin anion is on N_{10} and, to a smaller degree, on C_7. The absorption spectrum of the flavin radical when it is bound to a flavoprotein sometimes corresponds to the neutral and sometimes to the anionic form of the unbound radical (Massey and Palmer, 1966).

The positions at which flavins participate in biochemical electron transfer depend on their redox potentials. Free FMN, FAD, riboflavin, and lumiflavin have nearly equal potentials (about -0.20v) at pH 7 (Clark, 1960). The potential varies with changes in substitution, and on binding to macromolecules (Clark, 1960; Penzer and Radda, in press). In flavoproteins the potential may be shifted in either direction, e.g. +0.067v for FMN on binding to the apoprotein of the old yellow enzyme, and -0.153 for FAD on lipoyl dehydrogenase.

The kinetics of reduction of flavins by sodium dithionite

(Burn and O'Brien, 1959), NADH (Singer and Kearney, 1950; Suelter and Metzler, 1960; Radda and Calvin, 1964), and dihydrolipoic acid (Gascoigne and Radda, 1967) have been studied. For NADH and dihydrolipoic acid reduction proceeds by a single two-electron step, but no conclusion on this point was reached for the dithionite reaction.

The autoxidation of reduced flavins has been followed by rapid reaction techniques and the results suggest two simultaneous mechanisms (Gibson and Hastings, 1962). In the first the flavin semiquinone reacts with oxygen by a free radical path, while the competing process involves a direct two-electron oxidation through a flavin peroxide (fig.1-3). Chemical evidence for the hydroperoxide intermediate is that N_{10}- substituted dihydroflavins are resistant to autoxidation while N_9- substituted ones are very sensitive (Berends, Posthuma, Sussenbach and Mager, 1966).

Reduced flavins are not easily autoxidised when bound to proteins of the mitochondrial electron-transport chain but they are reactive in many other flavo^proteins. There is no satisfactory explanation for this.

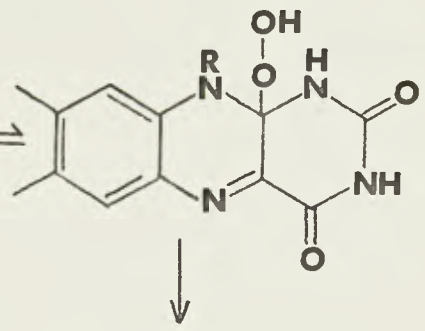
Reduced FMN and oxygen together can hydroxylate aromatic compounds (Mager and Berends, 1966). There is disagreement about the nature of the hydroxylating radical in this system, but it could be the flavin hydroperoxide.

Fig. 1 - 3

Autoxidation mechanisms

a) $FMN + FMNH_2 \rightleftharpoons 2\ FMNH^{\bullet}$

$FMNH^{\bullet} + \frac{1}{2}\ O_2 \longrightarrow FMN + \frac{1}{2}\ H_2O_2$

b) $FMNH_2 + O_2 \rightleftharpoons$ 

$\downarrow$

$FMN + H_2O_2$

One other striking feature of the chemistry of flavins is that the hydrogens of the methyl group at C_7 are active to a similar degree to those in p-nitrotoluene. They are exchanged in D_2O (Bullock and Jardetzky, 1965), condense with aromatic aldehydes (Hemmerich, Veeger, and Wood, 1965), and undergo oxidative dimerisation under non-solvolytic basic conditions (Ehrenberg, Müller and Hemmerich, 1967).

C) Formation of intra- and intermolecular complexes. The interactions of flavins with a number of organic substances have been known for many years. Their biochemical significance lies in two observations, that the flavin and adenine parts of FAD are in close intramolecular association and that the aromatic amino acids are among the substances capable of interacting with FMN or riboflavin. The former observation may be relevant to the coenzymic activity of FAD while the latter may partly explain the mode of interaction of flavins with proteins.

Evidence for the formation of intra- and intermolecular complexes will be discussed first. The absorption spectrum of FAD is different from those of riboflavin and FMN. The molar extinction coefficient of FAD at 260mμ is lower than the sum of the extinction coefficients of riboflavin and adenosine at this wavelength (Weber, 1950). There is also

a decrease in absorption at 375 and 450mμ, the latter being accompanied by a broadening of the band (Weber, 1950). Similar spectral changes are caused by amino acids such as tryptophan (Isenberg and Szent-Györgyi, 1958), or by purines and pyrimidines (Weber, 1950). These spectral shifts have often been recorded by difference spectroscopy, and broadening at long wavelengths has sometimes been mistaken for a new absorption band (Slifkin, 1963).

The fluorescence of FAD is less than one fifth of that of FMN. Similarly FMN fluorescence is quenched by many other aromatic compounds (Penzer and Radda, 1967), probably by complex formation. This has been clearly shown for purines (Weber, 1950) and indoles (Bereira and Tollin, 1967).

The optical rotatory dispersion of FAD differs from that of FMN alone and in an equimolar mixture with adenosine monophosphate (Gascoigne and Radda, 1965). The size of the FAD Cotton effect at 375mμ decreases under the same conditions that lead to an increase of fluorescence (Wentworth, 1966).

It is most likely that the planar aromatic systems in the complexes will interact best in a sandwich arrangement and the magnetic nuclear resonance of FAD confirms this

(R.H.Sarma, P.Dannies and N.O.Kaplan, 1968). Some workers (Harbury, LaNoue, Loach and Amick, 1959; Isenberg, Baird and Szent-Györgyi, 1961; Slifkin, 1963) have suggested that charge-transfer forces stabilise the complexes. Kosower (1966), however, has argued that the spectral and spin resonance data quoted as evidence for charge-transfer is inclusive. The stability of flavin-indole complexes is dominated by the entropy term in their free energy of formation, and hence it has been suggested that hydrophobic forces are involved (Pereira and Tollin, 1967). By varying the structure of the purine nucleotide in FAD analogues McCormick and his co-workers conclude that the complexes "may be characterised as of the donor-acceptor type as broadly defined. Such a classification involves mutual polarisability or partial charge-transfer effects" (Tsibris, McCormick and Wright, 1965).

It is important that in no other solvent than water have these complexes been detected, in spite of a careful search (Weber, 1966). In aqueous organic solvents the fluorescence of FAD compared to FMN increases (indicating unfolding) as the water concentration is decreased (Gascoigne and Radda, 1965a ; Velick, 1961). Even in formamide, which has a dielectric constant higher than

water, complexing is not significant (Hemmerich, Veeger and Wood, 1965; Weber, 1966). These observations underline the unique ability of water to stabilise complexes, and supports the suggestion that hydrophobic forces are important.

Molecular complexes between protonated flavins and phenols do show new absorptions that can be correlated with the donor ability of the phenol. Large single crystals of 1:1 complexes show an electrical conductivity that is increased on illumination. The photocurrent is composed equally of positive and negative carriers, and electron spin resonance shows that complete electron transfer can occur at lattice imperfection sites. These crystals probably consist of true charge-transfer complexes (Fleischman and Tollin, 1965; Ray, Guzzo and Tollin, 1965). Reduced FMN also forms charge-transfer complexes with NAD^+, but in this case the flavin acts as the donor (Sakurai and Hosoya, 1966).

The interaction of flavins with metals may be important in metalloflavoproteins, and it has been studied for the free coenzyme. The stability of oxidised flavin-metal chelates depends on the degree of back donation from the metal d-shell into the lowest antibonding orbital of the flavin (Müller, Hemmerich and Ehrenberg, 1968). Thus only

strong donor ions (e.g. silver and cuprous) form complexes in aqueous solutions. Reduced flavins have no metal affinity. The flavin semiquinone, however, is readily chelated by metals with d-electrons. The spectra of the complexes are very similar to that of the semiquinone anion (Müller, Hemmerich and Ehrenberg, 1968), which supports the suggestion that charge-transfer from the metal to the flavin is important. Studies of the radical paramagnetic relaxation show that the semiquinone can interact with metals in flavoproteins as well (Beinert and Hemmerich, 1965).

Flavoproteins

Over 50 enzymes have now been identified as flavoproteins. Their interactions with substrates and inhibitors, and their physical properties (spectra, molecular weight, etc.) have been more widely studied than their behaviour in simple chemical reactions. No X-ray analysis of a flavoprotein's structure has been published, nor has a complete amino acid sequence.

A start has been made on chemical studies of the amino acid oxidases to identify the active site and to discover how catalysis is achieved. Two types of experiment are possible. It is possible to compare enzyme activity

towards the oxidation of different amino acids because the amino acid oxidases do not have very narrow substrate specificity. The way reactivity depends on substrate structure may give a clue to the catalytic mechanism. Secondly it is possible to modify the protein chemically using reagents which are specific for different amino acid residues, and to correlate these reactions with changes in the enzyme's activity.

The activity of L-amino acid oxidase towards the oxidation of substituted phenylglycines (Radda, 1964), and of D-amino acid oxidase to both phenylglycines and phenylalanines (Neims, De Luca and Hellerman, 1966) have been analysed in terms of the Hammett equation. In every case biphasic Hammett plots have been obtained, showing that both strongly electron withdrawing and donating substituents reduce the activity of the enzyme. These plots are thought to be caused by a change in the rate determining step of the oxidation, rather than by competition between two different mechanisms.

Some protein modification studies using fairly unspecific reagents (e.g. trinitrobenzene sulphonic acid, which attacks free amino groups) have shown that when about half the amino groups have reacted D-amino acid oxidase has

lost all activity (Yagi, Harada and Kotaki, 1966). The enzyme is also inactivated by -SH reagents (Frisell and Hellerman, 1957). In both these reactions the protein is partly protected by FAD.

A more useful modifier may be glyoxal, which is thought to be specific for arginine residues. The modified enzyme has lost the ability to complex benzoate and is inactive. In the presence of benzoate the enzyme is protected both against loss of activity and loss of complexing ability (Kotaki, Harada and Yagi, 1962).

When D-amino acid oxidase is reduced by sodium borohydride in the presence of the substrate C_1^{14} labelled D-alanine the radioactivity is incorporated into the protein (Coffey, Neims and Hellerman, 1965). After acid hydrolysis of the adduct ∈-N-(1-carboxyethyl)-L-lysine can be identified in the hydrolysate (Hellerman and Coffey, 1967). This indicates that substrate interacts with the ∈-amino group of a lysine residue, presumed to be close to the active site. One mole of substrate binds per mole of coenzyme. The interpretation of these results in terms of a catalytic mechanism has recently been questioned following the discovery that it is possible to produce a fully active reduced enzyme-substrate adduct (Massey, Curti, Müller and Mayhew, 1968). It has also been shown that borohydride

reduces the flavin moiety in either D- or L- amino acid oxidase to give a fluorescent compound with its absorption maximum shifted to 410mμ (Massey, Curti, Müller and Mayhew, 1968). No positive identification has yet been made but it is possible that borohydride reduces the carbonyl groups on the pyrimidine ring of the flavin.

The positions and shapes of the two long wavelength absorption bands vary for different flavoproteins. Spectral studies of the interaction of D-amino acid oxidase with various aromatic compounds have led to calculation of the stoichiometry of complex formation (1:1,ligand:flavin) and to estimates of the dissociation constants (Massey and Ganther, 1965). Some flavoprotein spectra show fine structure in the visible absorption band (Massey and Ganther, 1965). This suggests by analogy with model studies (Harbury, La Noue, Loach and Amick, 1959) that in these compounds the flavin is in a largely non-aqueous environment.

Two spectrally distinct classes of flavoprotein semiquinones are found on reduction by dithionite, or irradiation in the presence of EDTA. The blue radical has a similar absorption to the semiquinone of a free flavin, while the red one has increased extinction at 370mμ (compared to oxidised flavin) and a new peak at 485mμ (Massey and Palmer,

1966) like the anion of a free semiquinone. The hyperfine structure of the electron spin resonance spectra of flavoprotein semiquinones is not well resolved. This is a consequence of the slow thermal rotational relaxation of proteins. The signals from blue and red radicals can be distinguished, however, on the basis of their line shapes and saturation behaviour (Massey and Palmer, 1966).

The intermediates which can be spectroscopically identified during flavoprotein catalysis can also be divided into two types. Type 1 intermediates are characterised by reduced absorption at 380mμ compared to oxidised flavin, with a broad band at 530-600mμ (Wellner, 1967). These intermediates do not show electron spin resonance signals, but they are important in flavoprotein catalysis. Type 2 intermediates are formed more slowly, have spectra corresponding to either red or blue radicals, and give spin resonance signals (Wellner, 1967), but they do not seem to be significant as intermediates during catalysis.

A possible explanation of the way type 1 intermediates are formed and react is that they occur after transfer of an electron from substrate to the flavin, but whilst the two can interact so that the potentially unpaired electrons remain with opposed spins (Massey and Gibson, 1964). Other

explanations in which similar spin coupling between two flavin radicals occurs have also been postulated (Wellner, 1967).

This Thesis

The most interesting features in the chemistry of flavins from a biochemical viewpoint concern their function as coenzymes. When bound to proteins flavins catalyse reactions which, when free in their ground states, they have no tendency to do. When electronically excited, however, free flavins catalyse many of the same reactions as flavoproteins, and so the photochemistry of flavins may give hints about the mode of action of flavoproteins. The main purpose of the work described in this thesis has thus been to learn more of the photochemistry of flavins, particularly in the reactions where substrates are oxidised.

The experiments in chapter 2 involve absorption spectra. Their purpose was to find both the relative and absolute directions of the absorption oscillators of the two long wavelength absorption bands of a flavin. This both aids a prediction of the distribution of electrons (and hence reactivity) in the excited states, and can lead to a better understanding of the reasons for spectral shifts when the environment is changed (Lippert, 1957).

Chapter 3 deals with the processes which deactivate the first excited singlet and the lowest triplet of a flavin. These were studied by emission spectroscopy. The comparatively unusual phenomenon of E-type delayed fluorescence (Parker, 1968) occurs for flavins in a rigid matrix at room temperature (Radda and Watson, 1968). By measuring the temperature dependence of delayed fluorescence and of phosphorescence it has been possible to calculate the activation energies of several of the deactivation processes.

The work described in chapter 4 is aimed at discovering the mechanism of anaerobic photoreduction of flavins. The inhibition of photoreduction by several aromatic compounds has been studied and compared with the reactivity of FAD.

Chapter 5 is concerned with the aerobic chemistry of flavins. In part this involves the same reaction as anaerobic photoreduction, with the effects of oxygen confined to triplet quenching and the reoxidation of reduced flavins. It has been possible to calculate several of the rate constants in the photoreduction scheme proposed in chapter 4 from measurements of the effect of oxygen concentration on reaction rate. In addition illuminated flavins can excite oxygen so that it oxidises some aromatic compounds. Some aspects of this reaction have been studied and preliminary experiments have been made to see if it could be applied to

the selective modification of amino acid residues in a protein.

Finally, in chapter 6, a different type of investigation is described. The experiments were again photochemical, but the primary light absorption was by spinach chloroplasts rather than a flavin. The behaviour of a series of flavin derivatives in the presence of chloroplasts (both during and immediately after illumination) was studied by fluorimetry. The order of reactivity of the flavins was compared with those found for several simpler model reactions, with the aim of discovering the nature of the interaction with chloroplasts.

Reports of parts of this work have been published, and copies are bound at the back of the thesis.

CHAPTER TWO

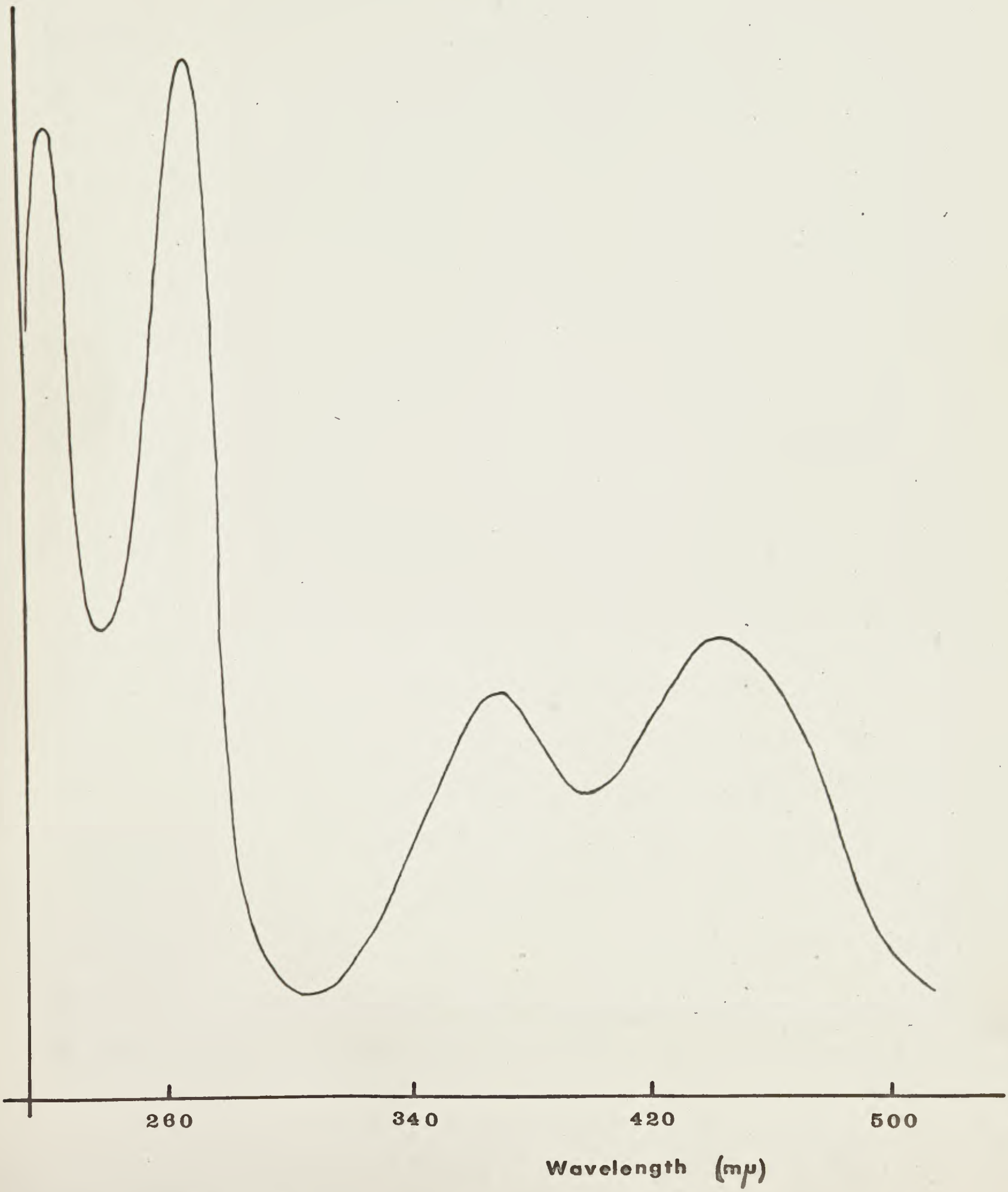
Flavin Absorption Spectra

Introduction

The two long wavelength absorption bands of the flavin chromophore are centred around 375 mμ and 445mμ (fig.2-1), so they are clear of any of the absorptions of proteins. In a flavoprotein the positions and shapes of these bands are changed. A better understanding of the nature of the electronic transitions would help in the deduction of how the chromophore binds to apoprotein from an analysis of the spectral shifts which occur.

There have been several approaches to a theoretical understanding of flavin absorption spectra. One is to derive the energies, intensities, and (sometimes) angles of the transitions by molecular orbital calculations. The Hückel approximation has sometimes been employed (Karreman, 1961), but it has subsequently been found that the self consistent field (SCF) method gives results in better accord with experimental measurements (Fox, Laberge, Nishimoto and Forster, 1967). Using a semi-empirical SCF method Kurtin and Song (1968) have also calculated transition energies, moments, and oscillator strengths for the 6,7-dimethylisoalloxazine nucleus. The relative oscillator strengths for the two long wavelength bands are in agreement with those found

Fig. 2-1 ; Absorption spectrum of FMN in dilute aqueous solution at neutral pH



experimentally for some isoalloxazines, but it is hard to assess the accuracy of the transition energies because the spectrum of 6,7-dimethylisoalloxazine is unknown. If it is similar to that of lumiflavin the transition energies calculated for the two long wavelength absorptions are too high. The angle between these transitions (θ) is calculated to be 42°. Other workers, who also use the SCF approximation calculate θ to be 27° (L.S.Forster reported in Tollin, 1968) and Grabe finds θ to be approximately 80° (private communication to Dr.G.K.Radda).

An experimental approach to the measurement of θ is to find the fluorescence polarisation of the chromophore when it is excited in each of the absorption bands. The Levshin-Perrin formula, $p = \frac{3\cos^2\phi - 1}{\cos\phi + 3}$ (where p is the fluorescence polarisation and ϕ is the angle between absorption and emission oscillators), enables calculation of ϕ (and hence θ) providing that the sample studied contains a random array of rigidly held molecules (Jablonski, 1935). These criteria have not been fully met by any of the published studies (Penzer and Radda, 1967; Kurtin and Song, 1968); in the first case pure glycerol at 25° was the solvent, and in the second a 9:1 glycerol:water mixture at -10° was used. In neither case is it certain that molecular motion was

negligible on the time-scale of fluorescence decay (the lifetime of the first excited singlet of FMN in water at pH 7 and 23° is 4.9 nanosec. (Chen, Yurek and Alexander, 1967) and measurements made on samples sent to the TRW Instrument Company agree reasonably with this value). Assuming that both absorption oscillators lie in the plane of the molecule there are still two solutions for θ from a particular pair of ϕ-values, $\phi_a + \phi_b$ and $\phi_a - \phi_b$. Different values of ϕ can be deduced from the experiments depending on whether it is assumed that the supporting matrix is rigid or not. Hence the values of θ which can be calculated from the data so far reported vary from 10° to 50°.

The variation of the polarisation of flavin fluorescence with the wavelength of excitation shows that the 375mμ and 445mμ bands are each dominated by a single electronic transition because p remains constant across each absorption (Weber, 1966). This conclusion is in accord with the calculations of Fox and his coworkers (Fox, Laberge, Nishimoto and Forster, 1967), who nonetheless predict the presence of subsidiary absorptions in this region of the spectrum with extinction coefficients about 10% of those of the main bands. The presence of some of these transitions has gained experimental support from the magnetic circular dichroism spectra of flavins (Tollin, 1968).

Varying substituents in the isoalloxazine ring system causes shifts in the positions of the absorption bands and changes of extinction coefficient (Müller and Hemmerich, 1966; Müller, Walker and Hemmerich, 1966). It is likely that the directions of the absorption oscillators, and hence the angles between them, will be altered too. However the facts that for most substituents the overall shapes of the spectra are unchanged, and that the ratios of the fluorescence polarisations over the two long wavelength bands vary little (Wentworth, 1966), make it unlikely that there are large changes in the directions of the absorption oscillators. Hence it should be possible to deduce the approximate orientations of the transition moments by observing the effects that different substituents have on the positions and extinctions of the absorption bands. Although there is a great deal of data on the spectra of flavin derivatives, no comprehensive survey or interpretation of it has been published.

In this chapter two experimental methods for measuring Θ are described. The first is fluorescence polarisation; the second is measurement of dichroic absorption spectra of partially ordered films of a flavin. The order is introduced into a random array of chromophore molecules held in a rigid

matrix by bleaching some of the flavin with polarised light. An attempt is also made to assign directions to the 375 and 445mμ absorption oscillators on the basis of the spectra of as many substituted flavins as possible.

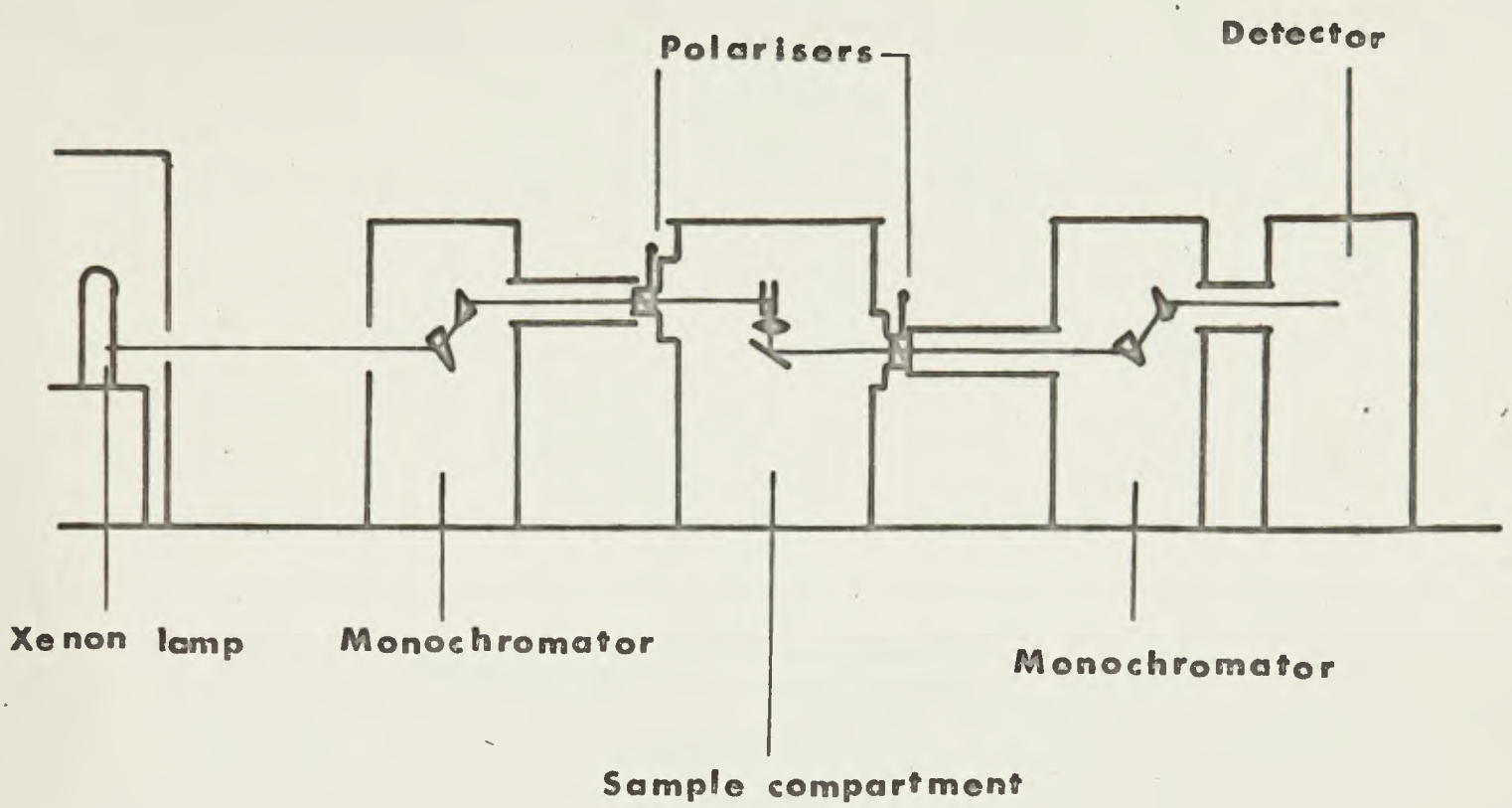
Measurements and Accuracy

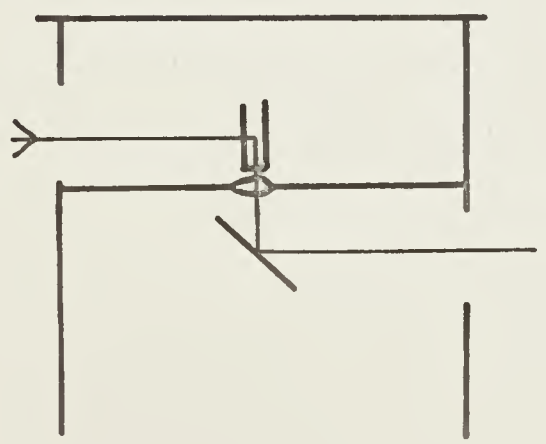
Polarisation of fluorescence was measured on a Zeiss PMQ II spectrofluorimeter, fitted with two Foucault prisms (12mm apperture) on either side of the sample compartment (fig.2-2). These prisms are transparent to light with wavelengths longer than 250mμ. They are both mounted so that they can be rotated through 90° in the plane perpendicular to the plane of propagation of the light. The exit prism was carefully oriented so that the electric vector of the light passing through it was vertical when it was at one extreme of its 90° arc, and horizontal when at the other. The entry prism was now adjusted to be accurately parallel or perpendicular to the exit one. If care was not taken with these settings polarisations lower than the true values were recorded.

Fluorescence polarisation, p, is defined by $p = \frac{V_V - H_V}{V_V + H_V}$

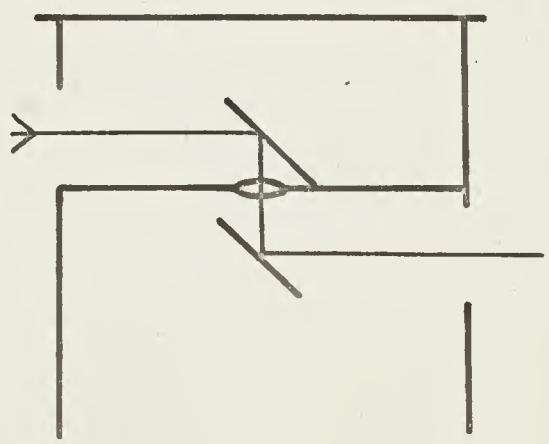
where the first letter of each couplet indicates the direction of polarisation of emission, and the second letter

Fig. 2-2 ; Diagram of the Zeiss spectrofluorimeter showing the two possible positions in which to view the sample





Normal

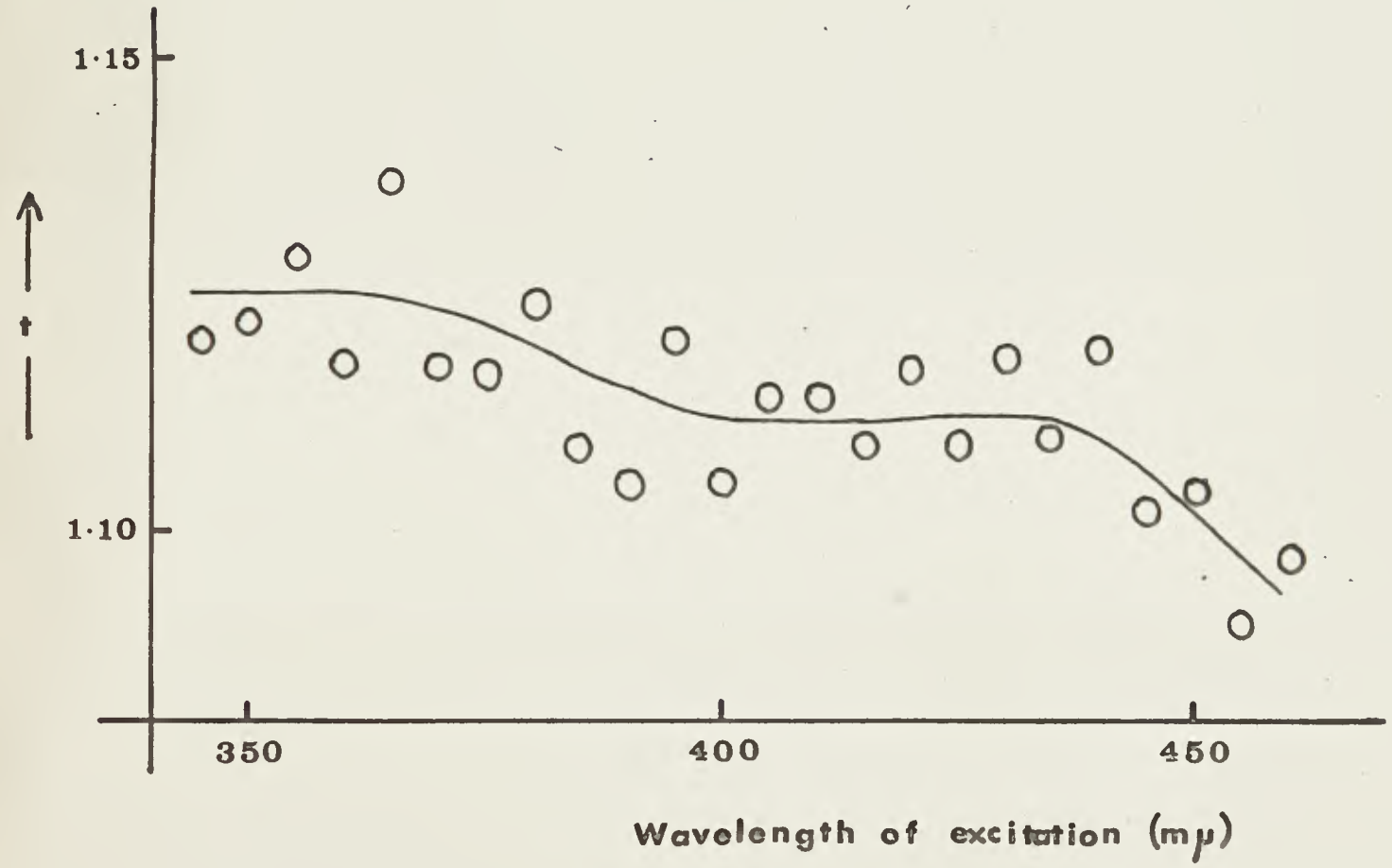


Angled

that of illumination. H means that the electric vector is parallel to the plane of propagation of light, V that it is perpendicular (Weber, 1960). In a perfect optical system $H_V = H_H = V_H$ when emission is observed at 90° from excitation. In practice no instrument is perfect because of polarisation caused by its mirrors, gratings, etc. It can be shown that if $t = \frac{V_H}{H_H}$ the corrected expression for polarisation is $p = \frac{V_V - tH_V}{V_V + tH_V}$ (Weill and Calvin, 1963). The wavelength dependence of t for the Zeiss is shown in figure 2-3. The theoretical limits for p are +0.5 (for a completely random array of chromophores in which the excitation oscillator is parallel to the emission oscillator) and -0.3 (for a rigid random array with excitation and emission oscillators perpendicular) (Weber, 1953).

The error in polarisation of fluorescence measurements depends on the intensity of emission, the value of p, and the background scatter. Consider a highly fluorescent sample with a high p-value, studied on an instrument with negligible intrinsic polarisation. The readings of V_V and H_V are 70 and 25 respectively, each subject to an error of $\pm$ 0.5. As the fluorescence intensity falls this error increases. The polarisation calculated is 0.474 $\pm$ 0.01 (i.e. an error of $\pm$2%). If the polarisation were lower, so

Fig. 2-3 ; Wavelength dependence of the fluorescence polarisation correction factor (t) for the Zeiss fluorimeter
(the sample was FMN (extinction 0.2) in glycerol at 25°)



that V_v and H_v become 55 and 45 (still subject to the same error), p = 0.100 ± 0.01 (i.e. an error of ± 10%). Better accuracy cannot be obtained on single beam spectrofluorimeters because of the variations in fluorescence intensity caused by small lamp fluctuations.

The solutions studied in this work were FMN dissolved in glycerol or a glycerol/water mixture to a concentration of about 3×10^{-5}M. The glycerol used was quoted as 98% pure; no attempt was made to dry it. Every polarisation was measured at least twice and the different results fell within 3% of the final average. The alignment of the polarisers on the instrument was checked before each series of readings. The slit widths used (0.5 to 1.5mm) were such that the band-widths passed by the monochromators were less than 40mμ. Even at the longest wavelength of excitation used no scatter was observed at the observation wavelength.

Polyacrylamide films were made by polymerising acrylamide solutions with N,N'-methylene-bisacrylamide (BIS) as a cross-linking agent. Ammonium persulphate (APS) was the initiator and N,N,N',N'-tetramethylethylene diamine (TEMED) was present as an accelerator. The physical properties of the films vary with the concentrations of the components. The conditions used were those which produce a dry brittle

polymer (Watson, 1968).

A stock solution containing 62.5 gm acrylamide, 0.8 gm BIS, and 0.35 gm TEMED in 192 ml of doubly distilled water was prepared. About 1mg of a flavin was dissolved in a 10ml aliquot of this solution, and then 0.15 ml of a 10% (by weight) solution of APS was added. In about two minutes the mixture became too viscous to manipulate, so during that period it was pipetted onto glass slides (2 - 3 ml per slide). These samples were dried for at least 48 hr. over anhydrous silica gel in a dessicator. The dry films were then clear and brittle, but not planar because they shrink away from the slide during the drying. They could easily be removed from the slides.

This technique has the disadvantage that it subjects the molecule to be included in the film to a high concentration of radicals and to a raised temperature (60-70° under Watson's conditions) during the polymerisation. Flavins are not usually degraded rapidly at 70°, but various adducts and semi-reduced species might be formed in the presence of some radicals. The absorption spectra of flavins in polyacrylamide films are the same as those found for aqueous solutions, and so any degradative or reactive processes occurring during polymerisation are not very important.

Absorption spectra of the films were measured on an SP 500 mk.2 spectrophotometer using a polyacrylamide film without chromophore as the blank. Dichroic spectra were measured using a polaroid filter. By rotating the polaroid through 90° it was possible to measure optical densities for light polarised in two mutually perpendicular directions. Before reaction the spectra of a film were identical for both polaroid positions.

Photobleaching of a flavin in a polyacrylamide film was achieved by subjecting it to repeated flashes of white light from a photographic flash gun. A cut-out filter was used to remove all wavelengths shorter than 400mμ , and a polaroid filter was used for polarised bleaching. The spectra of a flavin after polarised bleaching were now different for the two polaroid positions (parallel and perpendicular to the direction of polarisation of the bleaching light). The spectra of a bleached film were unchanged over a period of 24 hr. This shows that reoxidation of the products by oxygen and rotation of the flavin chromophore within the film are negligible.

The accuracy of optical density measurements on polyacrylamide films is only $\pm$ 0.01. This large error is caused by several factors: it is impossible to guarantee

that the blank film exactly matches the sample; the films, though clear, are not perfectly homogeneous in thickness or refractive index; because of the filtering effect of the polaroid film all readings are taken at abnormally large slit widths, with subsequent loss of spectral purity.

Theory

A) Fluorescence polarisation. The angle between the absorption and emission oscillators (ϕ) can be calculated from the relation $p_o = \frac{3\cos^2\phi - 1}{\cos^2\phi + 3}$, where p_o is the polarisation measured for a completely rigid but randomly oriented array of chromophore molecules. This simple expression cannot be applied when there is significant molecular rotation during the lifetime of the excited state, or when there is energy transfer (which can lead to depolarisation) between the excited molecules (Weber, 1953). In the absence of concentration depolarisation energy transfer is negligible.

P_o cannot be measured directly for a fluid solution. The extent of depolarisation due to isotropic rotation is given by $\left(\frac{1}{p} - \frac{1}{3}\right) = \left(\frac{1}{p_o} - \frac{1}{3}\right)\left(1 + \frac{3\tau_o}{\rho_o}\right)$, where p is the measured polarisation, τ_o is the lifetime of the excited state, and ρ_o is given by $\frac{4\pi\eta r^3}{kT}$, where η is the viscosity of the solution and r is the radius of the chromophore (Perrin, 1926 and 1929).

Thus under conditions where τ_o is constant $\frac{1}{p} \propto \frac{T}{\eta}$. A plot of $\frac{1}{p} : \frac{1}{\eta}$ at constant temperature can be extrapolated to $\frac{1}{\eta} = 0$ to give $\frac{1}{p_o}$. The theory for non-isotropic rotations (Weber, 1953) shows that the same extrapolation gives $\frac{1}{p_o}$, but the $\frac{1}{p} : \frac{1}{\eta}$ graph is no longer linear.

B) Dichroic bleaching. The measurement of the directions of electronic absorption oscillators by means of the dichroic bleaching of a rigid array of chromophores has not been reported before. Thus it has been necessary to develope the theoretical treatment required to find the angle between absorption oscillators from experimental measurements. In contrast to other methods of preparing partially ordered films (Gratzer, Holzwarth and Doty, 1961; Sackmann, 1968) dichroic bleaching produces a degree of order that can be calculated.

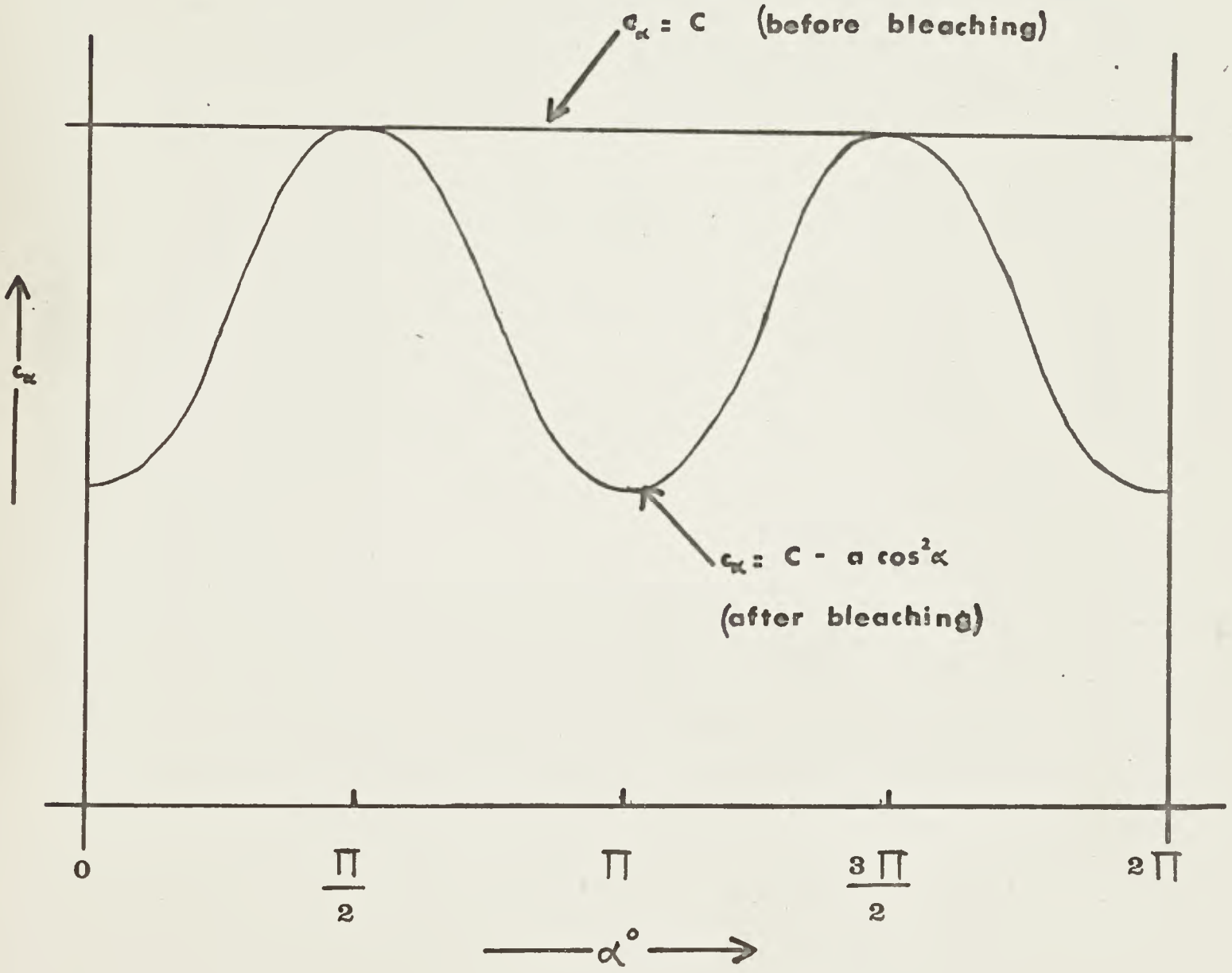
Consider a solution containing randomly oriented molecules of a flavin firmly held in a rigid matrix. Let θ be the angle between the dipole vectors for the bands centred at 375mμ and 447mμ, and let the angle between the 447mμ vector and a fixed reference direction in the solution be α. To a first approximation the system can be considered in two dimensions because the contributions of absorption oscillators directed out of the plane perpendicular to the direction of

propagation of light are smaller than those in plane. The inaccuracy of a two dimensional treatment compared to the more rigorous three dimensional approach is small (appendix 3). In any case the value of Θ found will be a mean value because in a condensed phase it varies from molecule to molecule due to local interactions. Let $c_\alpha . d\alpha$ give the number of molecules whose 447mμ vectors lie in the angle from α to $\alpha + d\alpha$ (considering only two dimensions).

The probability for absorption of polarised light is proportional to the square of the cosine of the angle between the direction of polarisation of the radiation and the absorption oscillator of the chromophore. The probability of photochemical reaction is directly proportional to the probability of absorption. Thus if photochemical reaction of a chromophore in a rigid matrix is induced by polarised light the angular distribution of unreacted molecules is no longer random (fig.2-4).

If the direction of the linearly polarised light used for bleaching is parallel to $\alpha = 0$, and if the wavelength of this light is such that it only illuminates the 447mμ band of a flavin, then the number of molecules with 447mμ vectors lying between α and $\alpha + d\alpha$ is given by $c_\alpha . d\alpha = (C - a \cos^2\alpha) d\alpha$,

Fig. 2-4 ; Simplified dichroic bleaching theory



where $C = c_\alpha$ before bleaching (and is thus independent of α) and a is a constant which depends on light intensity, period of illumination, etc.

Assuming that the products of bleaching do not absorb at 375 or 447mμ the extinctions at these wavelengths after bleaching are proportional to the following functions.

i) 447mμ parallel to bleaching, $\int_o^{2\pi} (C - a\cos^2\alpha)\cos^2\alpha . d\alpha$

ii) 447mμ perpendicular to bleaching, $\int_o^{2\pi} (C - a\cos^2\alpha)\sin^2\alpha . d\alpha$

iii) 375mμ parallel to bleaching,

$$\int_o^{2\pi} (C - a\cos^2\alpha).\tfrac{1}{2}\left\{\cos^2(\alpha+\theta) + \cos^2(\alpha-\theta)\right\}d\alpha$$

iv) 375mμ perpendicular to bleaching,

$$\int_o^{2\pi} (C - a\cos^2\alpha).\tfrac{1}{2}\left\{\sin^2(\alpha+\theta) + \sin^2(\alpha-\theta)\right\}d\alpha$$

(θ being the angle between the 375 and 447mμ oscillators).

Integrating these expressions, and simplifying them (see appendix 2)

$\frac{(i)}{(ii)} = \frac{4C - 3a}{4C - a}$ and $\frac{(iii)}{(iv)} = \frac{2(2C-a)-a\cos 2\theta}{2(2C-a)+a\cos 2\theta}$ (i) and (iii) can be (ii) and (iv) measured experimentally, and so 2θ can be calculated.

An alternative way to analyse the results of a polarised bleaching experiment is to consider the rates of bleaching of the 375 and 447mμ bands, rather than just the overall changes in optical density. The rates of bleaching at these wavelengths are proportional to the following functions (using the same nomenclature as before).

(v) 447mμ parallel to bleaching, $\int_0^{2\pi} \cos^4\alpha . d\alpha$

vi) 447mμ perpendicular to bleaching, $\int_0^{2\pi} \cos^2\alpha . \sin^2\alpha . d\alpha$

vii) 375mμ parallel to bleaching,

$$\int_0^{2\pi} \cos^2\alpha . \tfrac{1}{2}\{\cos^2(\alpha + \Theta) + \cos^2(\alpha - \Theta)\} . d\alpha$$

viii) 375mμ perpendicular to bleaching,

$$\int_0^{2\pi} \cos^2\alpha . \tfrac{1}{2}\{\sin^2(\alpha + \Theta) + \sin^2(\alpha - \Theta)\} . d\alpha$$

Integrating and simplifying these expressions (see appendix 2)

$\frac{(v)}{(vi)} = 3$ and $\frac{(vii)}{(viii)} = \frac{2 + \cos 2\Theta .}{2 - \cos 2\Theta}$

These methods have different merits. The first does not require exact reproducibility of flash gun position (relative to the film) or intensity. The second method needs both of these as illumination time is measured by the number of flashes to which a sample is subjected. The second method, however, gives more detailed information than the first. The extent to which scattered light causes unpolarised bleaching is indicated by the amount by which the ratio of the rates of bleaching at 447mμ (parallel:perpendicular) differs from 3:1.

Results and Discussion

A) Polarisation of fluorescence. The fluorescence polarisation of FMN in a glycerol/water mixture at 25° has

been measured as a function of the wavelength of excitation (fig.2-5). In contrast to the results of Kurtin and Song (1968) no fine structure was found on the spectrum between 340 - 460mμ . This may have been because the temperatures and solvents used in the two investigations were different. In any case the size of the fine structure is at the very limit of the accuracy of mamual polarisation measurements.

The polarisation of the long wavelength absorption of rigidly held riboflavin has been measured by plotting $\frac{1}{\eta} : \frac{1}{P}$ for solutions in differently composed glycerol/water mixtures (Weber, 1950). P_o for the 375mμ band has not been reported. This has now been measured by plotting $\frac{1}{P_{447}} : \frac{1}{P_{375}}$ for solutions of FMN in different glycerol/water mixtures (fig.2-6). $\frac{1}{P_{o,447}}$ is 2.0 (Weber, 1950), and so by extrapolation $\frac{1}{P_{o,375}} = 2.9$ (fig.2-6). The accuracy of this extrapolation is $\pm$ 0.1, so $P_{o,375} = 0.345 \pm 0.012$.

Applying the Levshin-Perrin formula to this value of P_o, $\phi_{375} = 29° \pm 1°$ is calculated. This formula gives $\cos^2\phi$, so it is not possible to deduce whether the direction of the absorption oscillator is +29° or -29° from the emission oscillator. Assuming that the value of $\frac{1}{P_{o,447}}$ is accurately

Fig. 2-5 ; Fluorescence polarisation spectrum for FMN (extinction 0.2) in glycerol at 25°

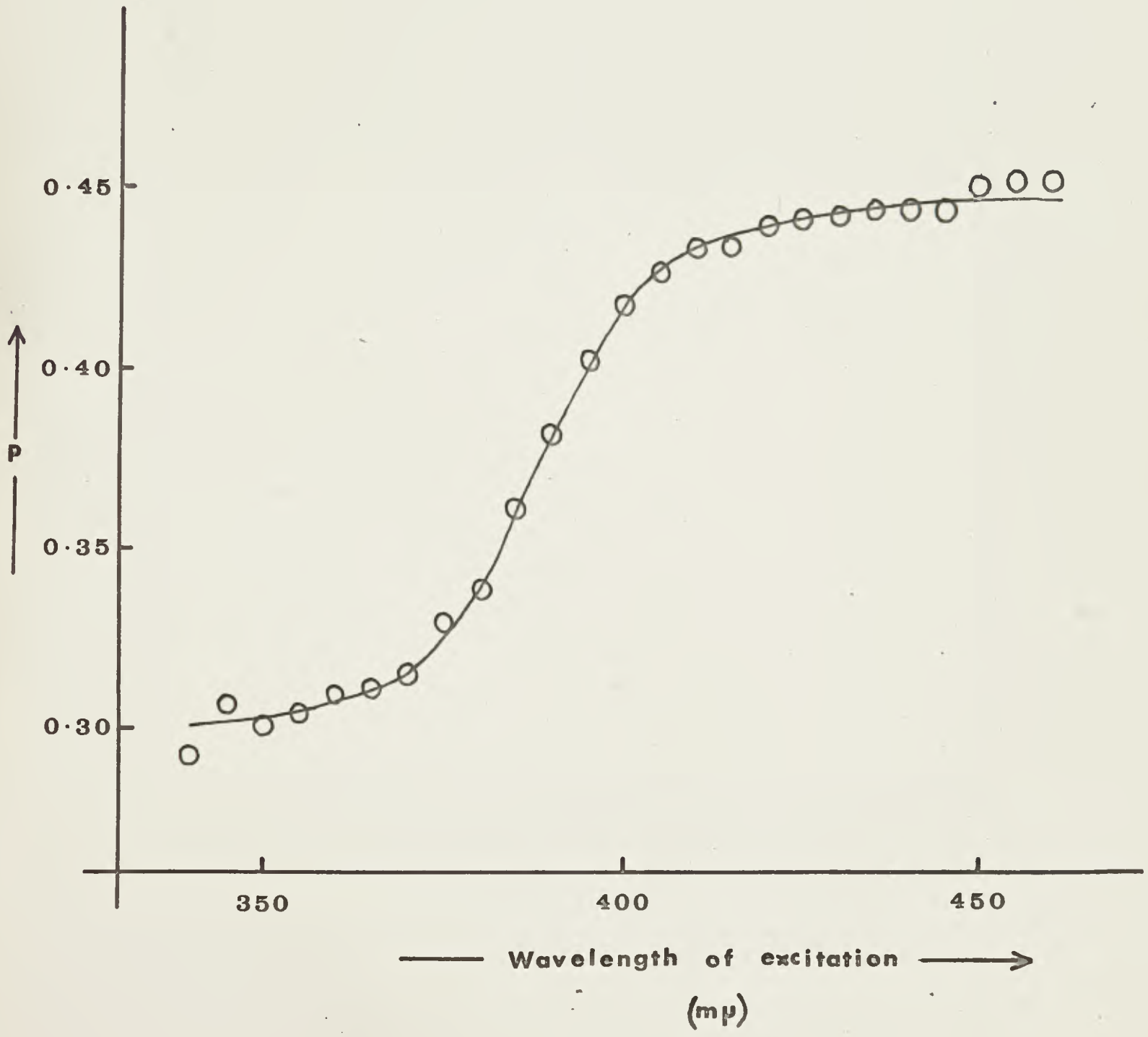
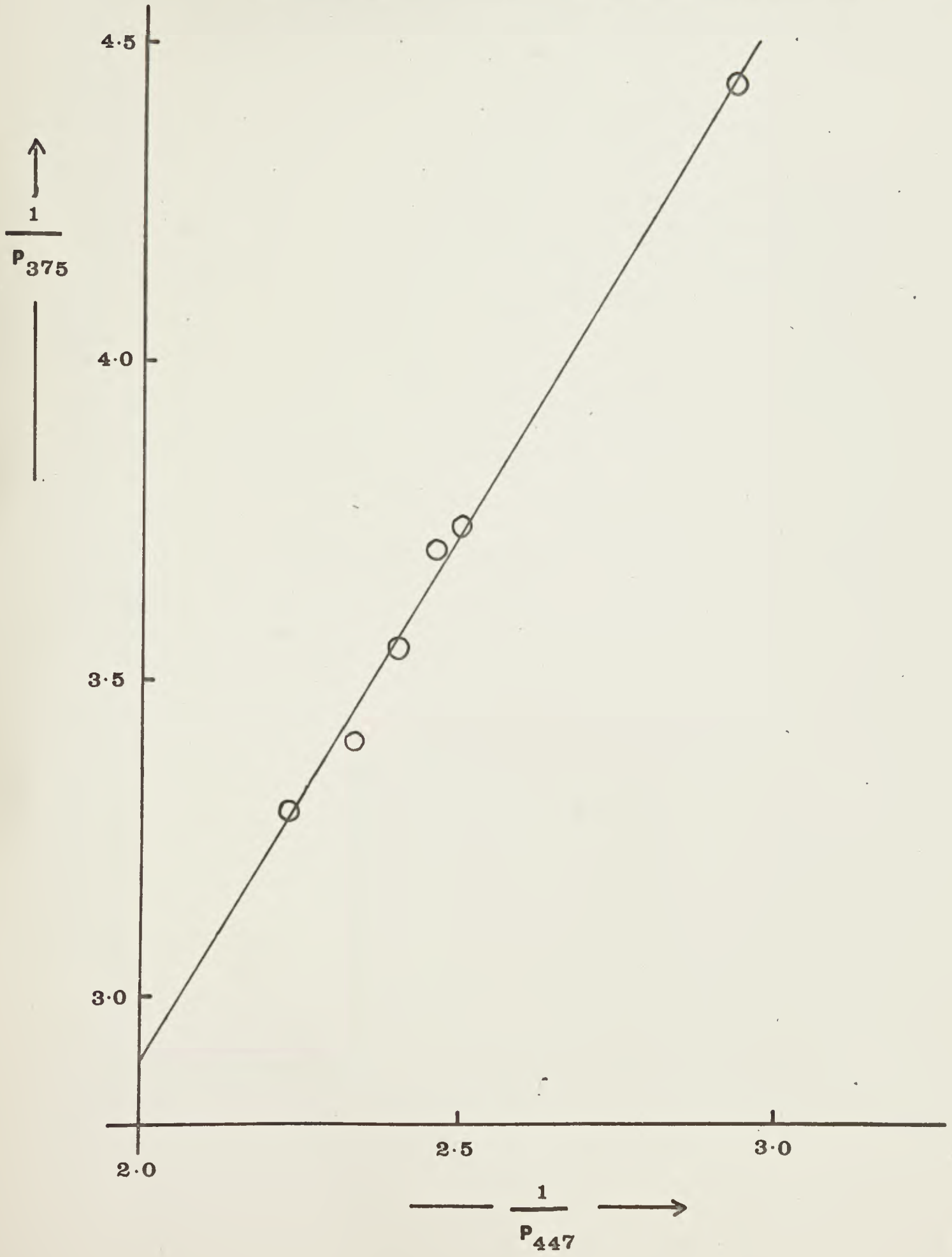


Fig. 2-6 ; Extrapolation to infinite viscosity by plotting $1/p_{375}$: $1/p_{447}$ for FMN solutions (extinctions 0.2) in various glycerol/water mixtures at 25°



equal to 2.000, as found by Weber, the visible absorption oscillator is parallel to the emission oscillator, and so ϵ is 29°. However an error of 0.05 in $\frac{1}{p_o}$ is quite possible, and this is equivalent to an error in ϕ_{447} of $\pm$ 8°. The error in ϕ_{375} is less than 1°.

Polarisation of fluorescence measurements thus suggest that the angle between the two absorption oscillators is 29° $\pm$ 8°.

Others work has suggested different values for ϕ_{375} and ϕ_{447}, 29° and 20° respectively for riboflavin (Kurtin and Song, 1968). The reason for the high value of ϕ_{447} is probably that their solvent system did not provide a rigid supporting matrix, and no effort was made to extrapolate to infinite viscosity. This is indicated by the facts that the fluorescence polarisation of lumiflavin was much lower than that of riboflavin, and that when similar glasses containing different proportions of glycerol were investigated different polarisations were registered. This being so it is surprising that a higher value of ϕ_{375} was not found.

B) Dichroic bleaching. A second technique for measuring ϵ was developed because of the inadequacies of the fluorescence polarisation method. Chromophores firmly held

in a polyacrylamide gel are illuminated with linearly polarised light of wavelengths which are absorbed by only one of the electronic transitions. This exciting light leads to the bleaching of some of the molecules which absorb it, thus leaving a partly oriented array of unbleached species. Because the way in which the probability of absorption of polarised light depends on the angle it makes with an absorption oscillator is known, an expression can be deduced for the distribution of orientations amongst the unbleached molecules. This is not the case with other techniques for producing partial order (Gratzer, Holzwarth and Doty, 1961; Sackmann, 1968).

The simple theories given on pp.29-33 rest on two basic assumptions. The first is that the exciting light is perfectly polarised, and is not significantly depolarised by scattering from the sample. The second is that product absorptions are negligible over the wavelength range studied. The validity of the first assumption can be tested by comparing the rates of bleaching at 447mμ perpendicular and parallel to the exciting light. If the ratio deviates from 1/3 then depolarisation has occurred. The relative rates for several flavins are shown in table 2-1, and they show that some depolarisation always occurs. The variation in the magnitude of the effect is not surprising because the optical homogeneity of the films was not constant.

Table 2-1

Relative rates of bleaching of flavin derivatives observed parallel and perpendicular to the direction of polarisation of the exciting light.

All reactions were in polyacrylamide films

Flavin	Rate ratio $(\frac{\text{perpendicular}}{\text{parallel}})$				Average
	430 mμ	440 mμ	450 mμ	460 mμ	
lumiflavin					
-film a	•40	•39	•49	•41	•42
film b	•85	•83	•83	•83	•83
film c	•28	•36	•40		•35
3-methyl lumiflavin	•59	•66	•62	•63	•63
riboflavin			•49		
FMN	•67	•68	•69	•70	•69
6-chloro-9-(N,N-diethylaminopropyl)-isoalloxazine					
film a	•52	•50	•49	•50	•50
film b	•50	•60	•45		•52

Assume that the exciting light consists of an unpolarised component superimposed on the polarised component. If the rate of bleaching parallel to the plane of polarisation is x, and that perpendicular to it is y, $y/x = 1/3$ when the unpolarised component is zero. When y/x is greater than 1/3 x and y are each composed of two parts, $x' + z$ and $y' + z$. x' and y' are the rates of bleaching caused by the polarised component of the exciting light, z is the rate of bleaching caused by the unpolarised component. Thus $y'/x' = 1/3$, and so $x' = 3y'$. The measured rate ratio, y/x, is therefore given by $(y' + z)/(3y' + z)$. Knowing x, and y from experiment x', y', and z can be calculated.

The values of z from 360 - 460 mμ have been calculated for a polarised bleaching experiment on lumiflavin for which y/x was 0.42 (table 2-2). It has been assumed that, for example $z_{370} = \frac{z_{450} \cdot \epsilon_{370}}{\epsilon_{450}}$, where ϵ is the extinction of an unbleached film. It can be seen that when applying the final optical density method for calculating 2θ the z corrections are very small compared to the readings being corrected. This is always the case, even for higher values of y/x, providing the overall extent of bleaching is small.

The second assumption, that product absorptions are

Table 2-2

Corrections for scattering during dichroic bleaching of lumiflavin (film a)

Figures in parenthesis give the number of flashes used to induce bleaching

Wavelength (mμ)	Initial extinction	Extinction after bleaching parallel to bleaching			Extinction after bleaching perpendic. to bleaching			Z*		
		(150)	(300)	(450)	(150)	(300)	(450)	(150)	(300)	(450)
460	1.11	1.00	0.92	0.86	1.08	1.03	0.99	0.009	0.025	0.037
450	1.16	1.06	0.98	0.91	1.13	1.06	1.04	0.019	0.031	0.037
440	1.08	0.99	0.90	0.86	1.05	1.00	0.98	0.009	0.025	0.031
380	0.89	0.85	0.82	0.79	0.88	0.86	0.85	0.011	0.023	0.030
370	0.98	0.94	0.91	0.89	0.97	0.96	0.94	0.010	0.023	0.029
360	1.00	0.96	0.95	0.94	1.00	0.98	0.97	0.010	0.021	0.026

Extinction corrected for Z parallel to bleaching			Perpendicular to bleaching		
(150)	(300)	(450)	(150)	(300)	(450)
1.01	0.95	0.90	1.09	1.06	1.03
1.08	1.01	0.95	1.15	1.09	1.07
1.00	0.93	0.89	1.06	1.03	1.01
0.86	0.85	0.82	0.89	0.88	0.88
0.95	0.93	0.92	0.98	0.98	0.97
0.97	0.97	0.97	1.01	1.00	1.00

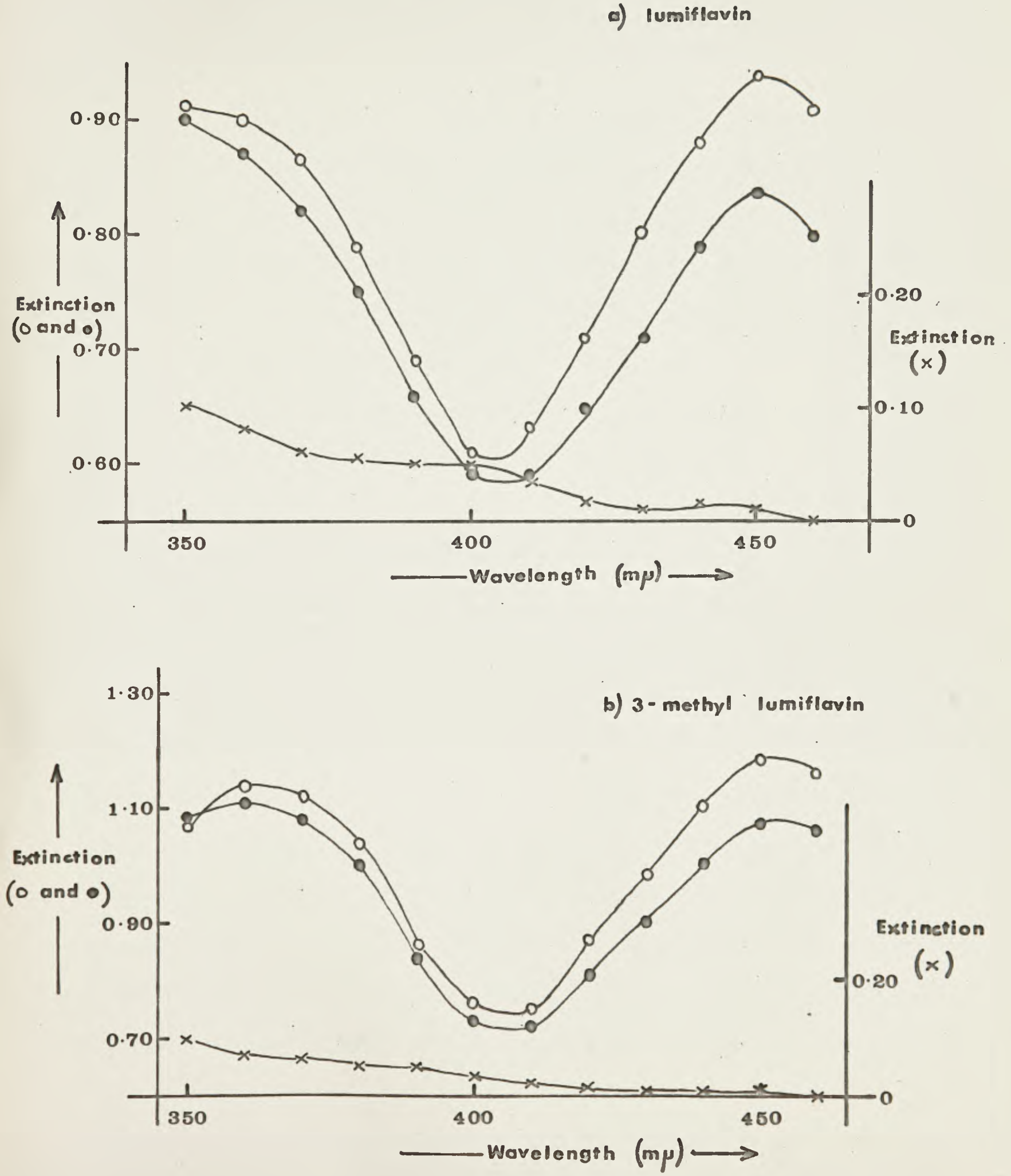
* $Z_{460-440}$ are calculated from the change in extinction perpendicular to bleaching (y) by applying $Z = {}^{y}/3.23$. $Z_{380-360}$ are calculated from $Z_{370} = Z_{450} \cdot \epsilon_{370}/\epsilon_{450}$, where ϵ is the extinction of the film before reaction.

negligible, has been tested for several compounds. For example, a film containing lumiflavin was subjected to normal unpolarised bleaching. By subtracting the spectrum of unreacted lumiflavin from that of the bleached film the absorptions due to the products are obtained (fig.2-7). By use of this spectrum a correction can be made for the absorptions of products after polarised bleaching, providing that the products possess no dichroism. This possibility may be excluded for the following reasons.

i) For reaction to occur the flavin must have at least limited freedom of movement to permit atomic reorganisation as the products are formed. The product molecules are probably oriented at random after such movements, in spite of the fact that the excited molecules were ordered before reaction.

ii) The absorption spectrum of the products (fig.2-7) is different from that of reduced flavin in aqueous solution (Holmstrom and Oster, 1961). The extinction coefficient of the film product is also twice that of reduced flavin at 380 mμ (product extinction at 380 mμ/concomitant fall in extinction at 450mμ is about 0.55 for a film, and 0.26 for aqueous solution). Thus in a film there may be several decomposition products of which reduced flavin could be one. The lack of shape of the product absorptions also argues against the

Fig. 2-7 ; Flavin spectra in polyacrylamide films, O before bleaching, ● after bleaching, × product absorption



formation of a single species. In these circumstances it seems unlikely that the overall product spectrum is strongly dichroic.

The corrections for product absorptions (assumed not to be dichroic) during photobleaching of a film containing lumiflavin are given in table 2-3. Although these corrections are greater than those for depolarisation by scattering (table 2-2), their effect on the final corrected optical densities is still small.

The corrected optical densities can be used to calculate θ by both the methods discussed in the theory section. The two calculations give values of θ in good agreement with each other. The accuracy for different measurements on the same flavin is generally better than $\pm 2^{\circ}$. The results for the limited number of flavins studied by this method show that there are only small variations in θ among the different compounds (table 2-4).

A graph of the dichroic ratio after bleaching as a function of wavelength (fig.2-8) can be compared with the variation of polarisation of fluorescence with wavelength (fig.2-5). Both graphs show that the functions are constant but different for each absorption band. This supports the assumptions made about the negligible dichroism of product

Table 2-3

Corrections for product absorption (assumed unpolarised) during dichroic bleaching of lumiflavin (film a)

Figures in parenthesis give the number of flashes used to induce bleaching

Wavelength ($m\mu$)	Extinction of the product			Corrected Extinction of unreacted lumiflavin (Extinction from table 2-2 - Extinction of products). Parallel to bleaching			Perpendic. to bleaching		
	(150)	(300)	(450)	(150)	(300)	(450)	(150)	(300)	(450)
460	0	0	0	1.01	0.95	0.90	1.09	1.06	1.03
450	.008	.014	.019	1.07	1.00	0.93	1.14	1.08	1.05
440	.009	.016	.021	0.99	0.91	0.87	1.05	1.01	0.99
380	.036	.072	.108	0.82	0.78	0.71	0.85	0.81	0.77
370	.036	.072	.100	0.91	0.86	0.82	0.94	0.91	0.87
360	.051	.089	.114	0.92	0.88	0.86	0.96	0.91	0.89

Table 2-4

Angle between the two long wavelength absorption oscillators (θ) for several flavins

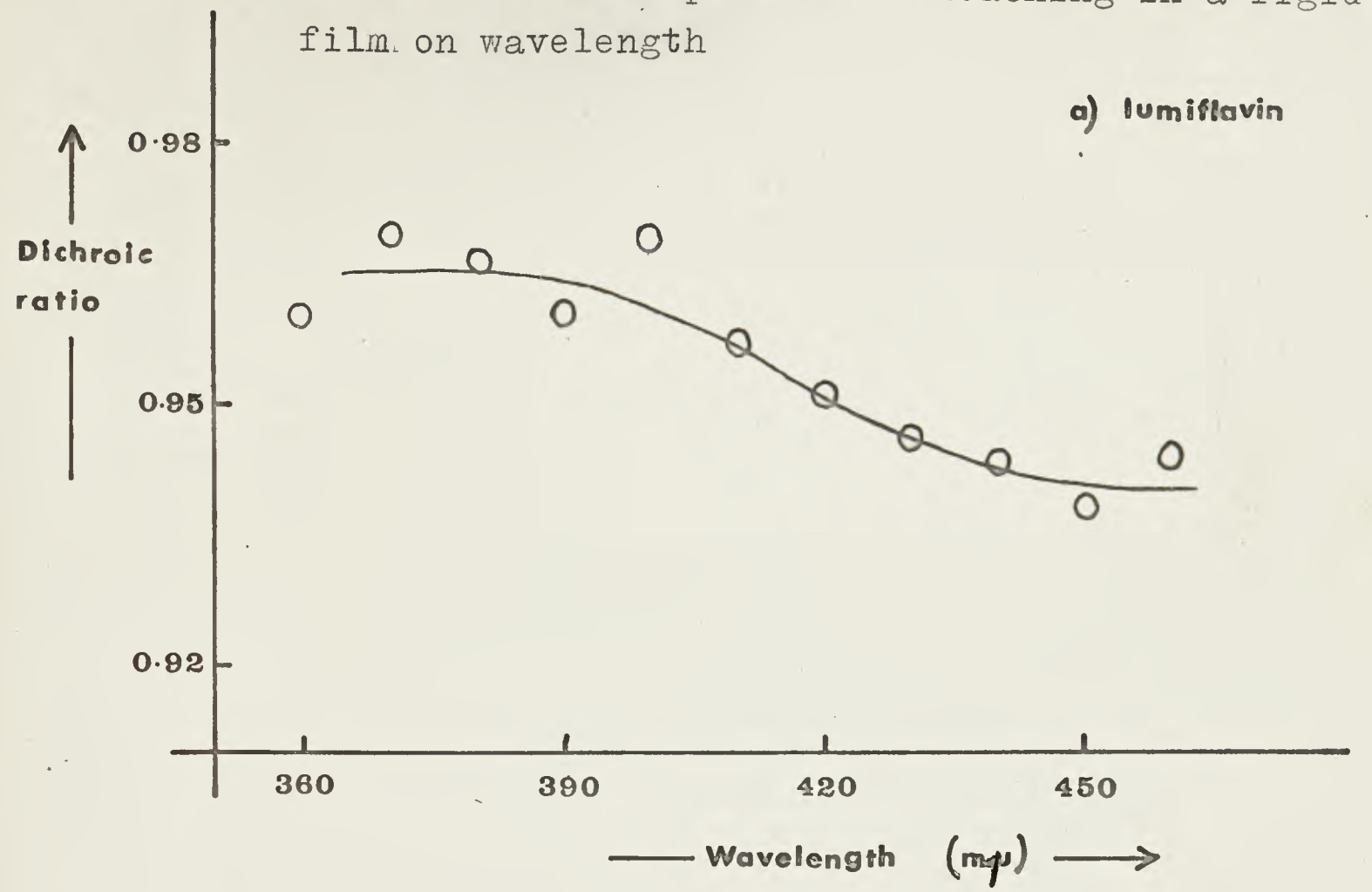
Flavin	No. flashes	Dichroic ratio ($\frac{\text{par. to bleaching}}{\text{per. to bleaching}}$) near u.v. band	Dichroic ratio ($\frac{\text{par. to bleaching}}{\text{per. to bleaching}}$) visible band	θ° (from dichroic ratio)
lumiflavin (film a)	150	.965	.936	28.5
		.965*	.937*	28.5*
		.964/	.938/	27.5/
	300	.957	.906	32
		.961*	.908*	33*
		.958/	.908/	32/
	450	.948	.874	33
		.950*	.881*	33*
		.943/	.880/	31/
lumiflavin (film b)	over 500	.948	.880	32.5
lumiflavin (film c)	200	.99	.95	42
3-methyl-lumiflavin	150	.980	.966	27
	300	.970	.929	33
	450	.965	.890	33
riboflavin	50	.982	.949	35
	100	.976	.931	35
	200	.962	.906	33
FMN	over 500	.882	.753	32
6-chloro-9-(N,N-diethylamino-propylisoalloxazine (film a)	150	.994	.935	42
	300	.988	.892	42
(film b)	200	1.0	.974	45

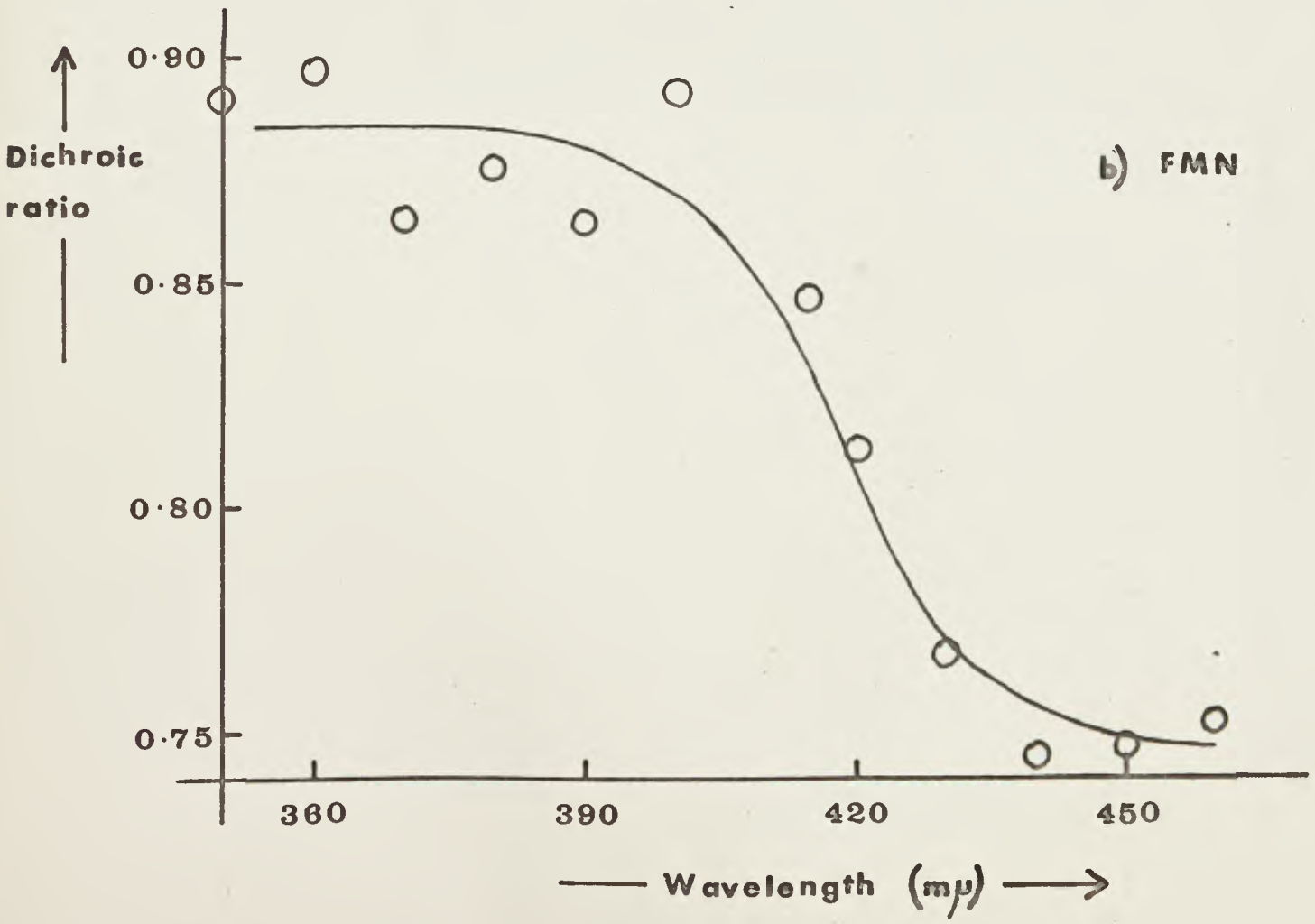
Lumiflavin (film a)-calculated from the rate of fall of corrected extinction - $\theta = 32^\circ$

* After correction for scattering only

/ After correction for scattering and product absorption

Fig. 2-8 ; Dependence of the dichroic ratio of flavin extinction after polarised bleaching in a rigid film on wavelength





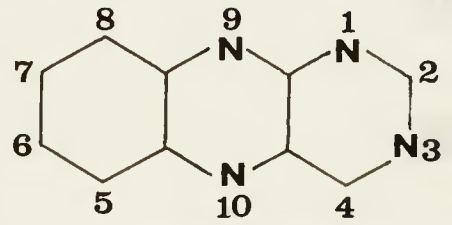
absorptions. If the assumptions were incorrect the dichroic ratio would vary in parallel with the change in extinction of the products.

Values of Θ found by dichroic bleaching (32° for FMN) agree well with those deduced from fluorescence polarisation. As the two methods are independent this is unlikely to be a coincidence.

C) Absorption spectra. It is not possible to deduce the absolute directions of absorption oscillators simply from their relative orientations. Some theoretical work suggests that the long wavelength transition is almost parallel to the long axis of the molecule with the near ultraviolet band as shown in figure 2-9 (Kurtin and Song, 1968), but no experimental confirmation of this has been forthcoming. Nevertheless a great deal of data on the spectra of flavin derivatives has been published (table 2-5), and it may be possible to learn about the directions of the transition moments from this.

The way substituents affect an absorption spectrum is usually dominated by mesomeric effects as inductive effects often change the energies of the ground and excited states similarly (Jaffé and Orchin, 1962). The effects are complex, and although there are simple empirical rules for the prediction of spectral shifts in some cases (e.g. the Woodward

Table 2-5
Absorption maxima of flavins



Flavin substituents								Absorption maxima of neutral species in water (figures in parenthesis are molar extinction coefficients) — mμ	Reference
2	3	4	5	6	7	8	9		
O	H	O	H	H	H	H	ribityl	335, 440	a
O	H	O	H	H	H	H	Me	348, 433	b
O	H	O	H	Me	H	H	$(CH_2)_2NEt_2$	356, 447	b
O	H	O	H	Me	H	H	ribityl	356, 440	a
O	H	O	Me	Me	H	H	ribityl	380, 450	c
O	H	O	Me	Me	H	H	ribityl	382, 448	d
O	H	O	Me	H	Me	H	ribityl	390, 440 (sh)	a
O	H	O	H	Me	Me	H	ribityl	375, 447 (12,000)	b
O	H	O	H	Me	Me	H	Me	375, 447	b

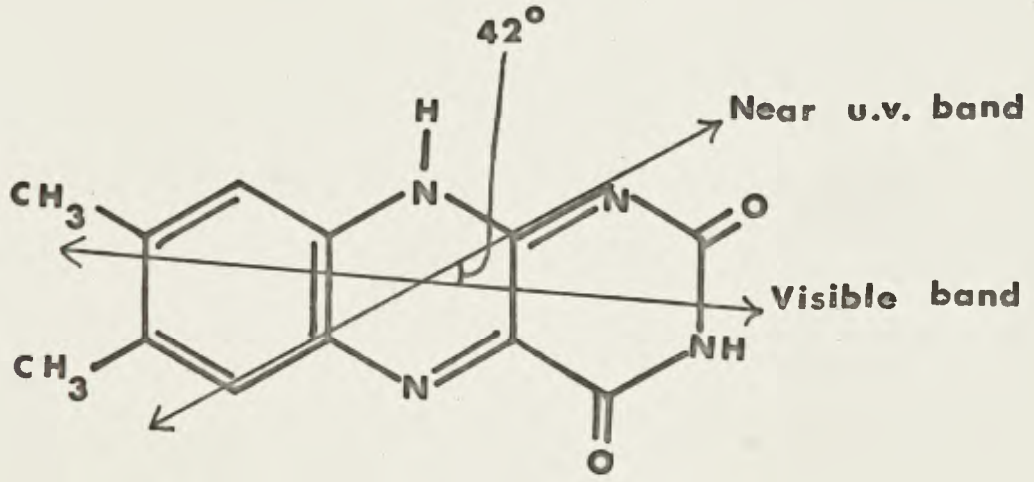
O	H	O	H	Me	H	Me	ribityl	372, 450	a
O	H	O	H	CH_2 CH_2 / CH_2		H	ribityl	359, 440	a
O	H	O	H	CH_2 CH_2 | | CH_2-CH_2		H	ribityl	363, 450	a
O	H	O	Me	Me	Me	H	ribityl	394, 446	d
O	Me	O	H	Me	Me	H	Me	369, 444	b
O	H	O	H	Cl	H	H	$(CH_2)_3NEt_2$	338, 446	b
O	H	O	H	H	Cl	H	$(CH_2)_3NEt_2$	361, 431	b
O	H	O	H	H	H	Cl	CH_3	385, 449	c
O	H	O	H	H	H	Br	CH_3	390, 451	c
O	H	O	H	H	H	Br	ribityl	395, 445	c
S	Me	O	H	Me	Me	H	Me	400, 494 (13,000)	e
O	H	S	H	Me	Me	H	Me	368, 494	e
NH_2	-	O	H	Me	Me	H	Me	358, 448	e
NH / $(CH_2)_2$-OH	-	O	H	Me	Me	H	Me	366, 451	e
$\begin{pmatrix} N \\ O \end{pmatrix}$	-	O	H	Me	Me	H	Me	368, 464	e

NHØ	-	O	H	Me	Me	H	Me	372,	470	e
H	-	O	H	Me	Me	H	Me	396,	456 (12,000)	f
OMe	-	O	H	Me	Me	H	Me	378,	442 (12,000)	f
OEt	-	O	H	Me	Me	H	Me	378,	442 (12,000)	f
SMe	-	O	H	Me	Me	H	Me	392,	454 (16,000)	f
O	⊖	NH_2	H	Me	Me	H	Me	358,	456	e
O	-	NH (CH_2) OH	H	Me	Me	H	Me	354,	468	e
O	-		H	Me	Me	H	Me	360,	468	e
O	-	NHØ	H	Me	Me	H	Me	362,	470	e
O	-	SMe	H	Me	Me	H	Me	368,	478	e

References

a, Kuhn, Velter & Rzeppa,1937; b,Penzer & Radda,1967a; c,McCormick,1967; d,Berezovskii & Radinova,1958; e,Müller & Hemmerich,1966;f,Müller,Walker & Hemmerich,1966.

Fig. 2-9 ; Theoretically calculated directions for the two long wavelength absorption oscillators of an isoalloxazine (taken from Kurtin & Song, 1968)



rules) there is no empirical system applicable to a polycyclic aromatic system like the flavin chromophore. It may still be possible to make useful deductions from i) comparison of flavin spectra with those of similar chromophores for which electronic assignments have already been made, ii) comparison of the spectra of flavin derivatives which differ only in the position of substitution of a single substituent, and iii) comparison of the spectra of derivatives which differ only in the nature of the substituent at a particular position. The reasons for spectral shifts which result from multiple changes in substitution are too complex to interpret.

The only flavin for which the detailed structure is known from X-ray analysis is the cation of 1,3,9 trimethylisoalloxazine (Kierkegaard, Norrestam, Werner, Ehrenberg, Erikson and Müller). Apart from a slight buckling in the pyrimidine ring the tricyclic nucleus is planar. There is no reason to believe that the neutral species (which is the one normally present in aqueous solution at neutral pH) is much different. It is thus reasonable to compare the spectra of flavins with those of tricyclic hydrocarbons and other heterocyclic molecules.

The spectra of cata-condensed aromatic hydrocarbons can be classified by Platt's method (Platt, 1949). The spectra of anthracene and acridine at neutral pH are similar (table

2-6). The bands around 350 mμ are assigned to the $^1L_a \leftarrow {}^1A$ transition, and the 250 mμ band to $^1B_b \leftarrow {}^1A$. These have been shown to have the expected transverse and longitudinal polarisations respectively for anthracene (Jones, 1945). For both anthracene and acridine the $^1L \leftarrow {}^1A$ transitions should have similar energies, but the ^1L_b band is much less intense (Klevens and Platt, 1949). Substituents round the ring may alter this situation. They may change the position of one or both of the bands (a bathochromic shift often corresponds to an increase in extinction as well) and they may profoundly affect the extinctions by altering the symmetry of the chromophore (Jaffé and Orchin, 1962).

Consider now some amino-acridines. Most of these have a different spectrum from unsubstituted acridine, with a new absorption around 400 mμ. This could be either of the $^1L \leftarrow {}^1A$ transitions shifted from its position in acridine. It is possible that in the 1,2,3 and 4-monoaminoacridines the 400 mμ band is $^1L_b \leftarrow {}^1A$, but in 9-monoaminoacridine it is $^1L_a \leftarrow {}^1A$. This is suggested because increasing the conjugation in the end rings usually favours longitudinally polarised transitions while a conjugative substituent in the 9-position directly aids a transversely polarised transition across the centre ring (Jaffé and Orchin, 1962). The different

Table 2-6

Absorption maxima of anthracene, acridine, and some acridine derivatives

Compound	Long wavelength Absorption maxima(mμ) figures in brackets are maxima of vibrational bands apparently associated with the same electronic transition
anthracene	252, (339,356,374)
acridine	249, 351
acridinium cation	254, 353
1-aminoacridine	263, (341,358), 414
1-aminoacridinium cation	290,357,529
2-aminoacridine	259, (338,355), 404
2-aminoacridinium cation	273,371,461
3-aminoacridine	262, (336,353), 410
3-aminoacridinium cation	272, (350,365), 454
4-aminoacridine	262, (343,360), 407
4-aminoacridinium cation	250,275,362,446
9-aminoacridine	260,389, (407,430)
9-aminoacridinium cation	259,326 (400,422)
1-hydroxyacridinium cation	272,355,454
9-hydroxyacridine anion	267,340, (411,435)
9-hydroxyacridine zwitterion	254, 294, (380,398)
2,7--diaminoacridine	263,293,391
3,6,diaminoacridine	262,284,395
4,5,diaminoacridine	269,447
2,6,diaminoacridine	272,350,439

All these values are taken from DMS, U.V.atlas of organic compounds, Butterworths and Verlag Chemie, 1966.

assignment of the band in 9-monoaminoacridine is supported by the spectral changes which occur on protonation of the ring nitrogen. The 400 mμ bands of 1,2,3 and 4-monoaminoacridines shift about 50 mμ to the red on protonation, but with 9-monoaminoacridine there is a blue shift of about 10 mμ (table 2-6).

A similar explanation can be given for the spectra of diaminoacridines. The $^1L_b \leftarrow {}^1A$ transition does not appear as a discrete band in the spectra of symmetrical diaminoacridines because its extinction is too low relative to that of the $^1L_a \leftarrow {}^1A$ band. This is itself shifted further to the red because of the increased conjugation of the chromophore. In contrast unsymmetrical 2,6-diaminoacridine does show the $^1L_b \leftarrow {}^1A$ band well red-shifted as expected.

The extrapolation from considering acridine spectra to those of flavins is a big one, and so it is not possible to deduce spectral assignments with complete certainty. If the flavin chromophore is considered as a tricyclic nucleus with two strongly conjugating substituents in the 2 and 4 positions it can be predicted that the $^1L_b \leftarrow {}^1A$ band is red-shifted from its position in acridine and enhanced in intensity, like in the 2 and 4-aminoacridines. Thus it is likely that the 450 mμ band of a flavin is the $^1L_b \leftarrow {}^1A$

transition, and hence it is polarised longitudinally. The 370 mμ band is $^1L_a \leftarrow {}^1A$, polarised transversely. These polarisations are not accurately in the directions suggested both because of the disymmetry of the molecule, and because of the directing effects of the various substituents and heteroatoms. There is also an element of charge-transfer even in the $\pi-\pi^*$ transitions of a flavin (Penzer and Radda, 1967). Spectral shifts in different solvents (Harbury, La Noue, Loach and Amick, 1959) indicate that charge transfer is greater in the 370 mμ transition than in the 450 mμ one.

When considering the spectral shifts between flavin derivatives small changes are not significant as they may result from effects other than changes in the energy levels of the excited states (e.g. changes in orbital overlap or band shape). However any substituent that extends the conjugation of the ring system should cause bathochromic shifts in the $\pi-\pi^*$ absorptions. In the case of bands with a transition dipole moment blue shifts may occur if there is a strong inductive effect to oppose the charge transfer.

Substituting methyl groups in the benzene ring of an isoalloxazine causes a red-shift of the long wavelength absorption band, but the position and number of the substituents do not have a great effect on the magnitude of

the shift. The near ultraviolet band has a larger shift in the same direction, with 5 and 8-methyl groups being more effective than 6 and 7 ones (table 2-5). This supports the tentative assignments made by analogy with the acridines.

The effects of substituting chlorine in the benzene ring are mostly similar to those found for methyl substitution (table 2-5), but the absence of a bathochromic shift in t he visible band of the 7-Cl derivative is inconsistent (see below).

Most of the 9-substituents studied are of the form $-CH_2R$, and so they do not conjugate with the unsaturated ring system. Thus it is not surprising that the spectral changes on altering these 9-substituents are insignificant.

Apart from substituting in the 3 position changes in the groups round the pyrimidine ring cause reorganisation of the conjugation of the chromophore. It still seems, however, that bathochromic shifts of the visible band are largest when the longitudinal conjugation is increased, while similar shifts of the near ultraviolet band are greatest for increases in transverse conjugation (table 2-5).

Finally, considering the effects of different substituents at the same position on the flavin nucleus, the only two positions where more than a few replacements have been

made are C_2 and C_4. In both positions, similar substituents cause bathochromic shifts of increasing magnitude as conjugation goes up (e.g. primary, secondary, and tertiary N) (table 2-5). However it seems that inductive effects are not negligible because replacing =O by =S, or $-NH_2$ by -H there are red-shifts in the visible band, in spite of the lower conjugating abilities of =S and -H. The explanation of this may be that the small transition dipole of the visible band is caused by charge transfer from the pyrimidine ring towards the benzene on excitation. This could also explain the surprising absence of a bathochromic shift in the 7-Cl derivative. Substituents in the 7-position can conjugate directly with the 2 and 4-carbonyl groups, thus tending to oppose the absorption by pushing electrons from the benzene ring to the pyrimidine.

The spectra of the ions of riboflavin show that protonation of N_1 causes a large reduction in the extinction coefficient of the visible band or a considerable hypsochromic shift. The small bathchromic shift of the near ultraviolet band may be caused by a true change in the energy of the absorption, or by the effect of coinciding with the visible band. These changes are consistent with the idea that the visible band is $^1L_b \leftarrow {}^1A$, because protonation must reduce the

conjugation of the pyrimidine ring, thus increasing the energy required for the longitudinally polarised transition. The near ultraviolet band is not so sensitive to the conjugation of the pyrimidine ring.

The riboflavin anion has lost the proton at N_3, but this does not make a great difference to the unsaturated system. There is thus little change in the visible band, and the blue-shift of the near ultraviolet band may be caused by the negative charge on the pyrimidine ring inductively opposing the transition dipole of the band.

The picture which is obtained from this consideration of flavin spectra is not completely clear, but several conclusions can be drawn with some confidence:

i) The angle between the absorption oscillators of the two long wavelength bands of a flavin is about 30°.

ii) The direction of the oscillator of the visible band is approximately parallel to the long axis of the molecule, with a small element of charge-transfer from the pyrimidine ring towards the benzene one.

iii) The near ultraviolet absorption band has a larger charge-transfer contribution, perhaps tending to donate electrons to the pyrimidine ring. If molecular orbital calculations are correct, this transition is approximately in the direction shown in figure 2-9.

CHAPTER THREE

The Physical Deactivation of flavin excited states

Introduction

The way in which a flavin can absorb light to produce excited singlet states has been discussed in the previous chapter. The several ways in which this excitation energy may be lost without leading to chemical changes are the subject of this chapter.

The possible routes for deactivation of excited singlets are outlined in figure 3-1. The efficiencies of some of these processes are known in general terms for typical aromatic chromophores. Deactivation of higher excited singlets to the first excited singlet (k_1) is a very efficient process (Pringsheim, 1949). This is confirmed for flavins by the observation that the quantum yield of fluorescence is independent of the exciting wavelength (Penzer and Radda, in the press). As fluorescence is the $^1A \leftarrow {}^1L_b$ transition this means that the $^1L_b \leftarrow {}^1L_a$ relaxation is 100% efficient under the conditions studied.

Fluorescence (k_2) is the only one of the three general types of excited singlet deactivation that has been studied directly. The quantum yield of riboflavin fluorescence in aqueous solution is 0.26 (Weber and Teale, 1957), but the yields of other derivatives vary from 0.01 to 0.50

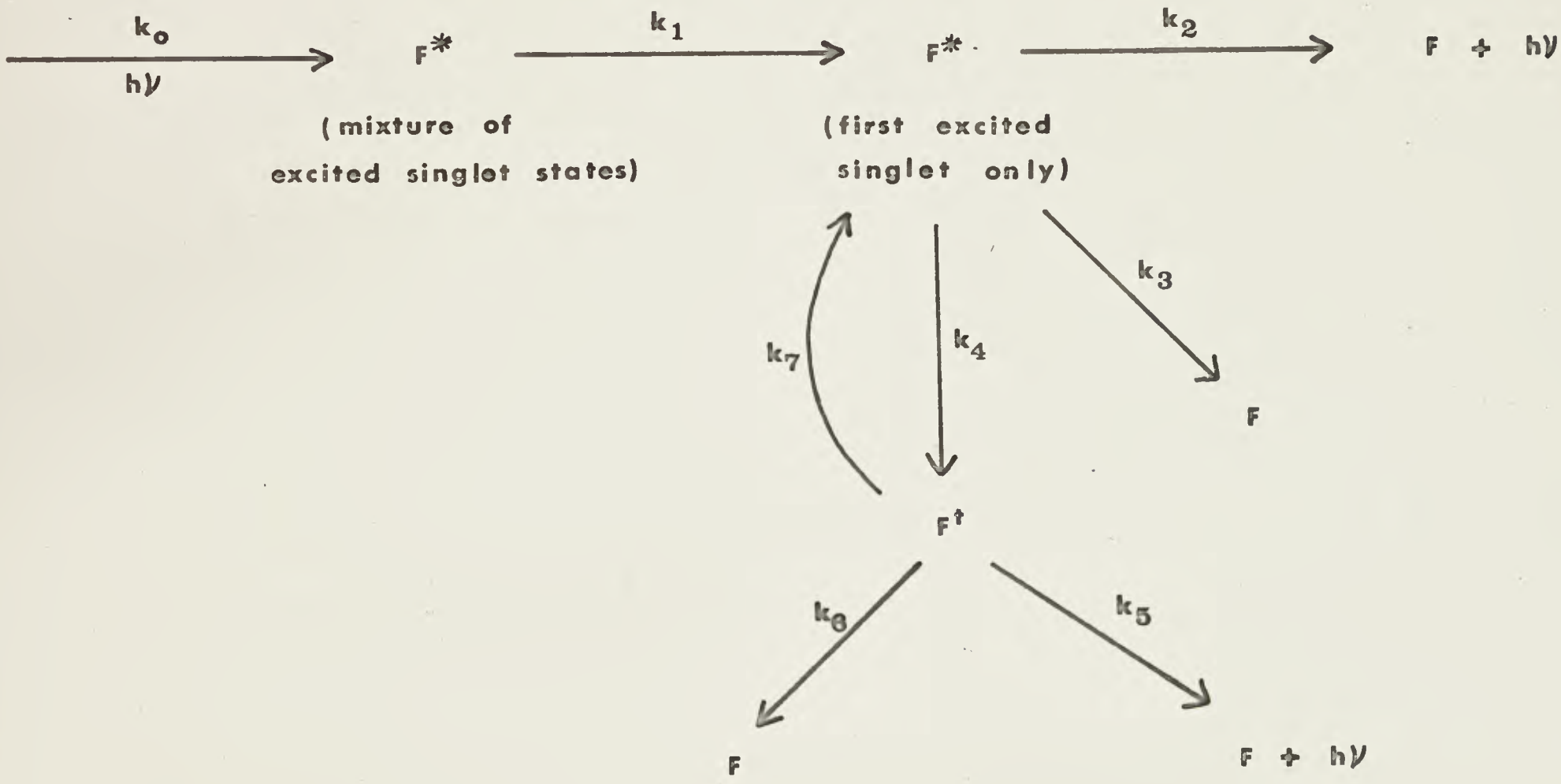


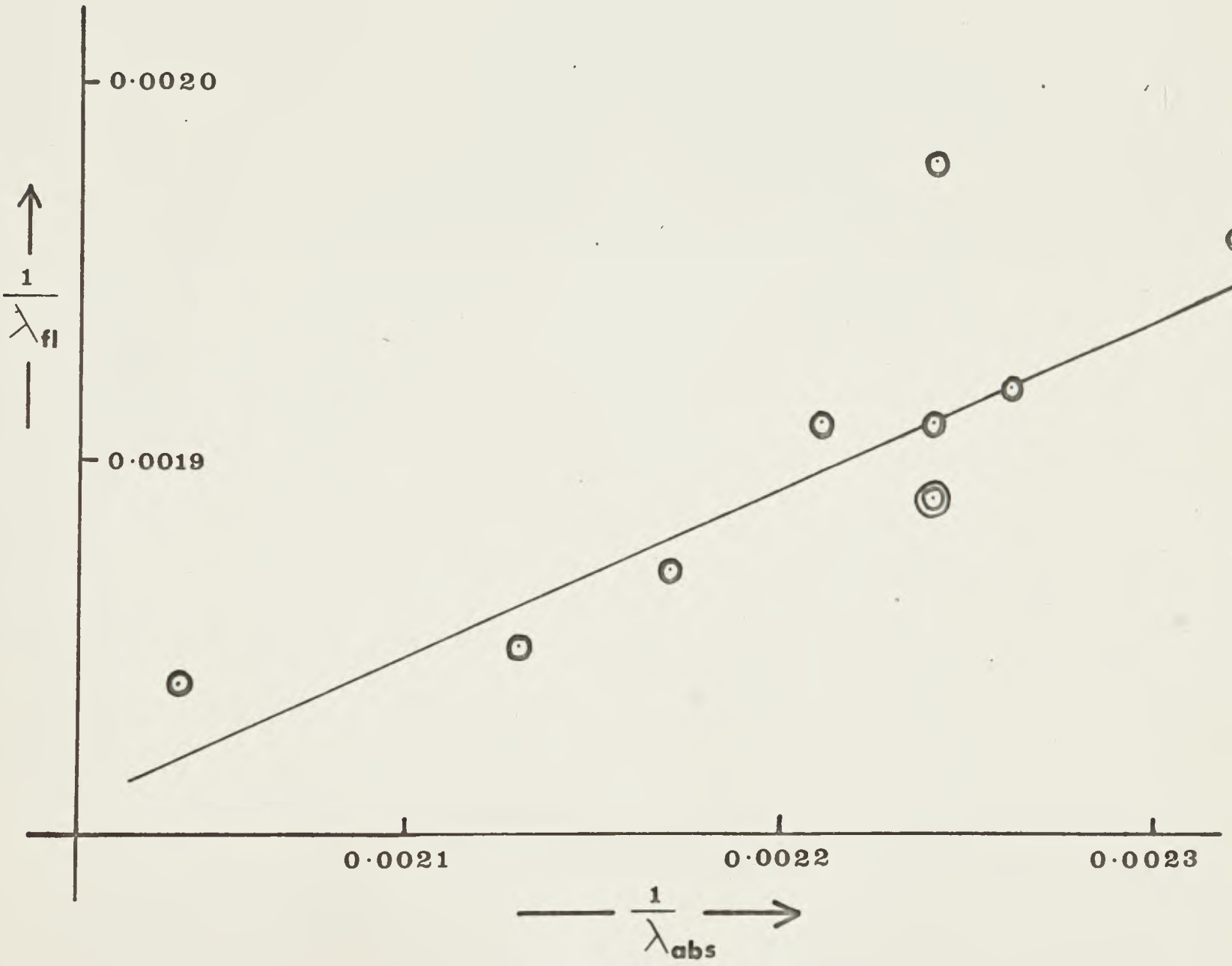
Fig. 3-1 ; Routes for the deactivation of flavin excited states

(Wentworth, 1966). Fluorescence is quenched either by complex formation with other aromatic molecules, or through collisional quenching by, for example, inorganic ions (Penzer and Radda, 1967a).

The positions of the fluorescence maxima and the quantum yields vary for flavin derivatives. Generally, if there is a bathochromic shift of the visible absorption band, the fluorescence maximum shifts in the same direction, but by a smaller amount of energy (fig.3-2). The difference between the absorption and fluorescence maxima is a measure of the solvation changes which follow electronic excitation, and so it also indicates the extent to which the electron distribution and geometry of the first excited singlet are distorted from those of the ground state. These effects are thus smaller for those flavin derivatives which are excited at lower energies.

The fluorescence lifetimes of a few flavins have been measured with a decay-time fluorimeter (Chen, Yurek and Alexander, 1967). The lifetimes of riboflavin, FMN and FAD are all similar, confirming that the very low quantum yield for FAD is caused by the formation of a non-fluorescent intramolecular complex (Weber, 1950; Gascoigne and Radda, 1965a). The lifetimes of a wider variety of derivatives have been measured (Wentworth, 1966) using the fluorescence

Fig. 3-2 ; Dependence of the position of the fluorescence maximum on the position of the absorption maximum for some flavin derivatives (taken from Penzer, 1966)



quenching method (Wawilov, 1929). For collisional quenching, $\frac{F_o}{F} = \frac{\tau_o}{\tau}$ (F_o and τ_o are the fluorescence efficiency and excited state lifetime in the absence of a quencher, F and τ are the same parameters in its presence). The theory was derived for perfect collisional quenching, and takes no account of charge interactions, solvation and other non-ideal behaviour in solution. It is an unreliable absolute method, but for a series of similar chromophores and a single quencher in the same solvent it is a reasonable comparative technique.

Changing the substituents on a chromophore alters its lifetime and quantum yield of fluorescence for several reasons. There may be a change in overlap between the orbitals of excited and ground states; there may be a change in the efficiency of intersystem crossing to the triplet state; there may be a change in the susceptibility to intermolecular quenching. It has not yet been possible to sort out the relative contributions of each of these effects to the change in quantum yield caused by a particular substitution.

Quantum yield (Q) and lifetime are related by the expression $\tau = \tau_i Q$ where τ_i is the intrinsic lifetime of the first excited singlet in the absence of all non-radiative deactivation processes. Thus the quantum yield increases with lifetime for a given emitter. Wentworth's observations

show that the same situation usually arises when comparing flavin derivatives. This means that τ_i ($= \frac{1}{k_2}$, fig.3-1) varies little for the different compounds. k_2 depends on the overlap between the orbitals of the ground and first excited singlet states of the flavin, and it is a similar integral that governs the extinction coefficient in absorption. There is little variation in the extinction coefficients of flavins without 5- and 8- substituents (Müller, Walker and Hemmerich, 1966; McCormick, 1967), and so the probability of emission (and hence k_2) is also likely to be fairly constant. Those derivatives with low extinction coefficients at 450 mμ all have low fluorescence yields (Berezovskii and Rodinova, 1958; McCormick, 1967).

The quantum yield of fluorescence of most flavins is around 0.25 for aqueous solutions at room temperature, and so the non-radiative processes (k_3 and k_4) must account for the deactivation of three quarters of the excited singlets. k_3 includes dissipation of the excitation energy into the vibrational modes of the chromophore and the translational modes of the solvent or added quencher. k_4 depends on the magnetic perturbations (including those caused by added paramagnetic ions) which reduce the forbiddeness of singlet to triplet intersystem crossing.

It has been possible in some cases to measure the rate of internal conversion to the ground state (k_3), and when care is taken to use pure solvents this rate constant is low (about 10^6 sec^{-1}) (Porter, 1968). Non-radiative deactivation from the excited singlet to the ground state is thus a relatively unimportant process. The same conclusion is drawn from the quantum yields of fluorescence and phosphorescence of 9-phenylanthracene, which add up to one (Horrocks, Medinger and Wilkinson, 1967).

There is still one deactivation mechanism which falls into the general class of k_3, which may be very efficient under favourable conditions, and that is energy-transfer. The simplest picture of this process is of a non-radiative transfer of energy from one molecule to another by resonance coupling (Förster, 1959). It is a long range process which may be efficient up to distances of 50 A° or more. The other requirement for effective energy-transfer is that the emission spectrum of the donor overlaps with an absorption band of the acceptor. The theoretical expression for the efficiency of energy tranfer depends on the assumptions which are made (Cellarius, 1967). Usually Förster's expression, which is applicable when there is rapid thermal equilibration of excited vibrational states, is appropriate. It is

$R_o^6 \approx \frac{9000(\ln 10)K^2 \phi_f}{128\pi^5 n^4 N} \int_o^\infty \frac{f_D \epsilon_A . d\bar{\nu}}{\bar{\nu}^4}$, where R_o is the donor-acceptor distance at which energy-transfer is as likely as spontaneous decay, K^2 is an orientation factor (about 2/3 for a random array), ϕ_f is the fluorescence efficiency, f_D is the fluorescence emission spectrum normalised to unity, ϵ_A is the molar extinction coefficient of the acceptor, $\bar{\nu}$ is the wavenumber in cm^{-1}, n is the refractive index of the medium, and N is Avogadro's number.

Aqueous solutions of flavins on which optical measurements can be made must usually be less than 10^{-3}M because of the high extinction of the chromophore. This concentration corresponds to an average intermolecular distance of about 100 A°. This is generally too great a distance for energy transfer, especially considering that the longwavelength absorption band of most flavins does not overlap much with the fluorescence emission.

Some molecules can be added to a solution of flavin which quench the first excited singlet. Sometimes this may be because of the formation of a non-fluorescent complex with the ground state (see p. 9). Aromatic quenchers often function by this route. Alternatively some species

(particularly ions such as I^-) quench by collision with the excited states. Whether the mechanism falls into the category of k_3 or k_4 is uncertain, though it is perhaps more likely to be the latter (Porter, 1968).

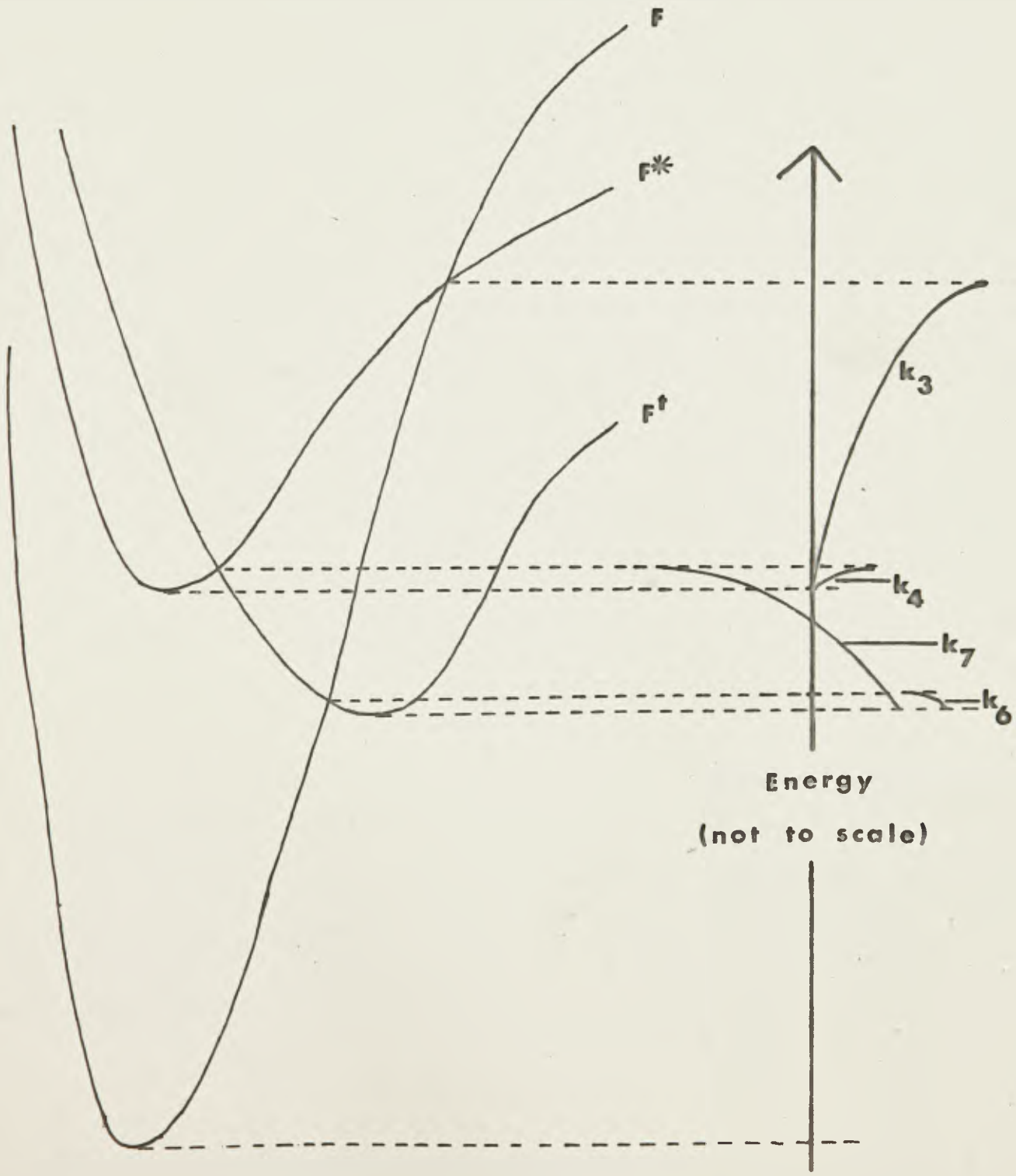
It is therefore apparent that deactivation of excited singlets via the triplet (k_4) is a very important process. The subsequent decay of the triplet may be by direct light emission (k_5), radiationless decay to the ground state (including energy-transfer) (k_6), or thermal re-excitation followed by intersystem crossing back to the first excited singlet (k_7). The sizes of k_5,k_6, and k_7 are different from the corresponding singlet deactivation processes, k_2, k_3, and k_4. k_5 is much less than k_2 because phosphorescence involves a forbidden transition, and k_7 is less than k_4 because of the activation energy requirements. Hence the times involved for triplet inactivation in aqueous solutions are typically about 1000 times longer than those for the excited singlet and in a rigid matrix lifetime is increased still further (Porter and Windsor, 1954).

The phosphorescence of flavins (k_5) was first reported in 1938 (Bhere and Castelli, 1938) and has since been the subject of several investigations (e.g. Lhoste, Haug and Hemmerich, 1966; Bowd, Byrom, Hudson and Turnbull, 1968).

In every case a rigid solvent was used, and in no case has delayed light emission from fluid solutions of flavins been observed. This is in accord with the finding that to detect delayed light emission from a solution the solvents and chromophores must be scrupulously purified and degassed, and the solvent should preferably be non-aqueous (Parker, 1968). Nevertheless the value of k_5, which is a true unimolecular process and is independent of temperature, may not be greatly affected by the rigidity of the solvent. The half life of phosphorescence in a glass is about 100 msec. (Bowd, Byrom, Hudson and Turnbull, 1968) and sets a lower limit to the rates of other significant deactivation processes in solution.

Radiationless internal conversion of the triplet to the ground state (k_6) and re-excitation to the first excited singlet (k_7) both involve intersystem crossing between the triplet and a singlet. The relative magnitudes of k_6 and k_7 depend on both the efficiencies of intersystem crossing, and the relative activation energies required before the crossing can occur (fig.3-3). k_6 is relatively more important than k_3 in spite of the change of spin because the radiative lifetime of the triplet is longer than that of the excited singlet, and also, perhaps, because the activation energy for intersystem crossing is less.

Fig. 3-3 ; Morse curves for F, F^*, and F^t. Energies are not to scale, and the curve for F^t is shifted laterally for clarity



None of the individual rates k_2 - k_7 have been measured for the flavin system. One report of the values of k_2, k_4 and k_6 is based on incorrect assumptions (Byrom and Turnbull, 1968).

This chapter contains the results of two series of experiments. In the first, singlet-to-singlet energy-transfer between flavins has been studied by observing the properties of flavins in thin films. The techniques of making the films and observing their properties had previously been developed (Penzer, 1966). Secondly, delayed light emission from flavins in a rigid matrix has been studied. In addition to phosphorescence, which is observed at low temperatures, E-type delayed fluorescence is seen at room temperature. This sort of emission is quite rare, but has been conclusively identified for this case. By measuring the dependence of the lifetime and intensity of both phosphorescence and delayed fluorescence on temperature it has been possible to estimate the activation energies of k_4, k_6, and k_7 for a series of flavins.

Measurements and Accuracy

A) Preparation of methyl cellulose films. 2.25 gm. of powdered methyl cellulose were gradually added to 100 ml of distilled water at 60 - 70°, with constant stirring. Under

these conditions a homogeneous suspension is formed which is free from bubbles. On cooling the suspension to 10° by surrounding the vessel with ice the methyl cellulose dissolved to give a clear, homogeneous solution. If any bubbles were present they were all on the surface of the solution, and were readily displaced by adding a drop of methanol to lower the surface tension.

The compounds to be included in the film were next dissolved in the methyl cellulose solution, stirring carefully to ensure homogeneity whilst avoiding the formation of bubbles. 3 or 4 drops of this sticky solution were spread on a microscope slide and allowed to dry for 24-48 hr. to form a film.

Most experiments were made with the film still stuck to the slide, but if required separately it was quite possible to peel the cellulose away from the glass.

B) Fluorescence measurements. Fluorescence and polarisation of fluorescence measurements were made on methyl cellulose films using the Zeiss spectrofluorimeter (see p. 24). The film (on its slide) was attached by adhesive tape to the normal cuvette basket which was placed in an angled position in the instrument so that front face fluorescence was measured at 90°.

The accuracy of fluorescence polarisation measurements on films in the angled sample position is not as good as that normally obtained for clear solutions. Scattering of light both from the surface of the film and from particles within it becomes significant, and probably causes some depolarisation. These two effects have been investigated (Penzer, 1966).

Measurements of the fluorescence polarisation of a solution of FMN in glycerol show that on changing from the vertical to the angled position in the fluorimeter the polarisation falls by less than 15%.

Depolarisation by light scattering was shown to be unimportant in two sets of experiments. a) The polarisation of FMN in glycerol (vertical position) fell by less than 10% in the presence of sephadex which scattered far more light than a film. In the angled position this depolarisation was even lower. b) The fluorescence polarisation of the flavoprotein lipoyl dehydrogenase (molecular weight about 100,000) is 0.43 both in water and in a concentrated solution of methyl cellulose. The fluorescence polarisation which is measured does not depend on whether the film is between the slide and the cuvette basket or on the outside of the slide.

Thus the polarisations measured for chromophores in methyl cellulose films are not more than 15% lower than their true values.

C) Delayed emission measurements. The rigid supporting matrix used in the studies of delayed light emission was a polyacrylamide gel, prepared by the method described on p. 26 .

The fluorimeter used for the delayed light emission measurements was specially designed for the purpose. The plans were based on C.A.Parker's instrument (Parker, 1964) with the aim of combining the maximum possible flexibility and sensitivity in a single instrument. The sensitivity was achieved by using a powerful light source, chopping the signal (200 or 800 cps), and detecting it with a good 11 or 13 stage photomultiplier. The signal from this tube could either be read directly or be amplified by a specially constructed phase-sensitive amplifier which dealt with signals down to 3 μv full scale deflection. The flexibility of the instrument was partly the result of a spacious sample-compartment arrangement which allowed a wide variety of optical allignments. Lifetime measurements were made possible by using synchronous motors to drive the choppers in the excitation and emission light paths. By rotating one of these so that the two chopping discs were either in or

out of phase it was possible to observe either prompt or delayed light emissions. The instrument was designed to be used in either a double or a single beam mode, but all the measurements in this thesis employed the single beam arrangement.

Figure 3-4 is a diagram of the fluorimeter; the legend gives details of the components. Two sets of discs were used with the synchronous motors, which ran at 50 cps. One pair had four slots per disc and the other sixteen. One of the major problems when studying delayed emissions at wavelengths where there was also prompt fluorescence or appreciable scatter of the incident light was obtaining perfect eclipsing of the discs so that only the delayed signal was detected. In this situation the 4-slot discs (in which the slot is a sector of about 30° with the dark patch 60°) were used because it was impossible to eliminate detection of some scattered light from the edges of the 16-slot discs. For maximum sensitivity with undelayed fluorescence and when there was no interfering prompt signal the faster chopping rate was preferred because a better signal/noise ratio was obtained.

The phase sensitive amplifier was triggered by the signal from a small photocell which detected light from a pilot lamp on the opposite side of the emission chopping disc. The

A xenon lamp (Bellingham & Stanley)

B prism monochromator (Hilger & Watts)

C Evershed Vignoles synchronous motors and chopping discs

D lens

E mirror

F sample

G lamp and photocell for triggering amplifier

H grating monochromator (Bausch & Lomb)

I photomultiplier (EMI 988 B); J power pack

K phase sensitive amplifier

L oscilloscope (Telequipment S 43)

M camera

N trigger

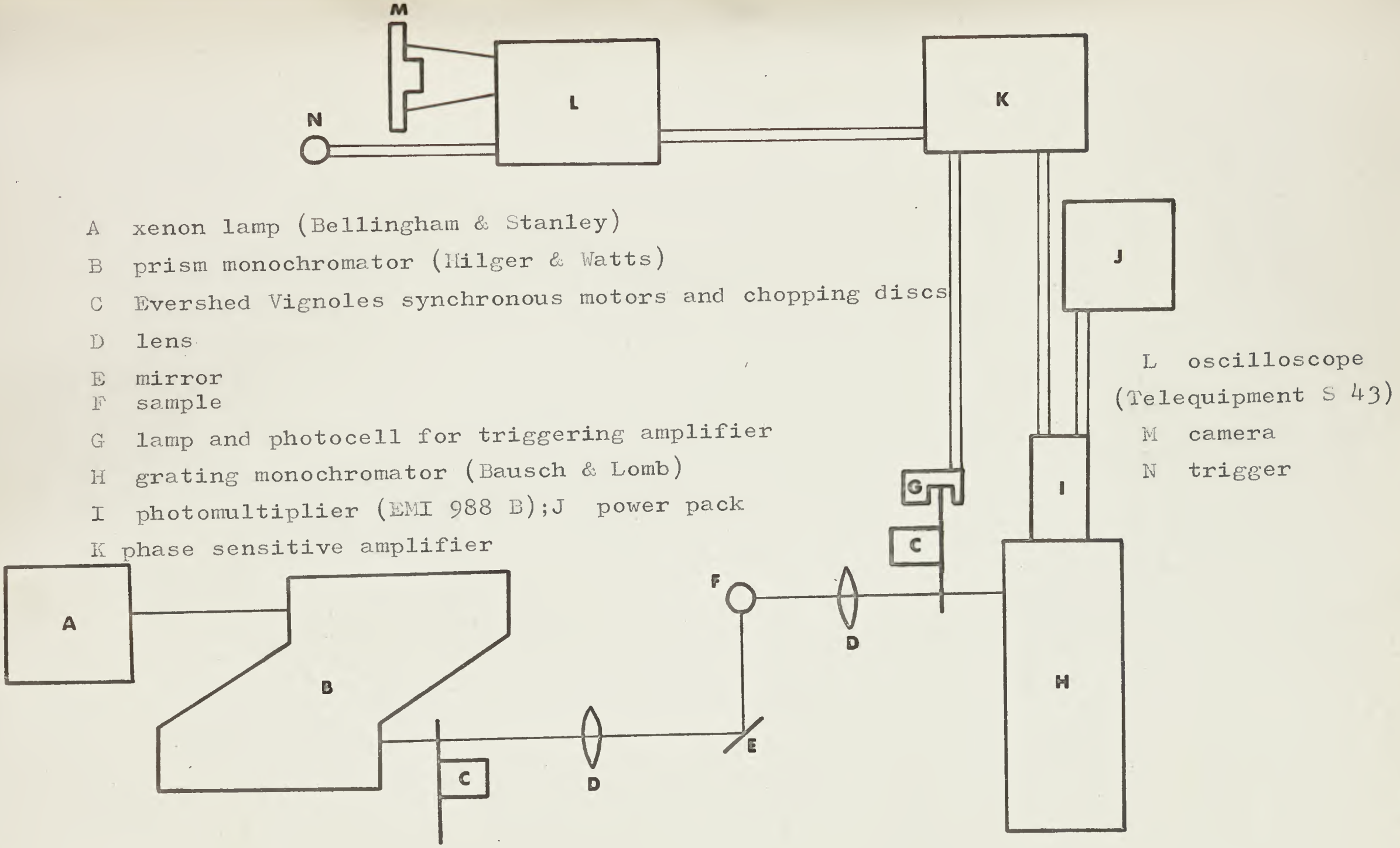


Fig. 3-4 ; Diagram of the fluorimeter used for delayed emission measurements

position of the cell and its lamp could be adjusted mechanically so that the triggering of the amplifier was perfectly in phase with the signal it detected.

The amplifier response time was slow (about 0.5 sec.) and so measurements of the lifetimes of delayed emissions could not be made with it in the circuit. For some of these experiments the photomultiplier signal was fed directly onto the y-plates of an oscilloscope, but it was also possible to increase sensitivity by using the amplifier with the slow rectification part of its circuit by-passed. The amplifier response, when displayed on an oscilloscope screen, now showed a chopped signal. Lifetime measurements on the same sample using the two modes of observation gave the same half-life, showing that the response-time of the amplifier was quite fast enough when connected in this way. The noise level on a signal varied from 5-20% depending on the intensity of the emission and the homogeneity of the sample.

The technique for lifetime measurements was as follows. The signal was connected to the y-plates of an oscilloscope, with the x-axis used as a time base. The trace was triggered manually, by discharge of a condenser, a few milli-seconds before the exciting light was removed from the sample by closing a shutter. The curved trace obtained in this way was photographed with a Polaroid camera (Thompson Land).

Knowing the time base of the trace the half-life of the emission could be measured directly from the picture. With a little practice it was possible to close the shutter about 5 msec after triggering the oscilloscope. The time taken in closing it was short (about 3 msec) compared to the lifetimes being studied (see fig.3-5 , which also shows a typical decay curve).

The temperature of the sample was measured with a copper/eureka thermocouple with its reference junction in ice/water. It was calibrated against a mercury in glass thermometer above 0° and using the boiling point of nitrogen and the subliming temperature of carbon dioxide as fixed points for calibration below 0° (fig.3-6). The potential between the junctions was measured with a Servoscribe potentiometric recorder. The junction was attached to a polyacrylamide film by adhesive tape, with a small amount of silicon grease to ensure good thermal contact with the sample.

The temperature of a sample was lowered by immersion in liquid nitrogen or acetone containing solid carbon dioxide. It was raised to temperatures above room temperature by warm air from a hair dryer. The films remained transparent at low temperatures and in liquid nitrogen or acetone, but they became opaque when heated much above 50°. Contact with

Fig. 3-5 ; Sample oscilloscope traces

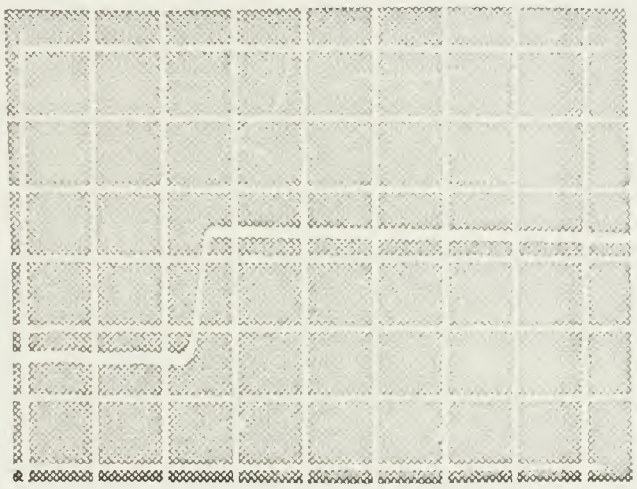
a) shutter closing - no amplifier in circuit

b) shutter closing - amplifier in circuit but with its rectifier by-passed

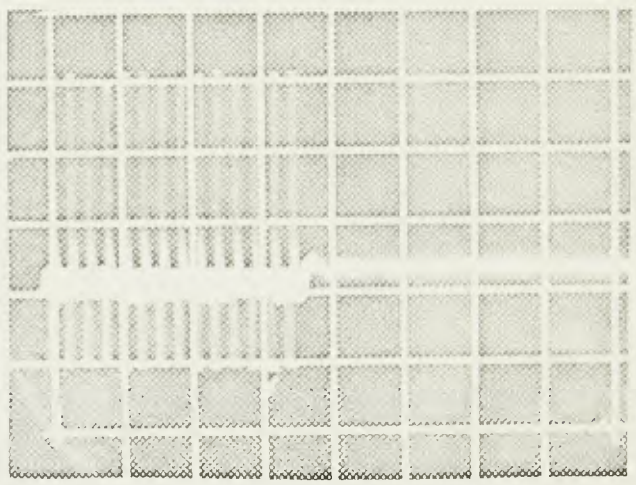
c) sample decay curve for delayed fluorescence

The line spacing on the x-axis of the grid corresponds to 20 msec.

a)



b)



c)

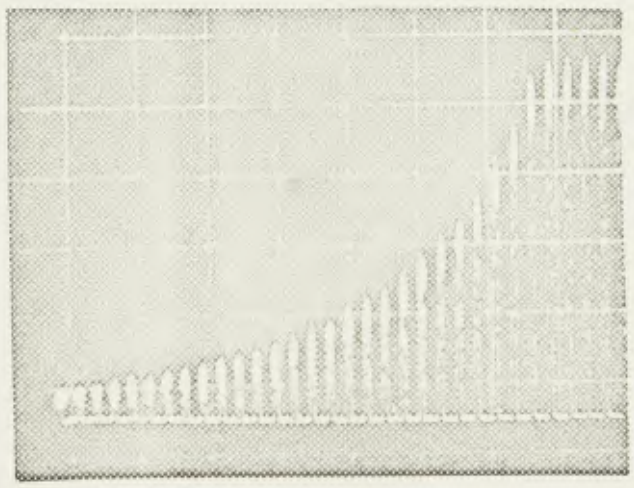
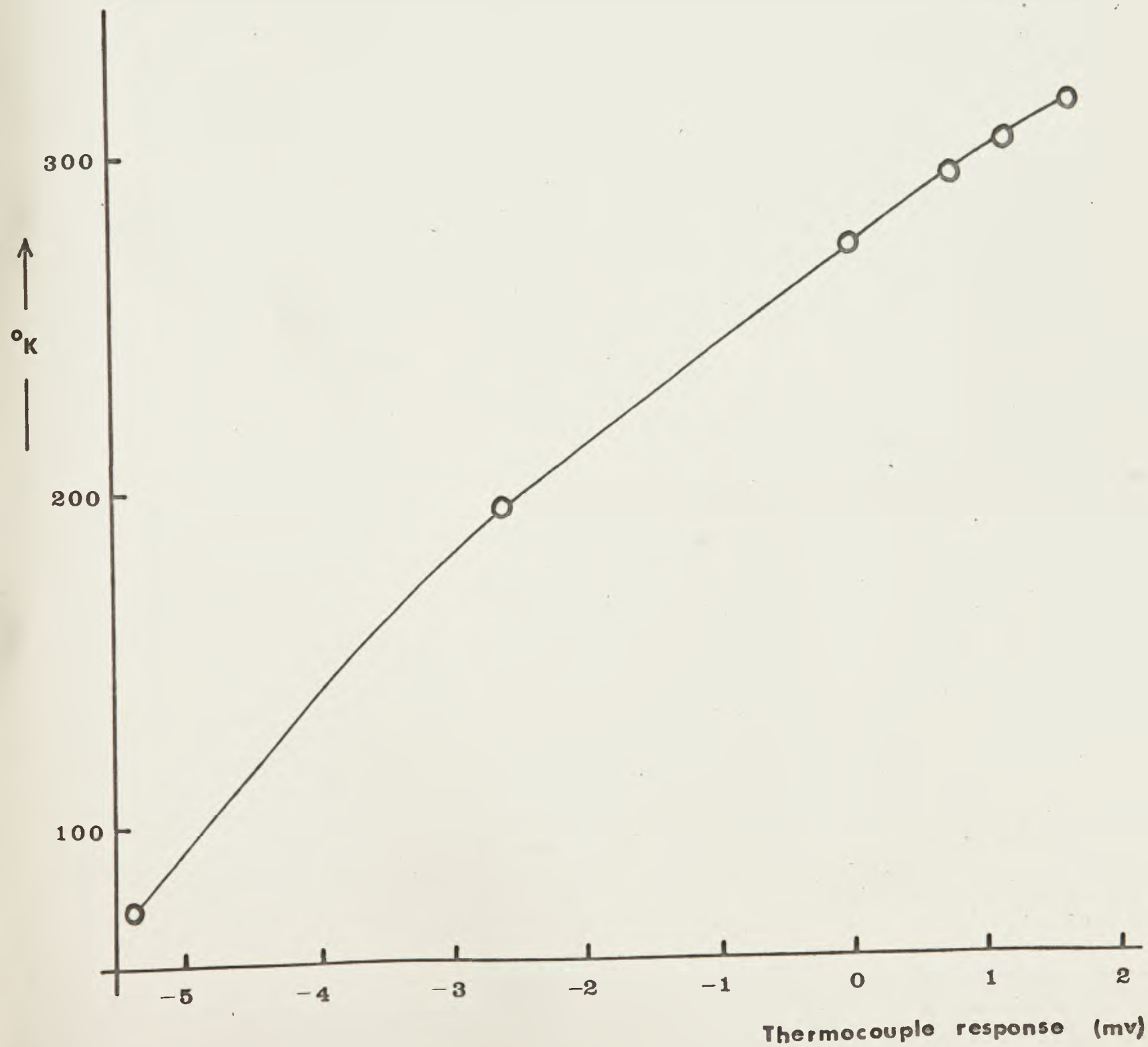


Fig. 3-6 ; Thermocouple calibration curve



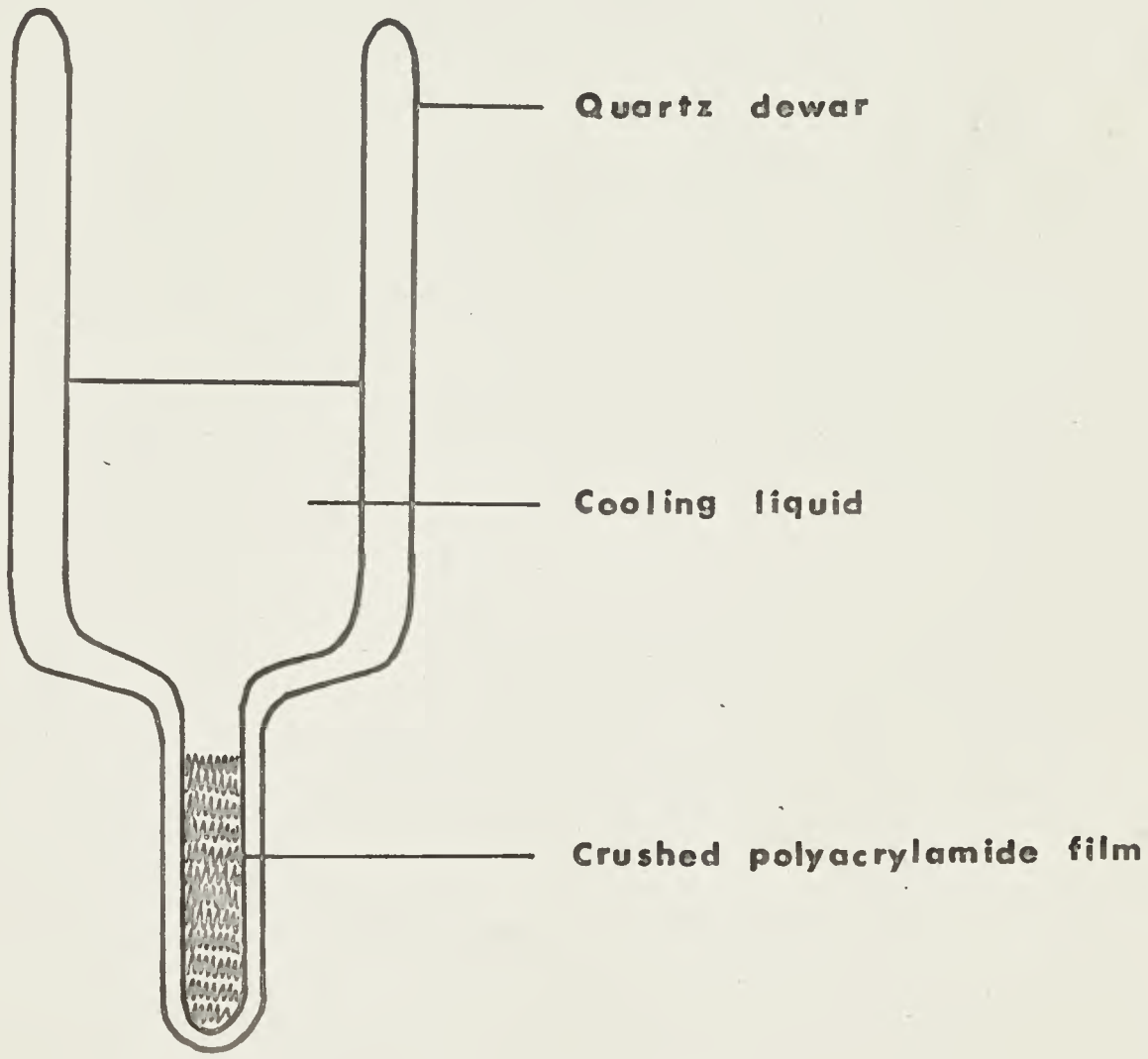
water also spoiled a polyacrylamide film, causing both softening and loss of clarity.

Studies of the temperature dependence of phosphorescence from liquid nitrogen temperatures upwards involved a modified experimental arrangement. A polyacrylamide film was crushed into small splinters which were placed in the observation finger of a quarz dewar (fig.3-7). The dewar was subsequently filled with either liquid nitrogen or an acetone/solid carbon dioxide mixture. Although the dewar was not moved during the readings on a particular sample the intensities at different temperatures were only semi-quantitatively comparable because of varying amounts of bubbling around the film. Temperatures were measured with the same thermocouple immersed in the liquid in the dewar.

The measurement of half-lives from photographs of the oscilloscope traces was accurate to about $\pm$ 7 msec. This figure is based on the length of time taken to close the excitation beam shutter and on the different half-life values obtained by separate measurements of the same picture. The main cause of the inaccuracy is the noise level of the signal.

Temperature measurements with the thermocouple were accurate to 0.5°, but there may have been further inaccuracy because of imperfect thermal contact between the film and

Fig. 3-7 ; Dewar used for low temperature delayed emission measurements



Scale approximately ½ true size.

the junction. Although the junction was attached to the film as close as possible to the patch being illuminated, there were temperature gradients between different parts of the sample. For these reasons the true temperatures of the part of the film being studied optically may have been several degrees different from those registered by the thermocouple.

The accuracy of intensity measurements at different temperatures depended on the strength of the signal, but generally readings could be taken from the photographs to ± 10%. The intensities emitted by different samples were never comparable because the optical properties of the films differed too much. The intensities from a given film at different temperatures could be compared, however, providing that the sample position was left unaltered for all the readings. Measurements at temperatures much below room temperatures were less accurate because of condensation of water onto the film. This difficulty was overcome when the film was crushed and surrounded by another liquid in a dewar.

Results and Discussion

A) The properties of methyl cellulose films. The thickness of a methyl cellulose film depends on the

concentration of the aqueous solution of methyl cellulose and on the way it is spread onto a glass slide. Typically values of 25-40μ have been measured with a micrometer screwguage.

Methyl cellulose is hygroscopic, and under normal atmospheric conditions it contains about 5% of water by weight (Green, 1963). This was detected in the films both by heating to constant weight and by infrared spectroscopy (Penzer, 1966). The water is held in capillaries within the cellulose. Its structure is probably little altered from that of the free liquid because the quantum yield of fluorescence of FAD in different strengths of aqueous methyl cellulose is unchanged (Penzer, 1966).

The structure outlined for methyl cellulose means that the macroscopic viscosity of aqueous solutions or films should be greater than the microscopic viscosity. This situation has been observed for other polymers (Weber, 1953). The fluorescence polarisations of FMN in glycerol/water mixtures are larger than those in methyl cellulose solutions of similar macroscopic viscosity (fig.3-8).

The fluorescence polarisation of FMN in methyl cellulose films depends on the concentrations of polymer and flavin, and on the extent of drying of the film (table 3-1).

Table 3-1

Fluorescence Polarisations of FMN in Methyl Cellulose Films

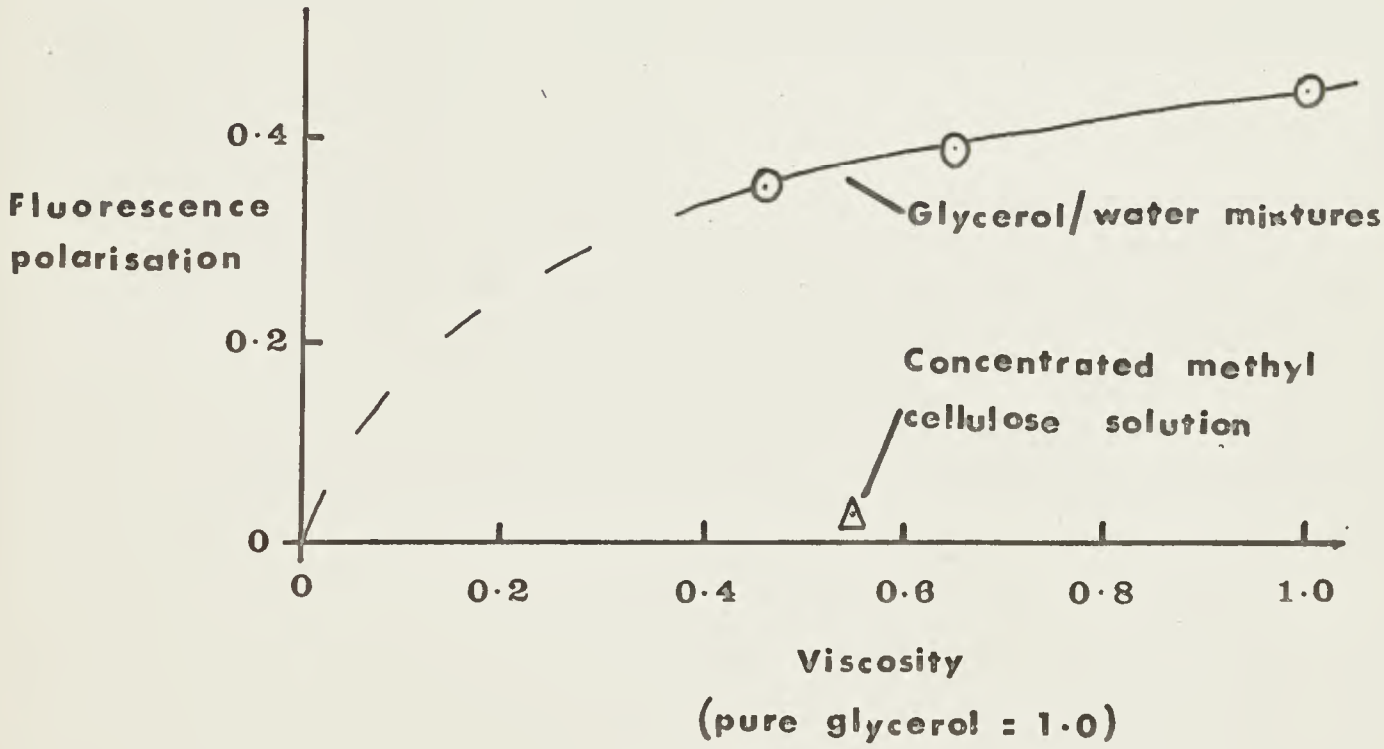
Optical density of FMN at 450 mμ	Fluorescence polarisation
0.2	0.28
0.2*	0.31
0.2⁄	0.25
0.2*⁄	0.28
0.5	0.29
~5.0	0.25
~10.0	0.20

* These films were dried for 12 hr. over calcium chloride

⁄ These films were made from solutions with half the methyl cellulose concentration normally used.

All measurements were made at room temperature.

Fig. 3-8 ; Dependence of FMN fluorescence polarisation on macroscopic viscosity (taken from Penzer, 1966)



Increasing the concentration of polymer reduces the rotational freedom of the chromophore, as does more thorough drying. The concentration of flavin has no effect on fluorescence polarisation up to an optical density of about 5.0. Above this value there is depolarisation. This could be caused by energy transfer or by reabsorption of the fluorescence by other molecules of the chromophore, leading to reduced polarisation on re-emission. The first of these possibilities is the more likely because the efficiency of the reabsorption mechanism should be related to the amount of light absorbed by the film. This is over 99% for all optical densities over 2.0, and so this kind of depolarisation should set in at lower extinctions than those for which it has actually been detected.

Attempts to determine the size of cavities in methyl cellulose have been made by observing the fluorescence polarisation of dyes with widely different molecular weights. The fluorescence polarisation of all the compounds studied increased by a factor of 2-3 on changing from pure water to a concentrated methyl cellulose solution (table 3-2). This implies that the pore size is larger than the largest of these chromophores (molecular diameter about 20A°). If this were not so the fluorescente polarisation should have increased by a larger factor for the bigger molecules.

Table 3-2

Fluorescence Polarisations in Aqueous Methyl Cellulose Solutions

Methyl cellulose concentration (g/l)	Fluorescence polarisation			
	FMN	FAD	Eosin	Rose Bengal
0	0.014	0.024	0.075	0.142
3.26	0.017	0.029	0.114	
6.41	0.015		0.145	
9.68	0.020	0.034	0.162	
12.83	0.022		0.177	
17.66	0.025	0.043	0.189	
22.50	0.029	0.043	0.195	0.204

All measurements were made at room temperature.

These results were first presented in a Chemistry pt.II thesis (Penzer, 1966).

The spectra of molecules included in a methyl cellulose film can only be studied at wavelengths longer than 350 mμ because of absorption by the support at lower values. The absorption spectra of flavins vary very little from aqueous solution to a film (fig.3-9). There are generally negligible changes in the visible band, but small hypsochromic shifts (375-368 mμ for FMN) in the near ultraviolet band. The fluorescence shifts are sometimes larger and are bath ~~hyps~~ochromic (see below). All the spectral effects are compatible with the film providing a slightly less polar environment than an aqueous solution (Harbury, La Noue, Loach and Amick, 1959).

Optical rotatory dispersion curves have been measured for flavins in methyl cellulose films (fig.3-10). The results cannot be completely trusted because it is known that the Bendix-Ericsson instrument can give rise to artefacts (Resnik and Yamoaka, 1966). The situation with regard to optical rotation measurements on flavins is discussed in appendix 1.

It is possible to calculate the period of rotation of a molecule from its polarisation of fluorescence (Weber, 1953). The results for FMN in various situations are shown in table 3-3. Although the value for FMN in a methyl cellulose film is longer than that in aqueous solution it is still

Table 3-3

Rates of Rotation of FMN in Different Environments

Solvent	Polarisation of fluorescence	$\cos^{-1}\sqrt{\overline{\cos^2 w}}$ *	ρ_0 (nsec.) ≠
Water	0.014	53.5°	.36
Concentrated methyl cellulose solution	0.029	52.5°	.77
Methyl cellulose film	0.28	35°	1.50
Glycerol	0.435	19°	83.5

* $\overline{\cos^2 w}$ is the average cosine square determined by the position of the emission oscillators at the times of absorption and emission. It is calculated from $\left\{\frac{1}{p} - \frac{1}{3}\right\} = \left\{\frac{1}{p_o} - \frac{1}{3}\right\}\left\{2/(3\overline{\cos^2 w} - 1)\right\}$ (Weber, 1966).

≠ ρ_o is the time after which the average angle, w, is such that $\overline{\cos w} = e^{-1}$. It is calculated from $\left\{\frac{1}{p} - \frac{1}{3}\right\} = \left\{\frac{1}{p_o} - \frac{1}{3}\right\} \times \left\{1 + \frac{3\tau_o}{\rho_o}\right\}$

τ_o is the fluorescence lifetime of the chromophore (Weber, 1953). All polarisations were measured at room temperature.

Fig. 3-9 ; Absorption spectra of FMN in a methyl cellulose film (a) and in aqueous solution (b)

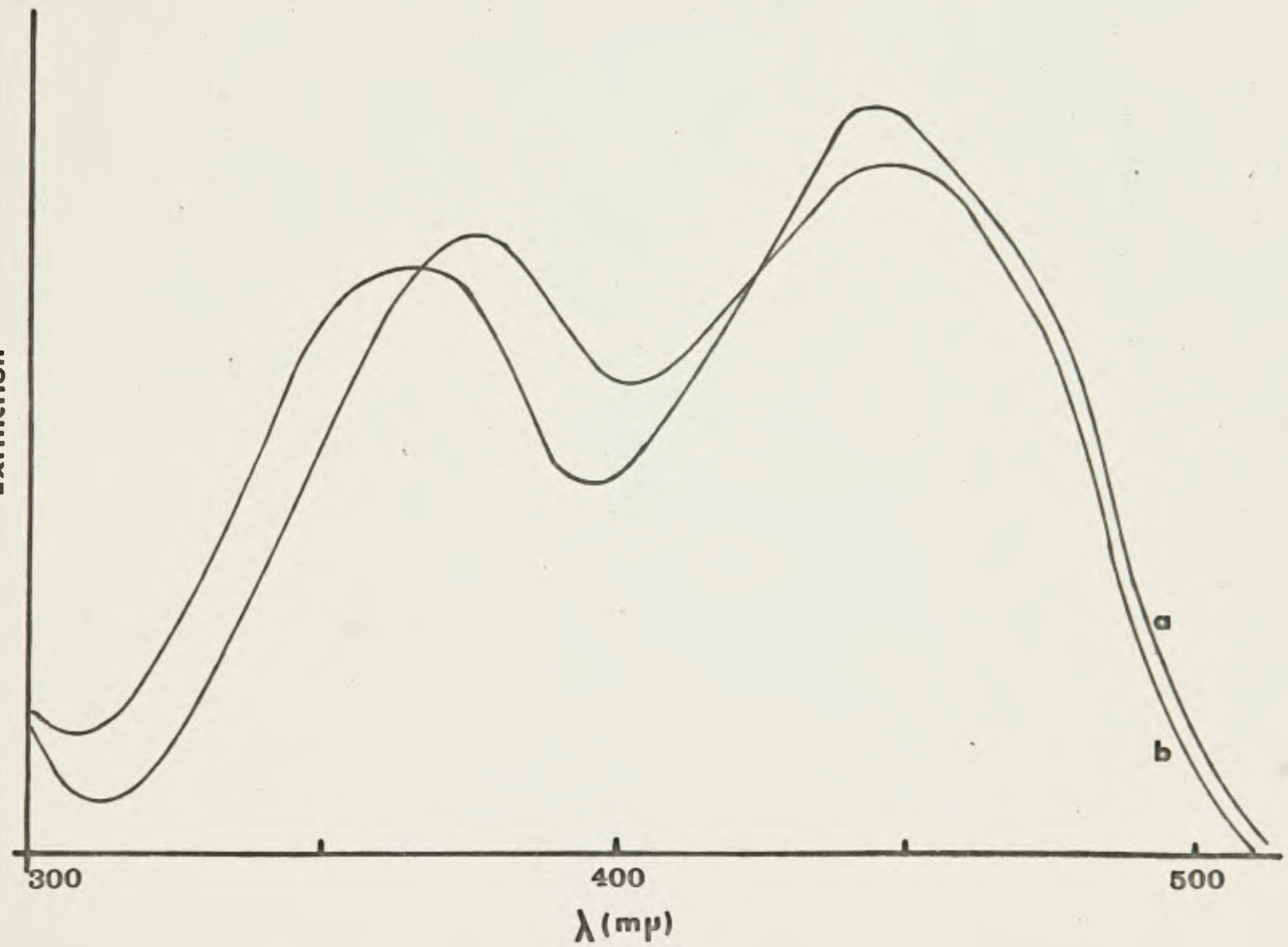
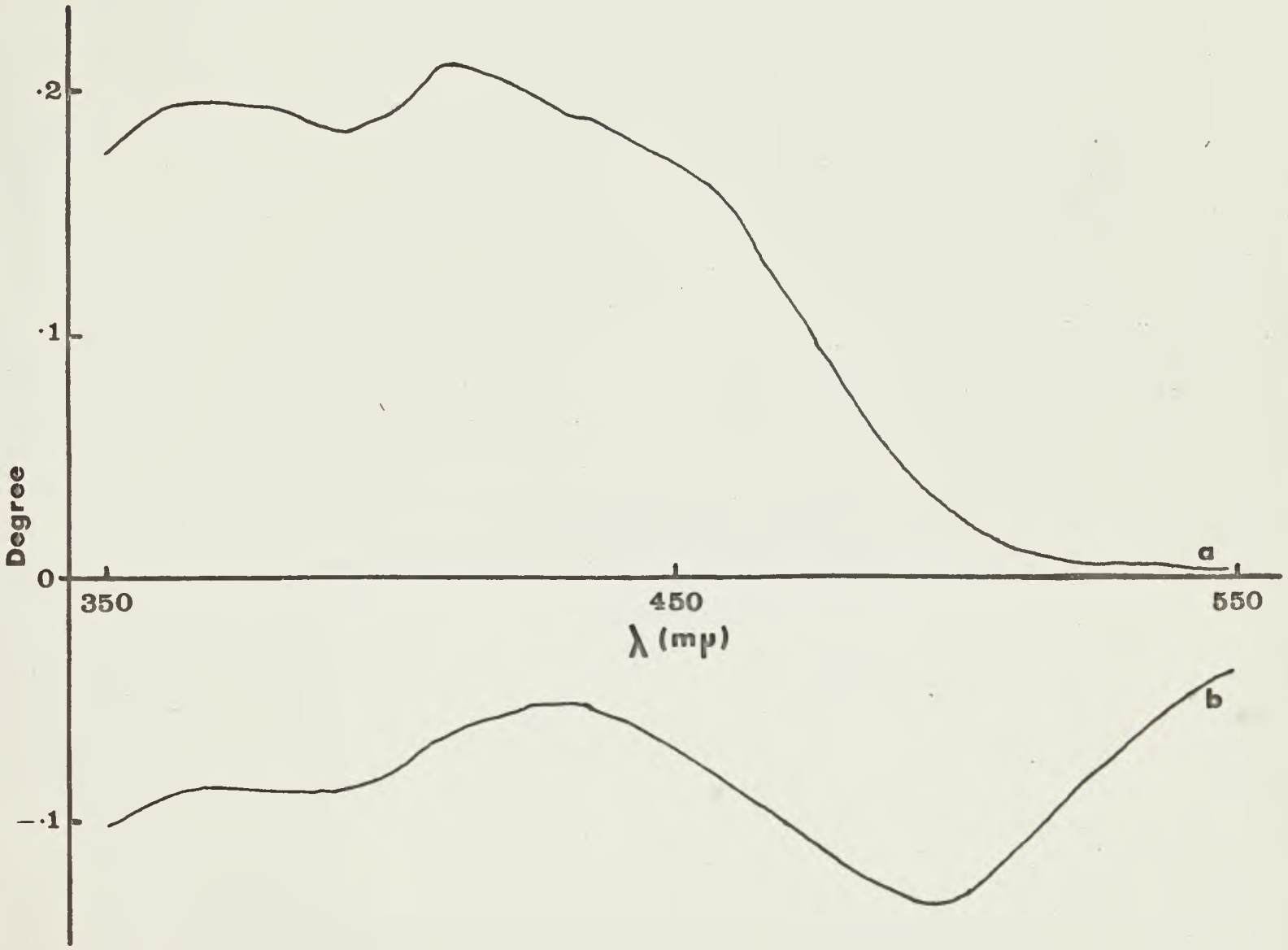


Fig. 3-10 ; ORD spectra of FMN (a - extinction 1.0) and FAD (b - extinction 0.5) in methyl cellulose films



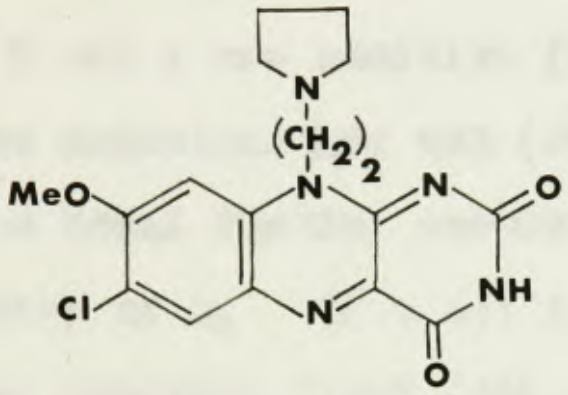
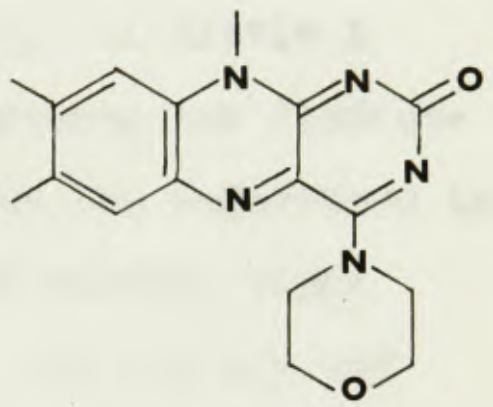
much too fast to allow dichroic bleaching experiments of the sort described in chapter 2. In confirmation of this it was found that when FMN in methyl cellulose was bleached using polarised light the reacted films showed no dichroism.

Diffusion of oxygen through methyl cellulose films is slow because after photoreduction by EDTA within a film the optical density of FMN only returns to its original value very slowly (more than 6 hr) (Penzer, 1966). Diffusion over short distances (up to 100 A°) may still be fast because in a similar matrix of ethyl cellulose photosensitization of singlet oxygen by other dyes followed by rapid reaction of the oxygen has been postulated (Bourdon and Schmuriger, 1966).

B) Energy transfer. Energy transfer between singlets (excited singlet (D) + ground state singlet (A) → ground state (D) + excited singlet (A)) is increasingly favoured as the fluorescence emission spectrum of D overlaps with an absorption band of A, and as the average distance between D and A is reduced. Two flavin derivatives which fulfill the first condition quite well are 6-chloro, 7-methoxy, 9-(ethyl-2'N-pyrrolidino)isoalloxazine (D) and 4-morpholinolumiflavin(A); their relevant spectral properties are shown in table 3-4. Methyl cellulose films provide a good matrix for the

Table 3-4

Spectral Properties of the Derivatives Chosen for Mixed Film Experiments

Derivative	D	A
		
Visible absorption maximum		
a) in aqueous solution	445 mμ	470 mμ
b) in a methyl cellulose film	445 mμ	475 mμ
Fluorescence maximum		
a) in aqueous solution	510 mμ	540 mμ
b) in a methyl cellulose film	530 mμ	570 mμ

fulfilment of the second condition because their thinness allows optical measurements to be made under conditions where the chromophores are closely spaced.

When mixed in a methyl cellulose film the absorption spectra of D and A are additive (fig.3-11) but their fluorescence emissions are not (fig.3-12). A little A (20% or more total flavin) completely quenches the fluorescence emission of D. In a 4:1 (D:A) film the reductions in intensity of exciting light (350 mμ) and emitted light (530 mμ) as a result of absorption by A are 15% and 10% respectively. This alone cannot explain the fluorescence quenching of D. On the other hand the fluorescence intensity of A is scarcely changed from a film containing only A to a film containing 80% D, in spite of the fact that under these conditions D absorbs about 80% of the incident light at 350 mμ. These results can only be explained by singlet-to-singlet energy transfer from D to A.

Using Förster's formula (see p. 53) the mean intermolecular distance at which energy transfer is as likely as other deactivation processes (R_o) can be calculated from the spectra of D and A. For this calculation the refractive index of a methyl cellulose film was assumed to be the same as that of water (1.33) and the quantum yields of fluorescence

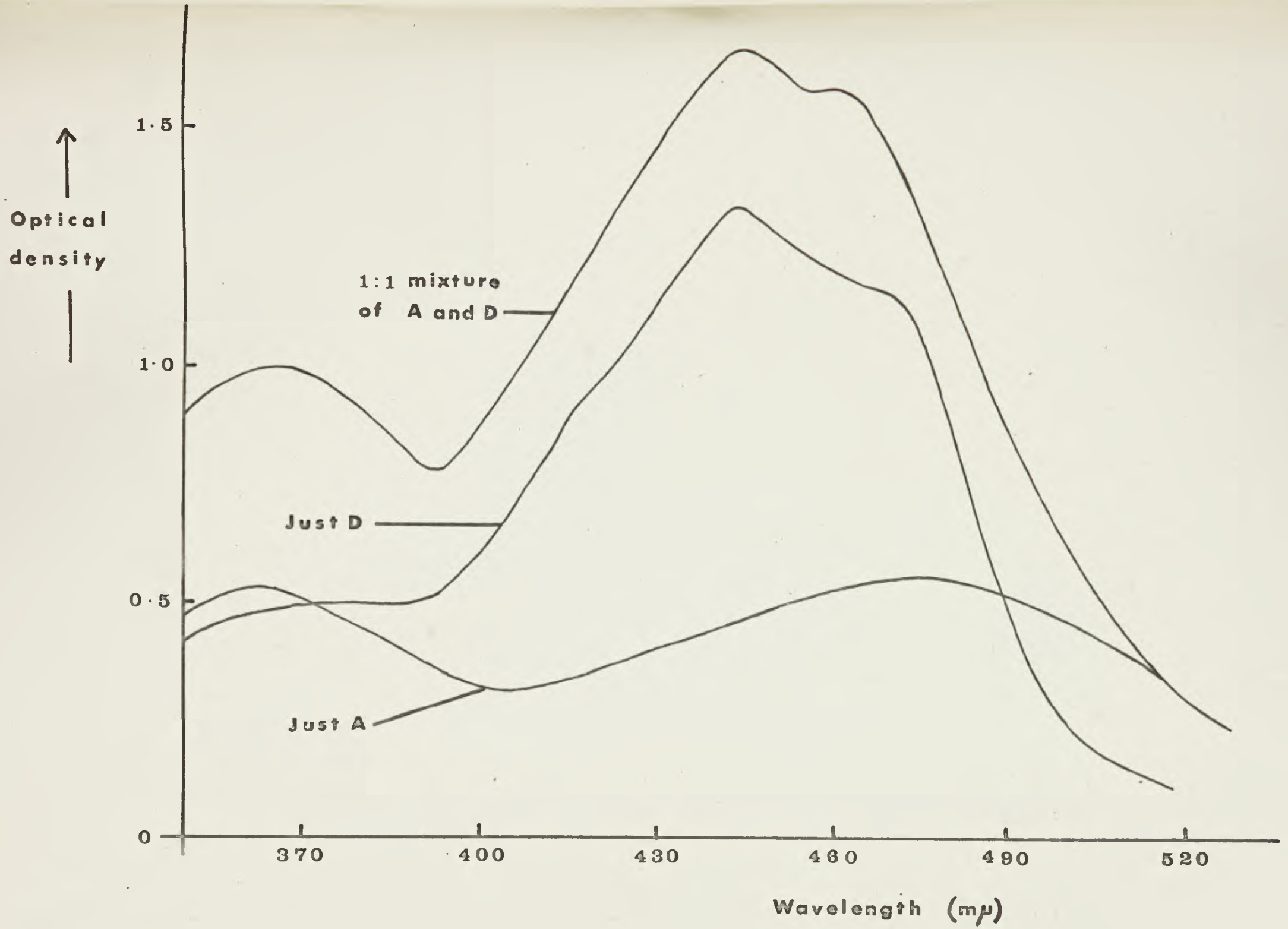
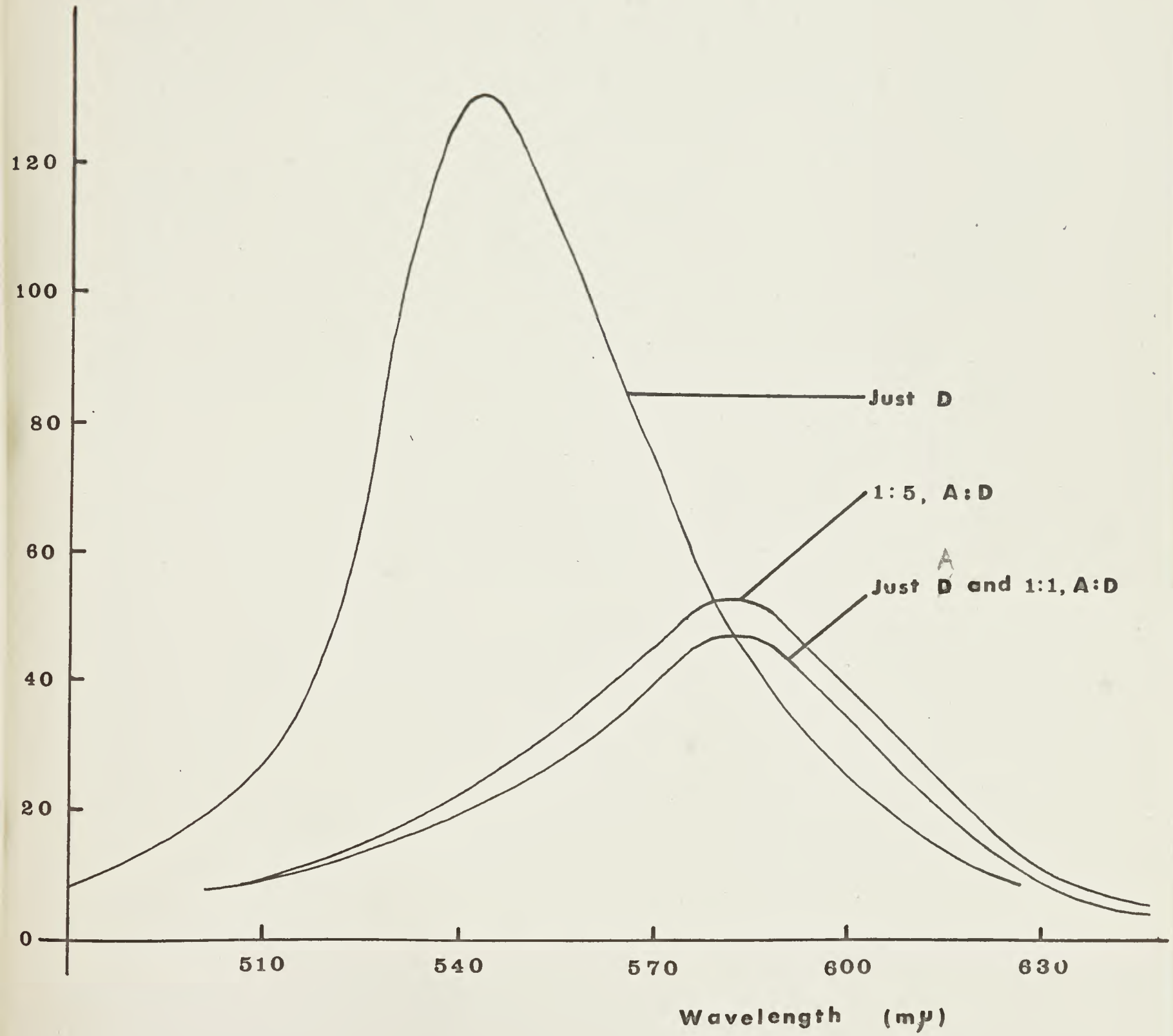


Fig. 3-11 ; Absorption spectra of D and A in methyl cellulose films (preliminary experiments were reported in Penzer,1966)

Fig. 3-12 ; Fluorescence emissions of D and A in methyl cellulose films
(preliminary experiments were reported in Penzer, 1966)



used were those found for aqueous solution (Wentworth, 1966). Overlap integrals were calculated from the spectra, areas being measured by weighing, with the fluorescence emission spectrum normalised to unity. R_o -values for FMN alone and for a mixture of D and A are 14 and 21 A° respectively. The average intermolecular distance in a mixed film with optical density 1.5 at 450 mμ is about 30 A°, and for FMN alone with an optical density of 0.5 the distance is 45 A°.

The polarisation of fluorescence of A in a film (optical density 0.55 at 475 mμ) is reduced by 15% in the presence of D when excited at 450 mμ . It can be seen from table 3-1 that the fluorescence of FMN is depolarised in films with extinctions greater than 5.0 at 450 mμ , where the average intermolecular distance is 20-25 A°. These results are consistent with the occurrence of energy transfer at mean intermolecular distances about 50% greater than the calculated R_o values. This discrepancy may be because of inaccurate assumptions of the refractive index of a film and the quantum efficiencies of the emissions, but it is unlikely that these explain the entire effect. It is probably untrue that the flavins are distributed completely at random throughout the film. The chromophores must congregate within the capillaries of the methyl cellulose network, and are thus held closer to their nearest neighbours

than the mean intermolecular distance calculated for the whole film. A similar situation seems to arise for dyes in ethyl cellulose where energy transfer at mean intermolecular distances of 120 A° has been detected (Bourdon and Schmuriger, 1966).

C) Delayed light emission. A flavin dispersed in a rigid polyacrylamide matrix has a delayed light emission at room temperature (Radda and Watson, 1968) which can be characterised as E-type delayed fluorescence (Parker, 1968). The position of the delayed emission maximum is the same as that of prompt fluorescence (fig.3-13) and the emission intensity is proportional to the intensity of the exciting light (fig.3-14). The two possible mechanisms of delayed fluorescence are triplet-to-triplet energy transfer (P-type delayed fluorescence) and thermal re-excitation of the triplet to the first excited singlet (process k_7 in fig.3-1, E-type delayed fluorescence) (Parker, 1968). P-type delayed fluorescence intensity is proportional to the square of the intensity of the exciting light because two molecules of the triplet are involved in its rate determining step ($F^t + F^t \rightarrow F^* + F$). Such a dependence is not observed so E-type delayed fluorescence is the mechanism.

Fig. 3-13 ; Fluorescence spectra of FMN
a) prompt fluorescence in aqueous solution
b) delayed fluorescence in a polyacrylamide film.

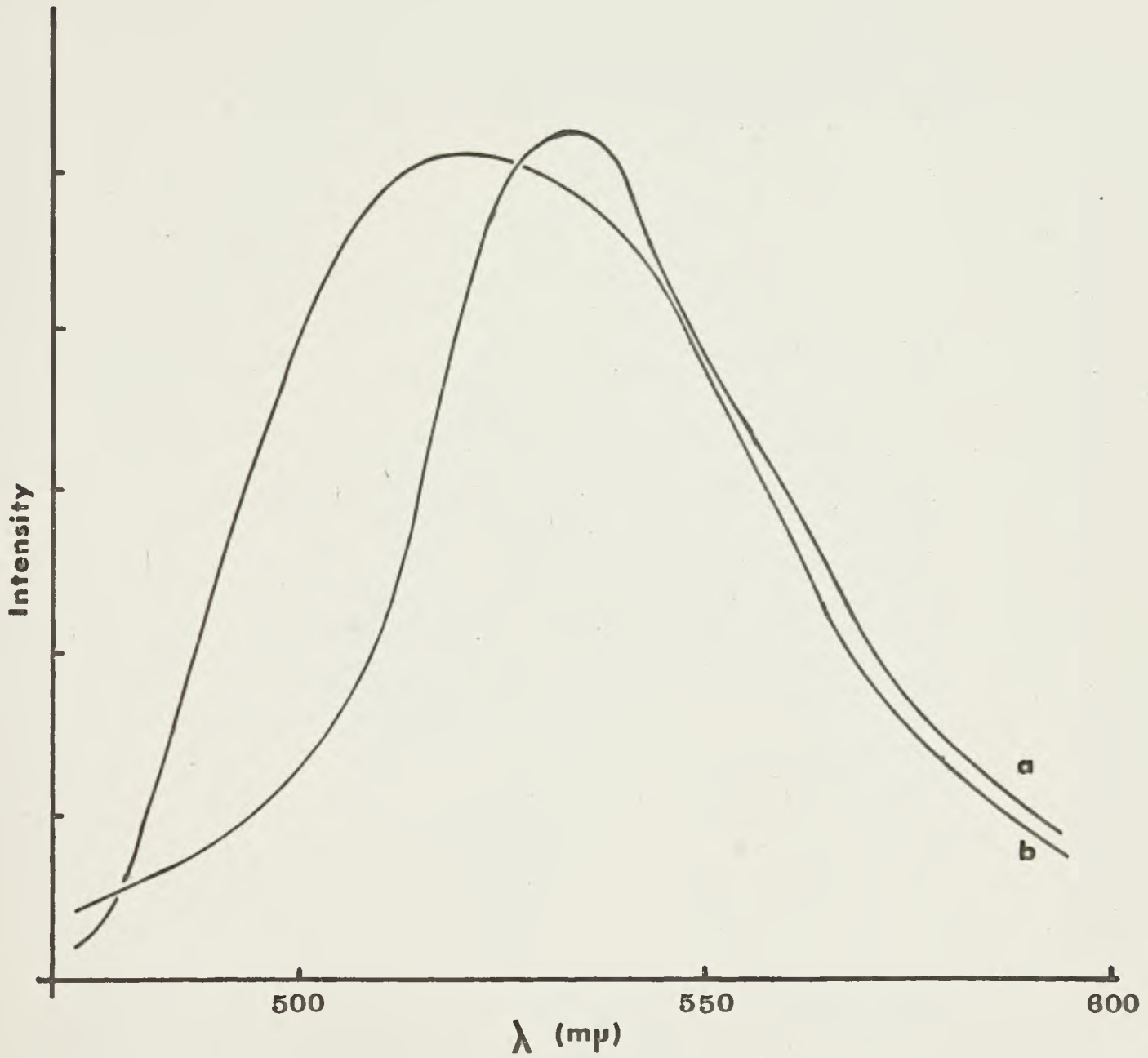
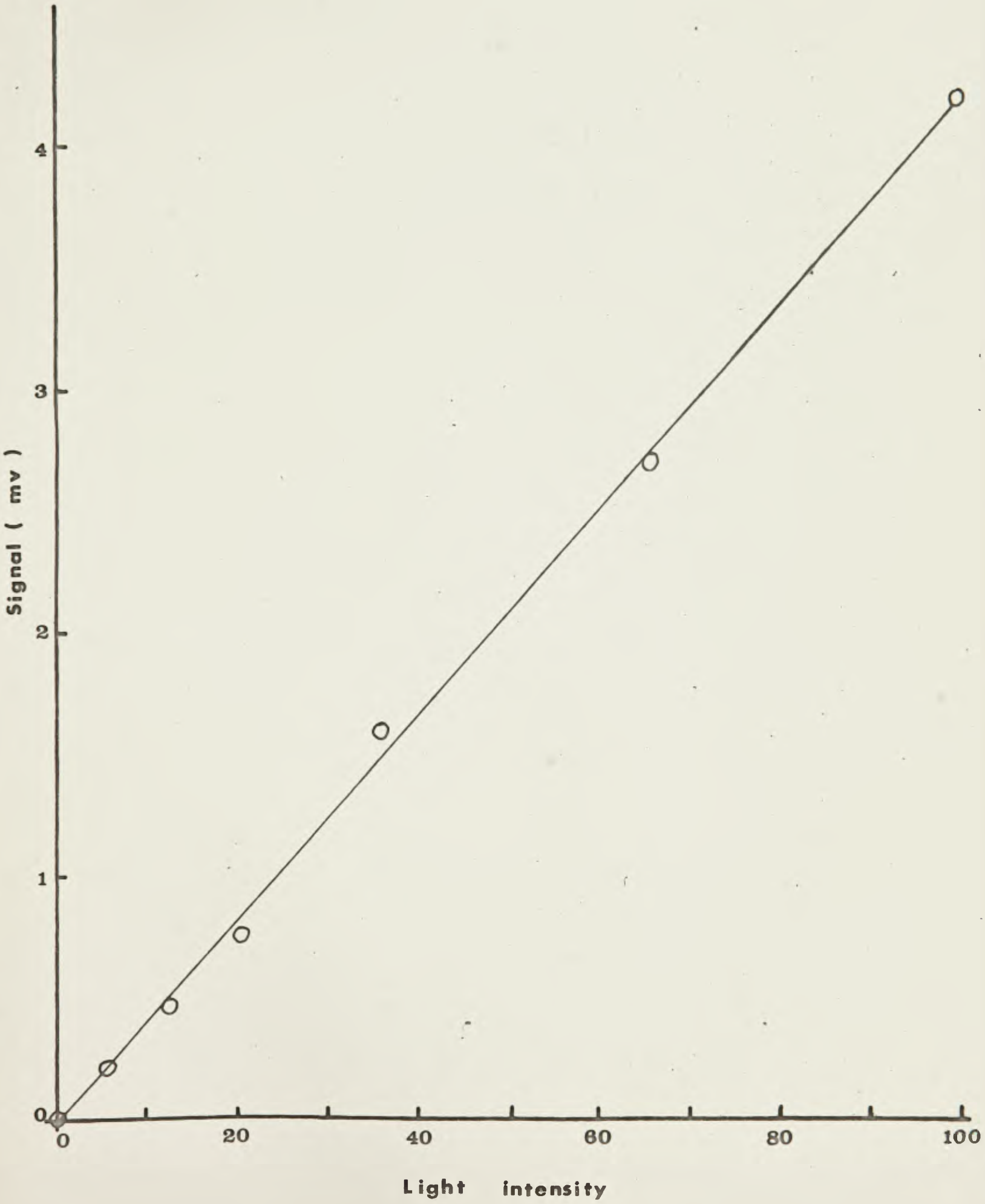


Fig. 3-14 ; Dependence of delayed fluorescence intensity on the intensity of excitation for FMN in a polyacrylamide film



The temperature effects on both the lifetimes and the intensities of the delayed emissions of several flavin derivatives have been measured (appendix 3). The decay curves of the emissions when the exciting light is cut off are exponential (fig.3-15). The first order rate constants of the decays k_c have been obtained from the reciprocals of the half-lives of the curves. These rate constants (typically 20 sec^{-1}) are composed of all the true and pseudo first order rate constants for triplet deactivation. Arrhenius plots of ln k_c:$\frac{1}{T}$ give combined activation energies for all these processes. The intensity of the delayed fluorescence, however, is proportional to $k_7.[F^t]$ (see fig.3-1). To calculate what this means in terms of the individual rate constants it is necessary to apply a steady state kinetic treatment to the concentrations of the two flavin excited states, the singlet (F^*) and the triplet (F^t).

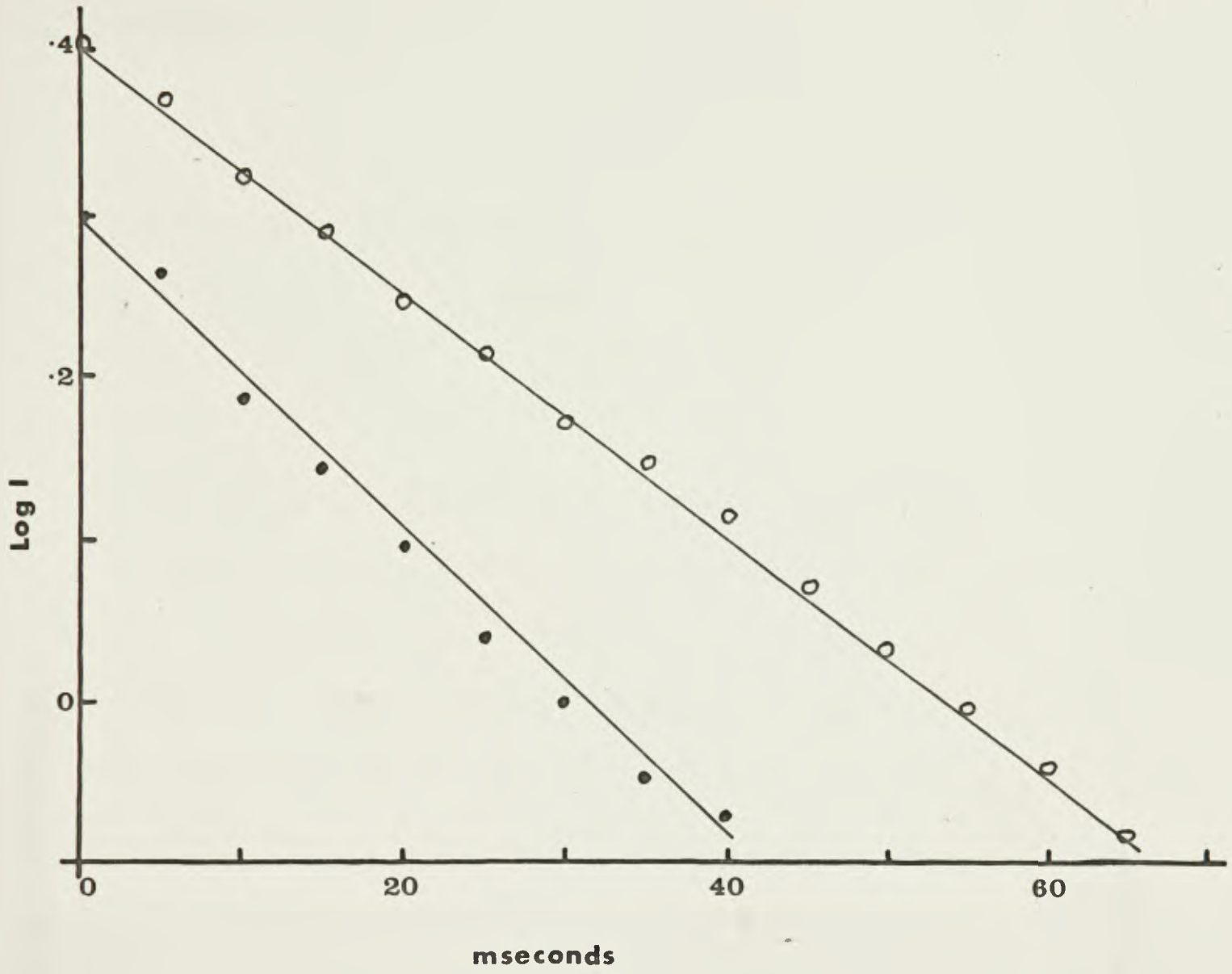
Steady state for F^*:

$$[F].k_0 + [F^t].k_7 = [F^*].(k_2 + k_3 + k_4)$$

The intensity of delayed fluorescence is very small compared to that of prompt fluorescence (the ratio is 1:575 for riboflavin at room temperature) and so to a good approximation $[F].k_0 + [F^t].k_7 = [F].k_0$. Therefore

$$[F^*] = \frac{[F].k_0}{(k_2 + k_3 + k_4)} .$$

Fig. 3-15 ; Semi-log plots of phosphorescence (• iso-riboflavin) and delayed fluorescence (O FMN) intensities (I) with time



Steady state for F^t :

$$[F^*].k_4 = [F^t].(k_5 + k_6 + k_7)$$

$$\text{Therefore } [F^t] = \frac{[F^*].k_4}{(k_5 + k_6 + k_7)} = \frac{[F].k_0k_4}{(k_2+k_3+k_4)(k_5+k_6+k_7)}$$

Thus the intensity of delayed fluorescence,

$$[F^t].k_7 = \frac{[F]k_0k_4k_7}{(k_2+k_3+k_4)(k_5+k_6+k_7)}$$

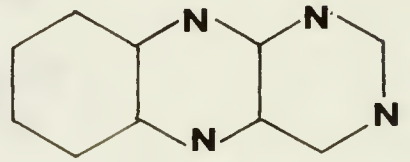
This expression is to be contrasted with the combined rate constant measured from the reciprocal of the half-life of decay, which is given by $k_c = k_5+k_6+k_7$.

Arrhenius plots for the intensity and half-life of delayed emission thus (for sample plots see fig.3-16) give different apparent activation energies because of the different ways in which the individual rate constants are combined. Table 3-5 contains the two activation energies for a series of flavin derivatives, along with their phosphorescence and fluorescence maxima, and the half-lives of the delayed emissions at 20°.

Very similar arguments apply when considering the effects of temperature on phosphorescence at low temperatures

Table 3-5

Activation Energies for the Delayed Fluorescence of some Flavin Derivatives: also their Emission Maxima, and the Lifetimes of the Delayed Emissions at 20°C



Compound number	Substituents							Fluorescence maximum(mμ)	Phosphorescence maximum(mμ)	Half-life A at 20°(msec)	B (kcal)	(kcal)
	2	3	4	5	6	7	9					
1	O	H	O	H	Me	Me	D-ribityl	525	598	40	1.6	4.3
2	O	H	O	H	Me	Me	D-ribityl phosphate	526	598	50	2.6	5.9
3	O	H	O	H	Me	Me	Me	525	600	55	2.4	5.0
4	O	Me	O	H	Me	Me	Me	527	597	55	2.2	5.5
5	O	H	O	H	H	H	$(CH_2)_2NMe_2$	513	580	40	2.5	3.1
6	O	H	O	H	Cl	MeO	$(CH_2)_2$-N	510	600	75	1.8	6.3
7	N O	-	O	H	Me	Me	Me	533	608	42	3.6	5.6
8	O	-	N O	H	Me	Me	Me	540	605	18	1.6	4.4

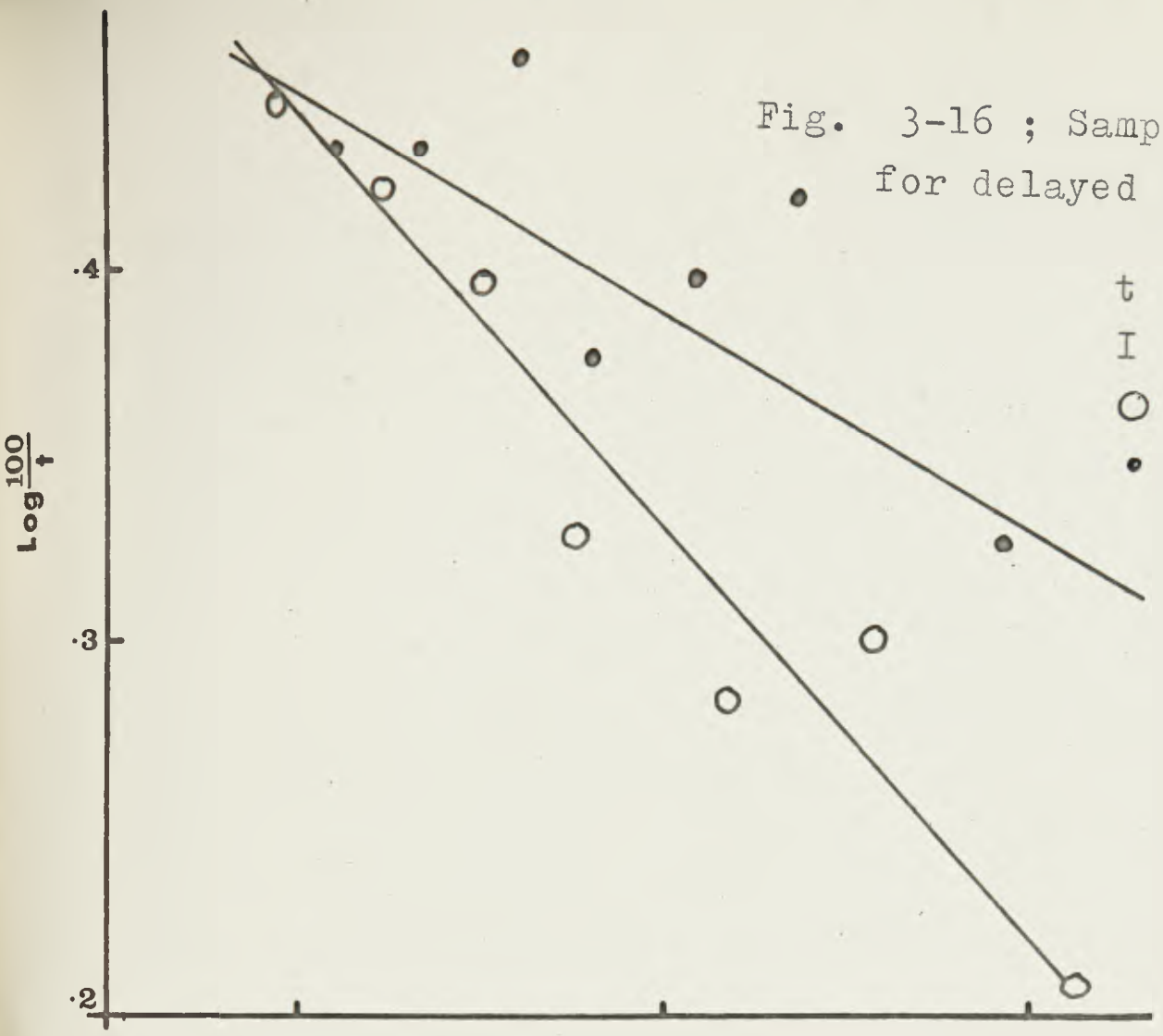
9	O	H	O	Me	Me	H	D-ribityl	527	590	24	3.4	3.1
10	O	H	O	Me	Me	H	peracetyl D-ribityl	531	590	30	2.3	3.5
11	O	H	O	H	Cl	H	$(CH_2)_3$ NMt_2	523	600	21	1.8	3.3
12	O	H	O	H	Me	H	$(CH_2)_2$ NMe_2	523	600	33	2.5	3.9
13	O	H	O (lumichrome)	H	Me	Me	-	445	550	No delayed fluorescence		

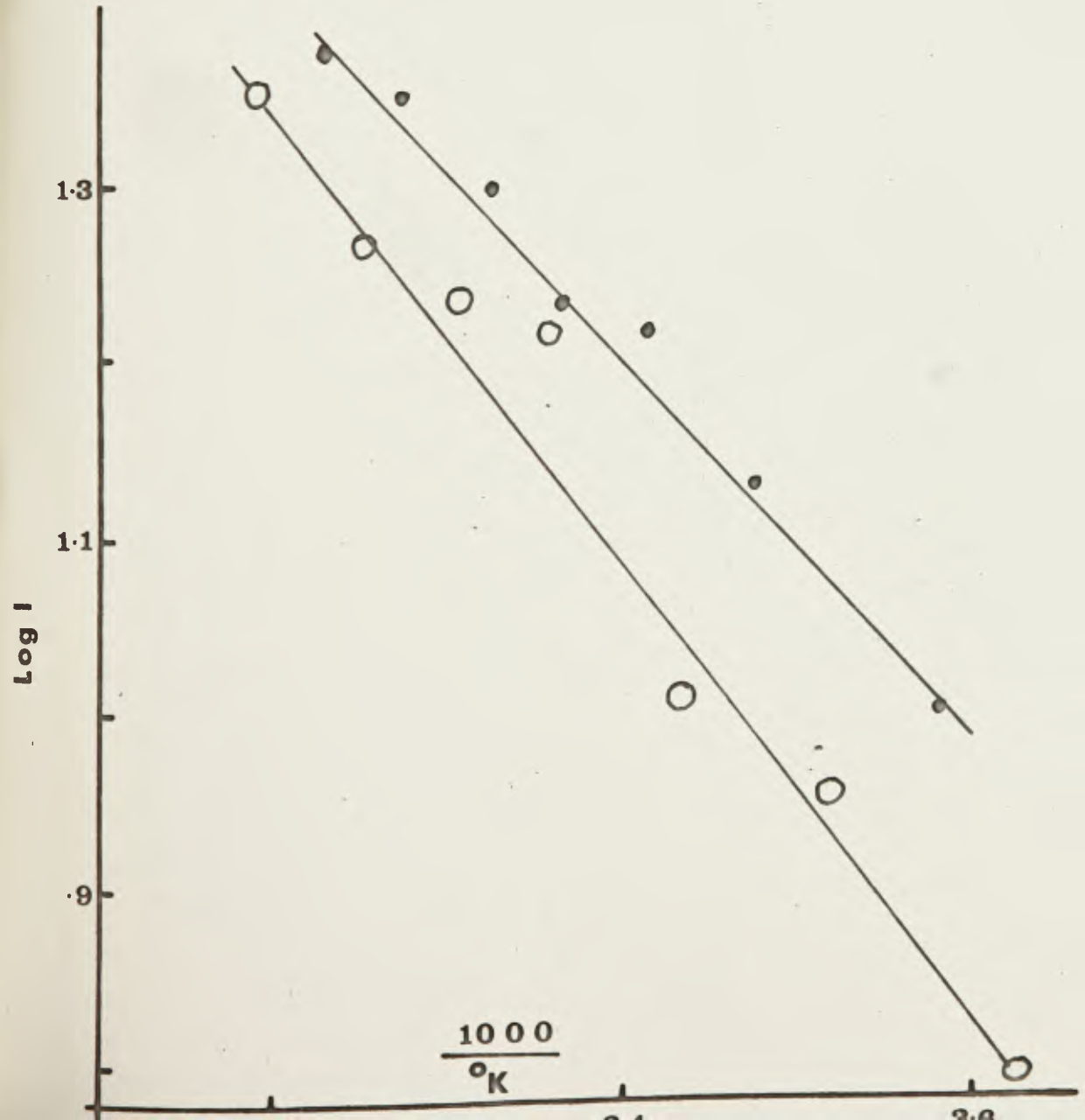
A is the activation energy calculated from the reciprocal of the decay half-lives;
B is the activation energy calculated from the intensity of delayed fluorescence.

All measurements were made on flavins in polyacrylamide gel films. The observation wavelengths were those where delayed emission intensity was a maximum.

Fig. 3-16 ; Sample Arrhenius plots for delayed fluorescence

t - half-life
I - intensity
○ - FMN
• - riboflavin





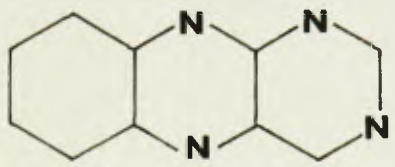
where delayed fluorescence is negligible. The activation energy obtained from the temperature of the half-lives again corresponds to a composite of all triplet deactivating processes. The intensity of phosphorescence is found by a steady state treatment to be $\frac{[F].k_0k_4k_5}{(k_2+k_3+k_4)(k_5+k_6)}$.

The results obtained for phosphorescence at temperatures where there was no delayed fluorescence are summarised in table 3-6. At temperatures where both delayed fluorescence and phosphorescence are measurable they have the same half-lives (as both result from decay of the flavin triplet), and in this temperature region the Arrhenius plots show a sharp curve (fig.3-17). This corresponds to the change from k_7 being negligible to the situation where its temperature dependence is dominant.

The accuracy of the activation energies is about $\pm$ 30% as the Arrhenius plots are not very good. The main uncertainty, especially for the delayed fluorescence measurements, is in the accurate measurement of temperature because of imperfect thermal contact between the film and the thermocouple. Different measurements on the same flavins in quite separately made films give results which agree within the error quoted. In spite of the comparatively large uncertainties in the values of the apparent activation

Table 3-6

Activation Energies for the Phosphorescence of some Flavin Derivatives; also the Lifetimes of the Emissions at 77° K.



Substituents							Half-life at 77°K(msec)	A (cal)	B (cal)
2	3	4	5	6	7	9			
O	Me	O	H	Me	Me	Me	155	65	
O	H	O	Me	Me	H	D-ribityl	83	120	-140
O	H	O	H	Cl	MeO	$(CH_2)_2$ 	190	120	-145
O	-		H	Me	Me	Me	59	50	-250

A is the activation energy calculated from the reciprocal of the decay half-lives; B is the activation energy calculated from the intensity of phosphorescence.

All measurements were made on flavins in polyacrylamide gel films. The readings were taken at the phosphorescence maxima of the derivatives.

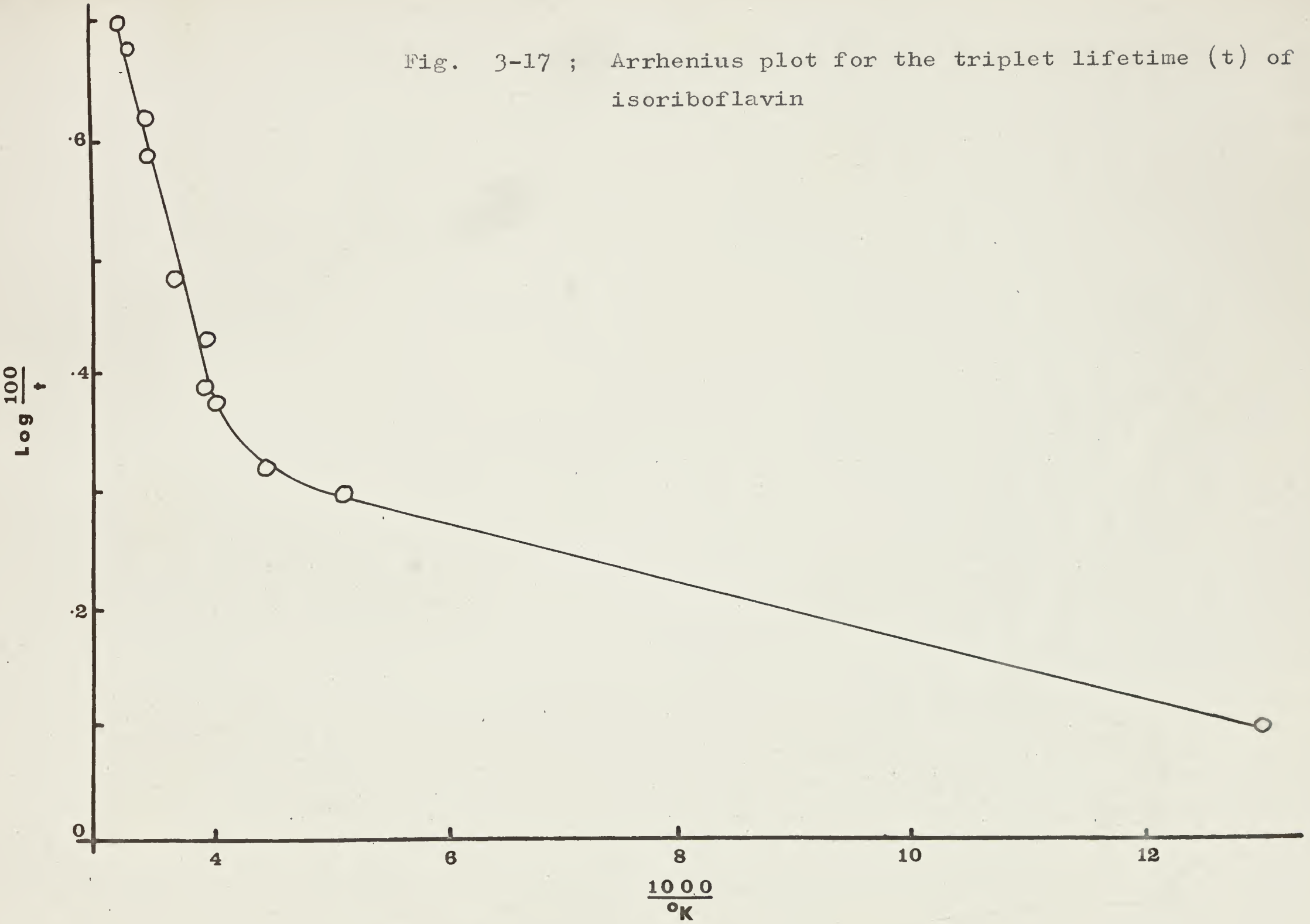


Fig. 3-17 ; Arrhenius plot for the triplet lifetime (t) of isoriboflavin

energies it is of great interest to have any direct experimental measure of these functions because this enables the estimation of the true activation energies of several of the individual processes denoted in figure 3-1.

Consider the temperature dependence of the individual rate constants. As long as the intensity of the exciting light is kept constant k_0,k_2, and k_5 are independent of temperature. They each depend only on the overlap between two wave functions, and do not possess activation energies. They may alter with temperature if the environment of the chromophore alters so as to change the orbital overlap, but in the case of a rigid supporting matrix this will be a small effect. The variation of prompt fluorescence intensity between 0° and 50° is too small to measure which means that either k_3 and k_4 are negligibly small, or they have very low activation energies. The former may apply to k_3 and the latter to k_4, which probably accounts for almost all non-radiative deactivation of the first excited singlet (Porter, 1968). In a solid matrix k_4 is probably lower than in a fluid solution because of the reduction in the magnetic perturbations which lower the forbiddenness of a singlet-triplet transition.

k_6 and k_7 remain as possibly dependent on temperature.

The activation energy of thermal re-excitation from the triplet to the first excited singlet can be found directly from the two activation energies measured for delayed fluorescence. The activation energy measured from the temperature dependence of delayed fluorescence intensity equals that of k_7 minus that of $k_5 + k_6 + k_7$. The activation energy found from the reciprocals of the half-lives is simply that of $k_5 + k_6 + k_7$, so the activation energy associated with k_7 is the sum of the energies measured from the two types of Arrhenius plot. The values obtained in this way are compared with those calculated from the difference between the spectral maxima of fluorescence and phosphorescence in table 3-7. The values are close, but they are not necessarily identical for two reasons. Ideally the energies of the zero-zero spectral bands should be used, and they may not be the same as those of the emission maxima. Secondly, a small activation energy may be required for intersystem crossing above the level of the first excited singlet, and the measured activation energy should be greater than the difference between the zero-zero bands by this amount.

If k_4 is entirely independent of temperature the activation energy from the reciprocal of phosphorescence half-life is that for $(k_5 + k_6)$ when delayed fluorescence is negligible. The activation energy from phosphorescence

Table 3-7

Comparison of the Energies Separating the Two Emission Maxima with the Sum of the Activation Energies of Delayed Fluorescence for some Flavin Derivatives

Compound number	Fluorescence maximum (λfluor) -phosphorescence maximum (λphosph) (Kcals/mole)	A + B (kcals/mole)
1	6.7	5.9
2	6.6	8.5
3	6.8	7.4
4	6.4	7.7
5	6.4	5.6
6	8.4	9.1
7	6.7	9.2
8	5.7	6.0
9	5.8	6.5
10	5.4	5.8
11	7.0	5.1
12	7.0	6.4

Mean ratio of A+B:(λ fluor - λ phosph) = 1.04

The compound numbers, and the meaning of the symbols A and B are the same as in Table 3-5.

intensity, however, is that for $\frac{1}{(k_5 + k_6)}$. Thus the energies from the two Arrhenius plots for phosphorescence should be numerically equal, but with opposite signs, and this is what is found (table 3-6 and fig.3-18). The accuracy of the measurements is insufficient to rule out the possibility that k_4 has an activation energy from 0 to 100 cals.

The values for the activation energies of $(k_5 + k_6)$ are all much lower than those for k_7, lying around 100-200 cals. The true value for k_6 alone cannot be deduced because it depends on the relative magnitudes of k_5 and k_6, but it will certainly be higher than the composite energy. It is possible to estimate $\frac{k_6}{k_5}$, which is greater than 1 even at the temperature of liquid nitrogen. This is deduced from the fact that delayed fluorescence intensity rises to at least twice the phosphorescence intensity at 77°K as the temperature is raised to room temperature. Thus even at the lower temperature k_6 must be greater than k_5, and so the activation energy measured for $(k_5 + k_6)$ must be quite close to that for k_6 alone.

None of the Arrhenius plots should ever be perfect straight lines because triplet deactivation is not the result of a single process with a single activation energy at any of the temperatures studied. The experiments were too

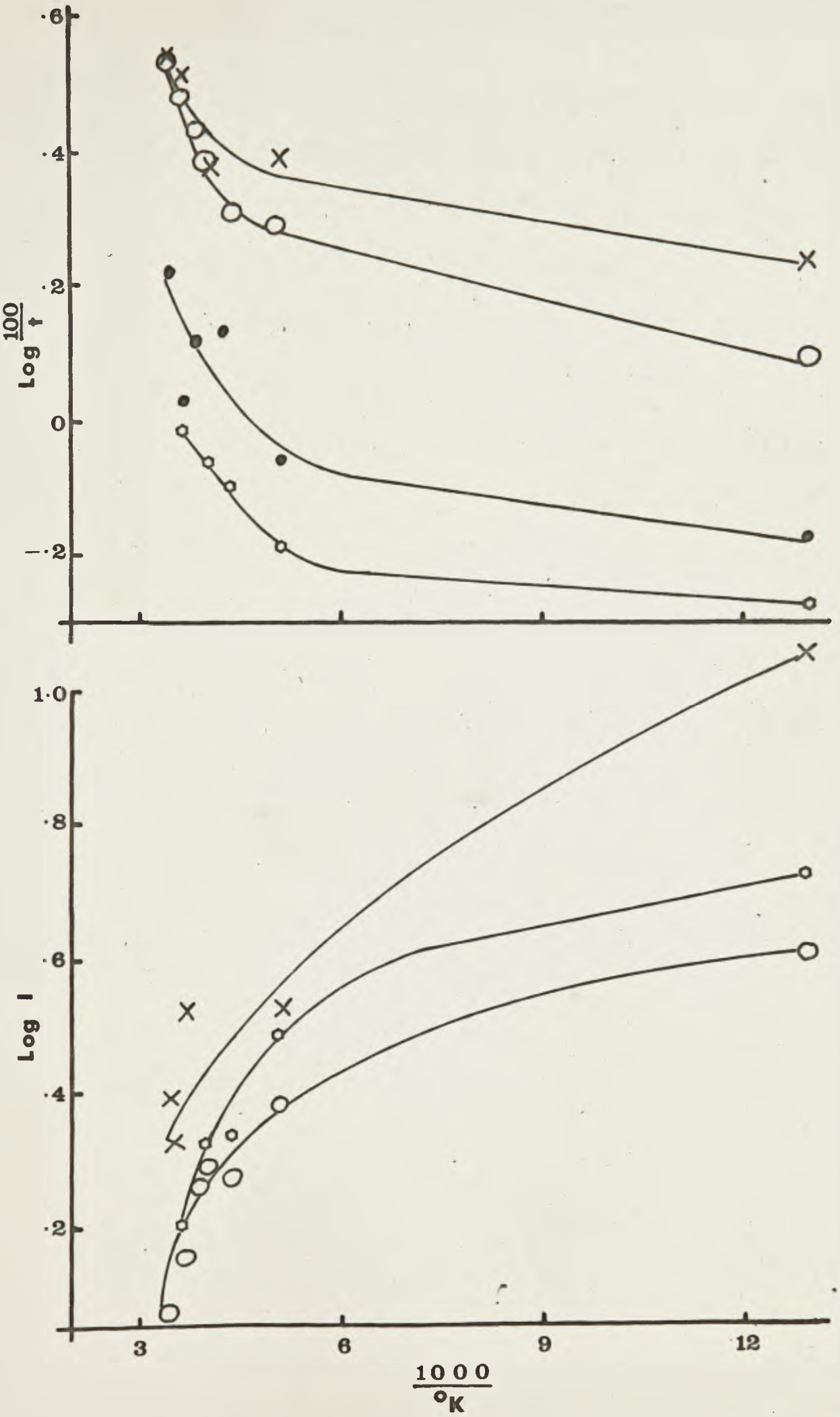
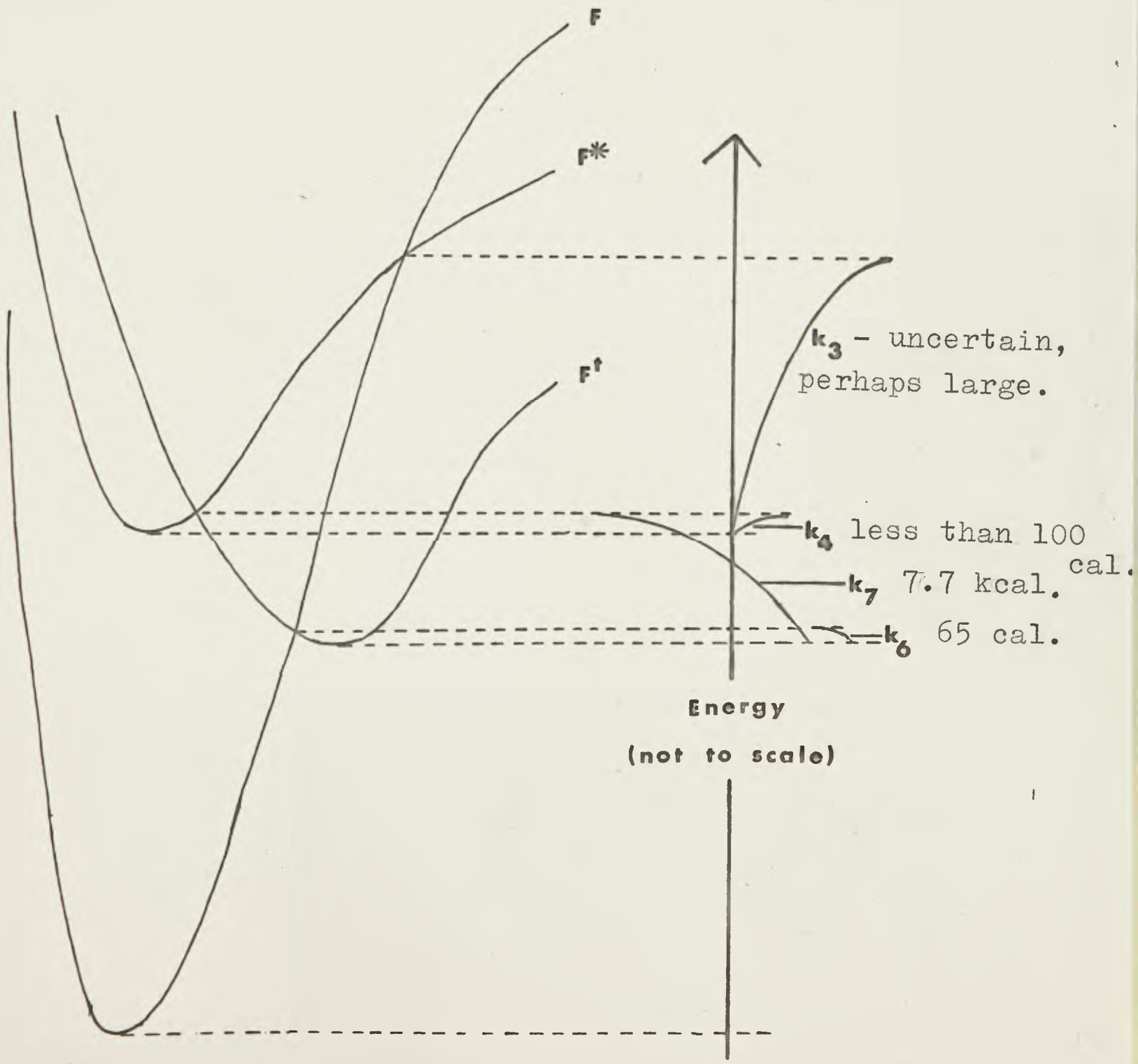


Fig. 3-18 ; Arrhenius plots for phosphorescence half-lives (t) and intensities (I) for cpds.4 (•),6 (○),8 (×) and 9(○) - table 3-5

inaccurate to allow the unequivocal detection of curvature except around the temperature of interchange from phosphorescence to delayed fluorescence. The activation energies which have been deduced, although only semi-quantitative, are of considerable interest as indications of the relative magnitudes of the energies required in the various mechanisms of triplet deactivation. The results for a typical flavin (3-methyl lumiflavin) are summarised in figure 3-19.

The behaviour of all the flavin derivatives shows no major variations or anomalies. The activation energies for triplet to first excited singlet re-excitation lie between 5.1 and 9.2 kcal, and the phosphorescence-delayed fluorescence changeovers occur at 250°K ± 50°.

Fig. 3-19 ; Activation energies for intersystem crossing between different electronic states of 3-methyl lumiflavin



CHAPTER FOUR

Anaerobic Flavin Photochemistry

Introduction

In addition to the non-radiative deactivation processes described in chapter 3 excited flavins may undergo chemical reaction. As with other aromatic dyes, almost all reactions involve the triplet state. Under anaerobic conditions flavin photochemistry may be split into two categories - photoreduction and reactions as a sensitizer.

Three situations must be distinguished when considering the anaerobic photoreduction of flavins. They are i) reaction in the absence of external electron donors (photobleaching), ii) reaction in their presence (photoreduction), and iii) reaction with an external donor which forms an adduct with the flavin (photoaddition). An anaerobic aqueous solution of riboflavin containing an added electron donor is reduced on illumination with ultraviolet or visible light. There is a concomitant loss of colour because the reduced flavin has negligible extinction in the visible region. On admitting oxygen to the reduced solution riboflavin is quantitatively restored (Oster, Bellin and Holmström, 1962). Illumination of riboflavin in the absence of donors leads to a slower fading which is only

partly reversed on reoxygenation. The products of this reaction contain lumiflavin and lumichrome, of which the spectrum of only the former resembles that of riboflavin (Oster, Bellin and Holmström, 1962; Moore, Spence, Raymond and Colson, 1963). Thus photobleaching is irreversible. With some substrates a photoaddition occurs which leads to precipitation of the product from solution when some 3-substituted flavins are studied. This product reacts more slowly than reduced flavins with oxygen (Hemmerich, Massey and Weber, 1967), but the reaction may be accelerated by illumination of the reoxygenated mixture.

The quantum yield (Q) of photobleaching of riboflavin (Q = 0.006 in aqueous solution at pH 6.8 -Holmström and Oster, 1961) is less than those found for photoreduction by EDTA (Q = 0.06 for 10^{-3}M EDTA at pH 7 and 20° - Radda and Calvin, 1964), NADH (Q = 0.25 for 10^{-3}M NADH at pH 7.0 and 20° - Radda and Calvin, 1964), or methionine (Q = 0.42 for 10^{-3}M methionine at pH 7.0 and 25° - Byrom and Turnbull, 1968). Photobleaching is also a little slower than photoaddition of phenylacetate (Q = 0.0077 for 10^{-3}M phenylacetate at pH 8 and 25° - Taylor, 1968).

A) Photobleaching. It has been suggested that photobleaching is reduction by water-splitting (Strauss and Nickerson, 1961:

Vernon, 1959), providing a chemical analogue for the water-splitting reaction in photosynthesis. The evidence quoted in support of the mechanism is i) hydrogen peroxide is produced during anaerobic photobleaching, ii) catalase (which destroys hydrogen peroxide) inhibits the reaction, iii) methionine sulphoxide is produced when methionine is added to the reaction mixture, iv) the riboflavin spectrum is nearly restored on reoxygenation, and v) the reaction does not occur in non-aqueous solvents like ethanol. Photoreduction is said to be faster than photobleaching because the added 'activators' reduce the energy required to break the OH bond in water by forming some kind of complex. Some degradation of riboflavin occurs during reoxidation, but only in the absence of activators (which can somehow protect the side-chain).

This evidence is unsatisfactory. i) The methods used to detect hydrogen peroxide are not unequivocal (Radda and Calvin, 1964; Enns and Burgess, 1965). ii) The effect of catalase is uncertain. Some authors have found it to accelerate rather than inhibit photobleaching, and it is in any case hard to envisage how decomposition of a reaction product can retard the reaction in which it is produced. iii) The production of methionine sulphoxide can be explained by a reaction in which methionine acts as an electron donor;

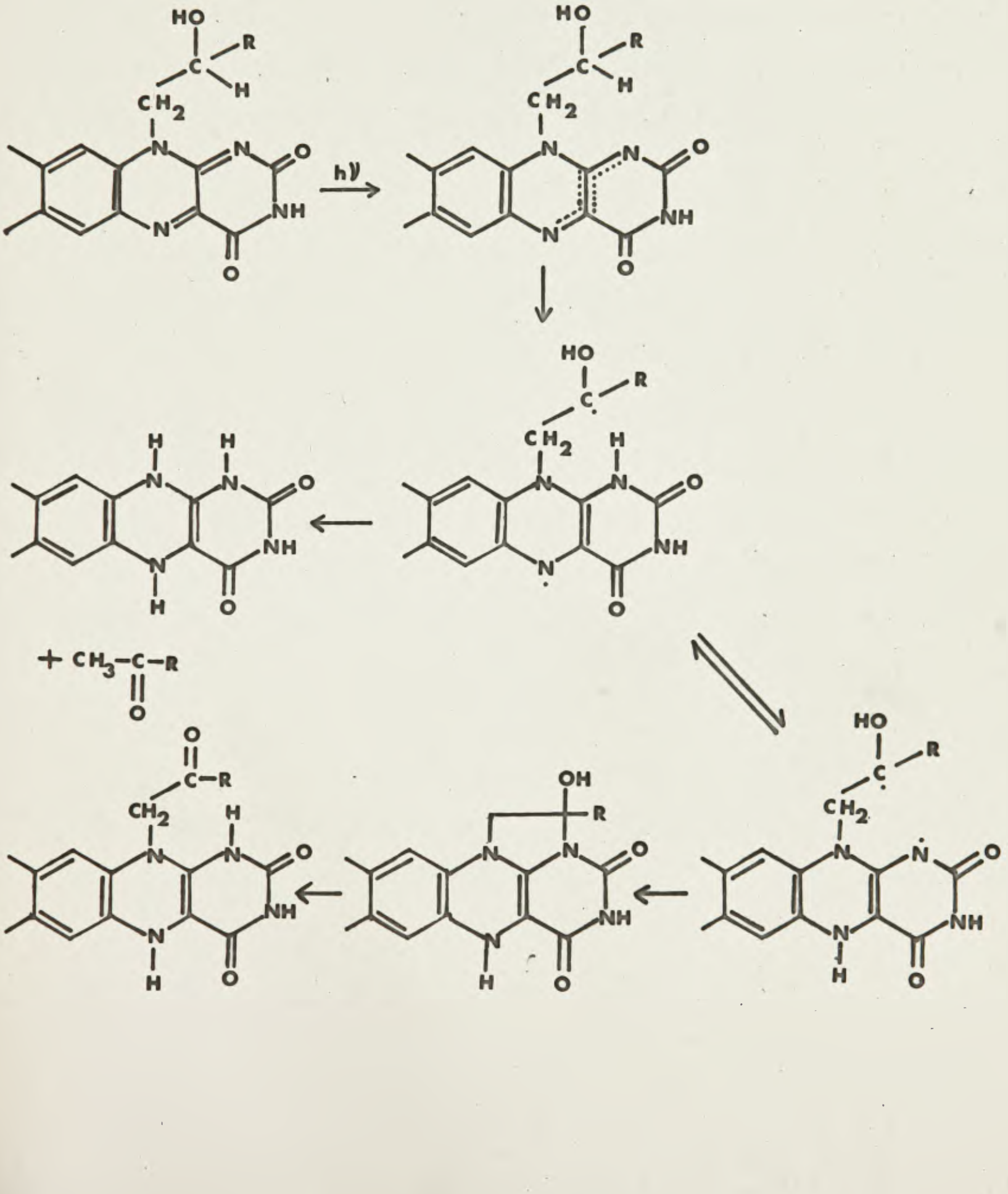
its oxidation product is subsequently hydrated to the sulphoxide. More recent studies have shown that the primary product of anaerobic photoreduction by methionine is 3-(methylthio)propionaldehyde, not methionine sulphoxide (Enns and Burgess, 1965b). iv) The near restoration of the riboflavin spectrum only shows that the isoalloxazine chromophore is not completely destroyed. v) It has now been shown that photobleaching <u>does</u> occur on illumination of ethanolic solutions of riboflavin (Radda and Calvin, 1964).

A more satisfactory mechanism has been suggested (Holmström and Oster, 1961; Moore, Spence, Raymond and Colson, 1963; Radda and Calvin, 1964) which involves intramolecular disproportionation with the ribityl side-chain reducing the isoalloxazine nucleus. There is plenty of evidence for participation of the side-chain in reduction. The products of photobleaching are lumiflavin, lumichrome and 6,7-dimethyl, 9-formylisoalloxazine (Smith and Metzler, 1963), so the side-chain has certainly reacted. There is a kinetic isotope effect ($k_H/k_D = 2.5$) for the photobleaching of 9-(2'-hydroxyethyl)-isoalloxazine and 2',2'-$^2H_2$ 9-(2'-hydroxyethyl)-isoalloxazine which shows that there is hydrogen abstraction from the side-chain during the rate-determining step (Moore, Spence, Raymond and Colson, 1963).

There is no isotope effect using D_2O as a solvent compared to water, suggesting that hydrogen is not abstracted from either water or the hydroxyl groups of the side-chain. Lumiflavin, which is not photobleached at all under normal conditions, is photoreduced in the presence of ribitol. This reaction is much slower than the photobleaching of riboflavin, indicating (for the latter) an intramolecular mechanism (Radda and Calvin, 1964).

The suggested mechanism for photobleaching is shown in figure 4-1. Although the 2' position is the most favourable for hydrogen abstraction, ω-hydroxyl chains of 3 - 6 carbon atoms will allow some reaction in the order 3>5>6>4 (Yang and McCormick, 1965). Radical stabilisation in the side-chain can be affected by nitrogen as well as oxygen (Radda, 1966). When there are no hydrogens in the α-position the hydroxyl hydrogen may be abstracted instead (Moore and Baylor, 1966). Detailed polarographic and spectral measurements on flavin analogues with 9-(2'-hydroxyethyl) and 9-formylmethyl substituents have shown that the latter is bleached the faster to give (probably) a tetracyclic leucoflavin and lumichrome in about equal amounts (McBride and Moore, 1967; McBride and Metzler, 1967). Although a bimolecular mechanism was suggested to explain this result, it is equally possible

Fig. 4-1 ; Mechanism of photobleaching

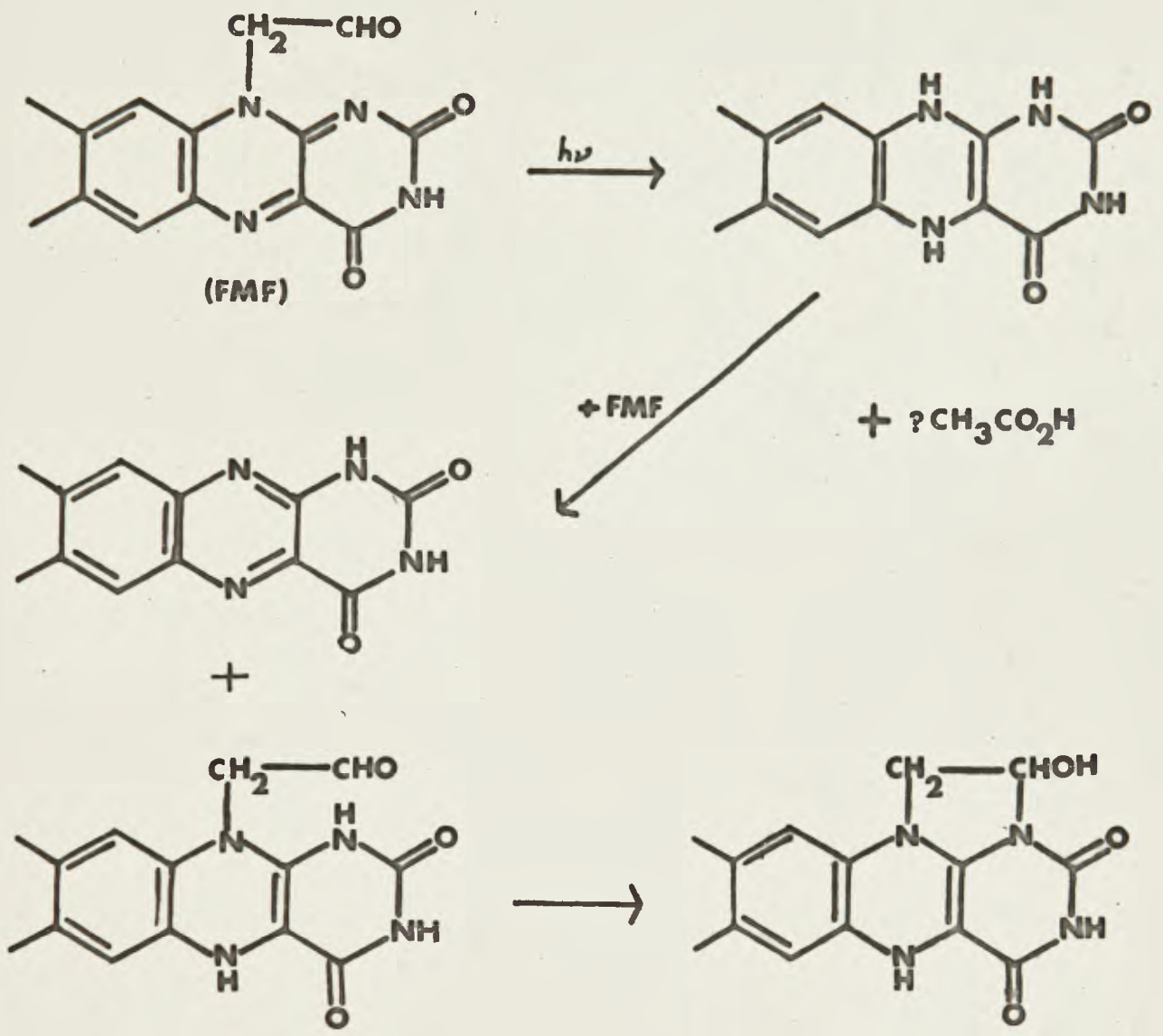


that the aldehyde is photobleached by abstraction of hydrogen α to the carbonyl group to give a reduced alloxazine. Because this has a more negative reduction potential than an isoalloxazine it reduces any oxidised flavin present. This reaction is followed by nucleophilic addition of N_1 of the reduced flavin to its side-chain carbonyl to give the leucoflavin (fig.4-2).

B) Photoreduction. Photoreduction differs from photobleaching because the reducing equivalents come from a second molecule, not from the flavin side-chain. The reactions of EDTA, NADH, and methionine have been most thoroughly studied, though many amines can fulfil their role. The stoichiometry of the EDTA reaction is uncertain, but one mole of glyoxylic acid is produced for each mole of flavin reduced (Enns and Burgess, 1965b). NAD^+ is the major product after reduction by NADH (Radda and Calvin, 1964), and methional after reduction by methionine (Enns and Burgess, 1965b).

Finer mechanistic details are similar for both photoreduction and photobleaching. The most studied of these is the nature of the flavin excited state. Small amounts of KI (about 10^{-6}M) significantly decrease the quantum yield of photoreduction, whereas the concentration required for

Fig. 4-2 ; Mechanism suggested for the bleaching of 9-formylmethyl lumiflavin

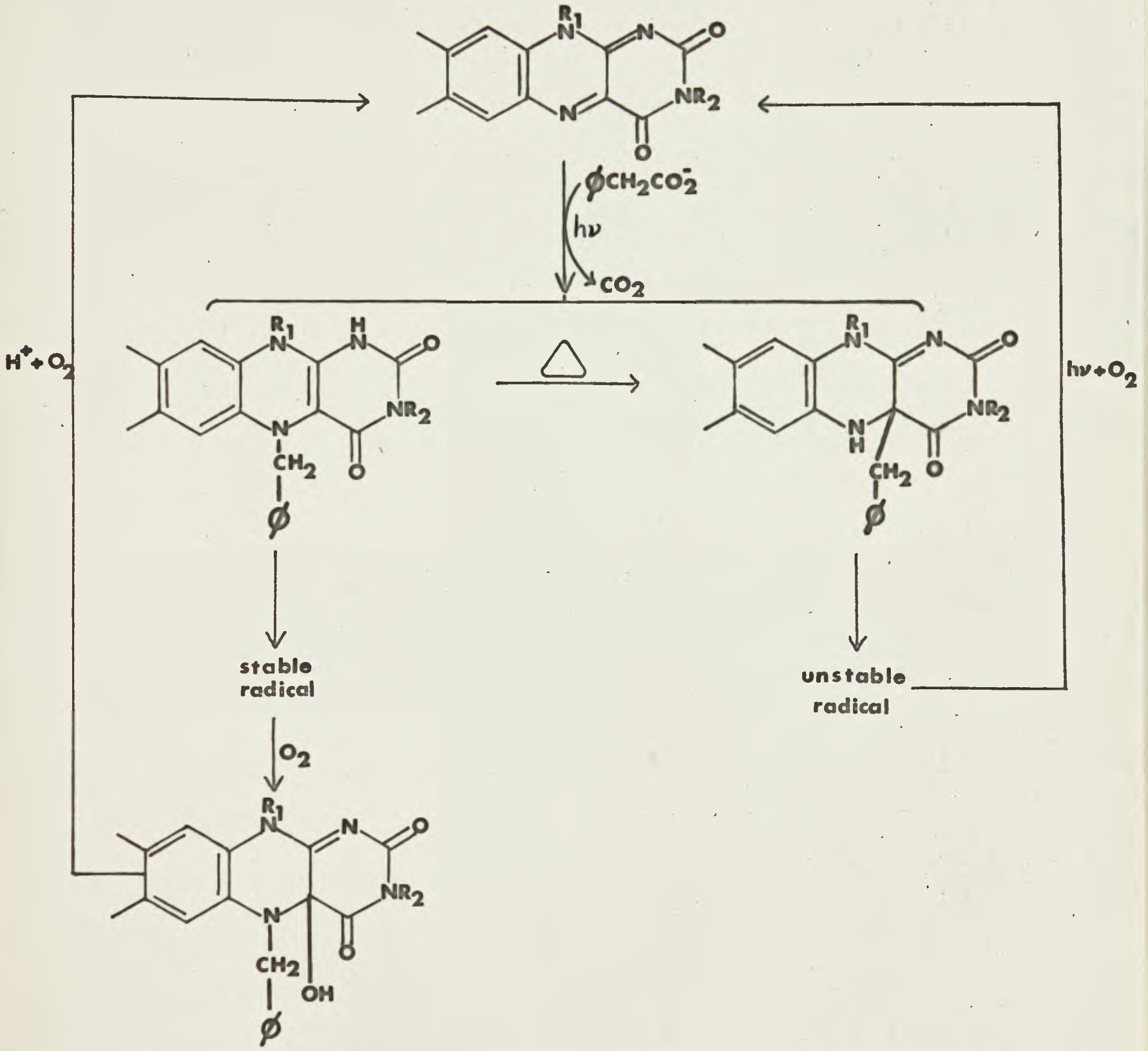


fluorescence quenching is 10^{-4}M (Holmström and Oster, 1961). Collision theory calculations suggest that the chemically active species has a lifetime of at least 10^{-4} sec (Holmström and Oster, 1961; Radda and Calvin, 1964). Paramagnetic ions inhibit photobleaching whereas diamagnetic metal ions do not. All these observations implicate a triplet as the active state of the flavin. An alternative interpretation, that the flavin semiquinone participates in the rate-determining step, is also possible because the radical's lifetime is of the order of 10^{-3}sec (Holmström, 1964), and it is known to form complexes with metals (Hemmerich, Veeger and Wood, 1965). It has been shown by flash photolysis that the flavin radical is an intermediate in both photobleaching and photoreduction (Holmström, 1964; Green and Tollin, 1968). An electron spin resonance signal has also been detected, but the time resolution of the method is too low to permit the study of the semiquinone as an intermediate (Radda and Calvin, 1964). Addition of KI however inhibits the rate of radical formation as well as that of the overall reaction (Holmström, 1962) which is the best evidence that the radical is not involved in the rate-determining step. Thus the reactive species must be the triplet, and the reaction probably proceeds by two one-electron steps.

The triplet state of lumiflavin has been studied in aqueous solution by flash photolysis, and a first order decay constant of about $10^4 sec^{-1}$ has been calculated (Knowles and Roe, 1968). At liquid nitrogen temperatures flavins phosphoresce at 605 - 630 mμ (Dhere and Castelli, 1938; Posthuma and Berends, 1966), and give rise to spin resonance signals at 1560 gauss. These correspond to forbidden $\Delta m = 2$ transitions. Measurements of the anisotropy of the spin-spin coupling tensor for several isoalloxazines show that the electron distribution in triplet states is not very sensitive to the substituent in the 9 position, but it does depend on the groups at C_6 and C_7 (Lhoste, Haug and Hemmerich, 1966). Photochemical reactivity in photoreduction is also more dependent on 6 and 7 substituents than on those in the 9 position (Radda, 1966).

C) Photoaddition. The scheme shown in figure 4-3 has been proposed for photoaddition on the basis of product analysis (Hemmerich, Massey and Weber, 1967). Subsequently a more detailed study has shown that this too is a reaction of the flavin triplet (Taylor, 1968). Unlike photobleaching and photoreduction, photoaddition is most favoured at acid pH

Fig. 4-3 ; Photoaddition reaction pathways



(Gordon Walker, 1968). The rate of photoaddition of phenylacetate to lumiflavin at pH 4 / the rate at pH 8 is approximately 3 (Taylor, 1968). The lack of a kinetic isotope effect with α,α-dideuterophenylacetic acid supports the proposed scheme (Taylor, 1968).

D) Photosensitization. Riboflavin can catalyse the photoreduction of Fe^{III} to Fe^{II}, in which the flavin is probably bleached in the usual way before the reduced flavin so formed reduces Fe^{III} (Rutter, 1958). A similar mechanism may explain the photoreduction of nitrate to nitrite in the presence of FMN and Mo^{VI} (Spence and Frank, 1963; Williams, 1961).

On illuminating an anaerobic mixture of riboflavin and ergosterol with visible light the steroid is dehydrogenated to give 6,6'-bisergostadienol (Tether and Turnbull, 1962). Triplet involvement is indicated by the action of phosphorescence quenchers, and the participation of flavin radicals has been suggested because the rate of reaction is independent of ionic strength. The ease of reaction decreases in the order FMN > riboflavin > lumiflavin = FAD = 0. This reactivity order was attributed to more favoured singlet-to-triplet conversion for the molecules with large

side-chains.

Flavins sensitize the cis-trans isomerisations of double bonds in pimaricin (an olefinic fungicide - Posthuma and Berends, 1966), stilbenes, and vitamin A derivatives (Gordon Walker and Radda, 1967). When either cis or trans-stilbene-4-carboxylic acid is illuminated in the presence of riboflavin an equilibrium mixture of 40% cis and 60% trans isomers is produced (Posthuma and Berends, 1966). Lumichrome is as effective as riboflavin, so neither the side-chain nor the isoalloxazine nucleus is vital here. Oxygen, paramagnetic ions, and KI all quench the reaction, so triplet involvement is again likely, and energy transfer between sensitizer and the olefin has been suggested.

E) Inhibition of photoprocesses. Many flavin photoreactions are inhibited by compounds other than generally accepted triplet deactivators like paramagnetic ions. Some compounds (e.g. tryptophan and serotonin) inhibit the photoreduction of other dyes (e.g. methylene blue and the phthalein dyes) as well as that of riboflavin. Others, notably the herbicides CMU and DCMU only affect flavin photoreactions (Radda and Calvin, 1963; Homann and Gaffron, 1964). More generally it is found that compounds expected to be good

electron donors towards a flavin, and which form ground-state complexes with it, are powerful inhibitors of photoreduction. Good electron acceptors (e.g. methyl viologen) and substances which do not form a complex with riboflavin have no effect (Radda, 1966). The concentrations of ground-state complexes are too small to explain the magnitude of the inhibitions, and so a direct interaction between flavin triplet and the inhibitor has been postulated (Radda, 1966).

A different kind of interaction is observed in the presence of some micellar macromolecules (polyvinylpyrrolidone, sodium decyl sulphate, etc.) which enhance the photosensitivity of many dyes. It is thought that an increased triplet lifetime is caused by an interaction between the excited flavin and the macromolecule (Kostenbauder, Deluca and Kowarski, 1965).

Most of this chapter concerns studies of the anaerobic photoreduction of flavins. The aims of the work have been to gain a detailed understanding of the mechanism of the reaction and its inhibition, and to find the features in the reducing substrate which lead to photoreduction rather than photoaddition. All the results have been discussed in terms of a detailed kinetic scheme.

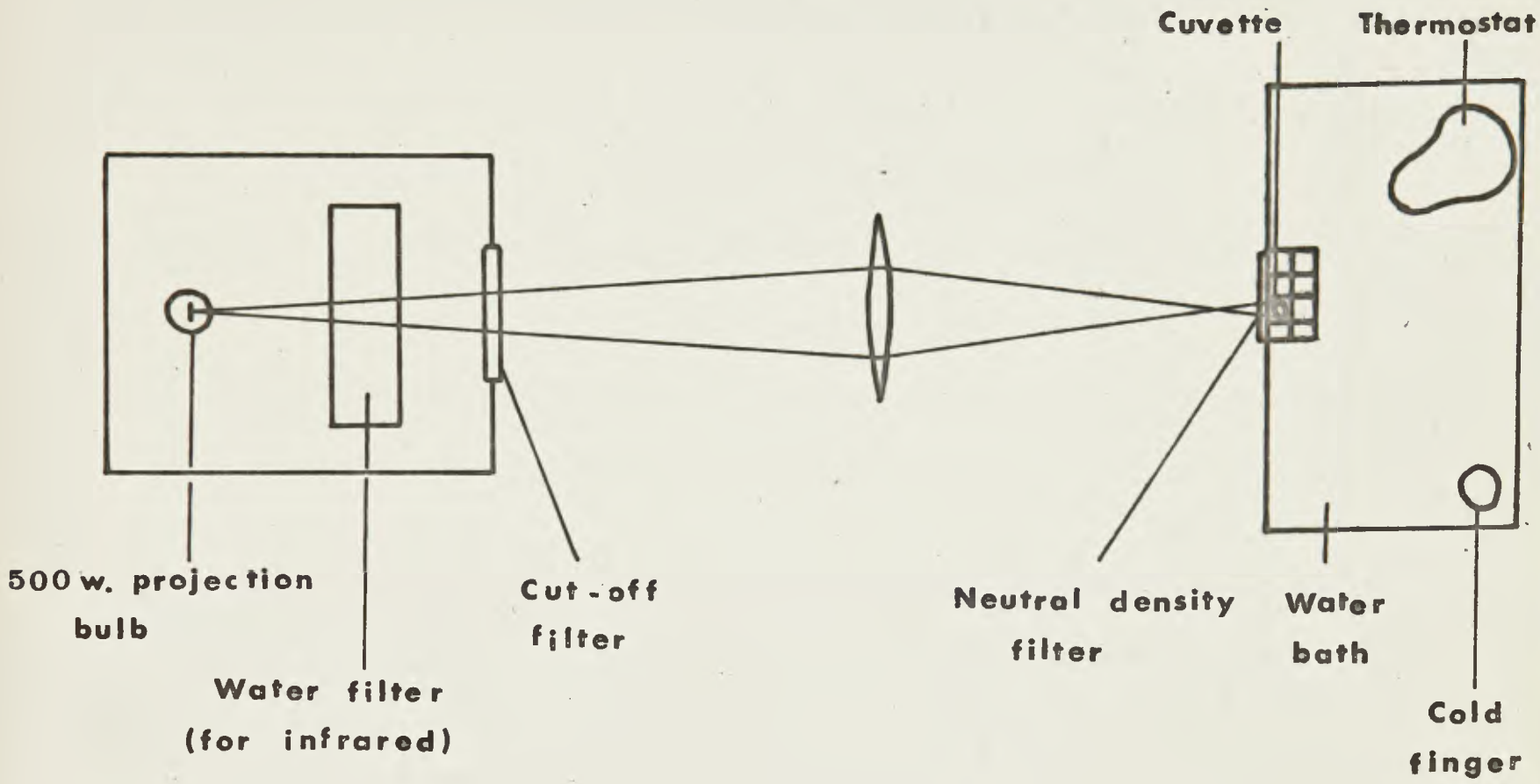
Measurements and Accuracy

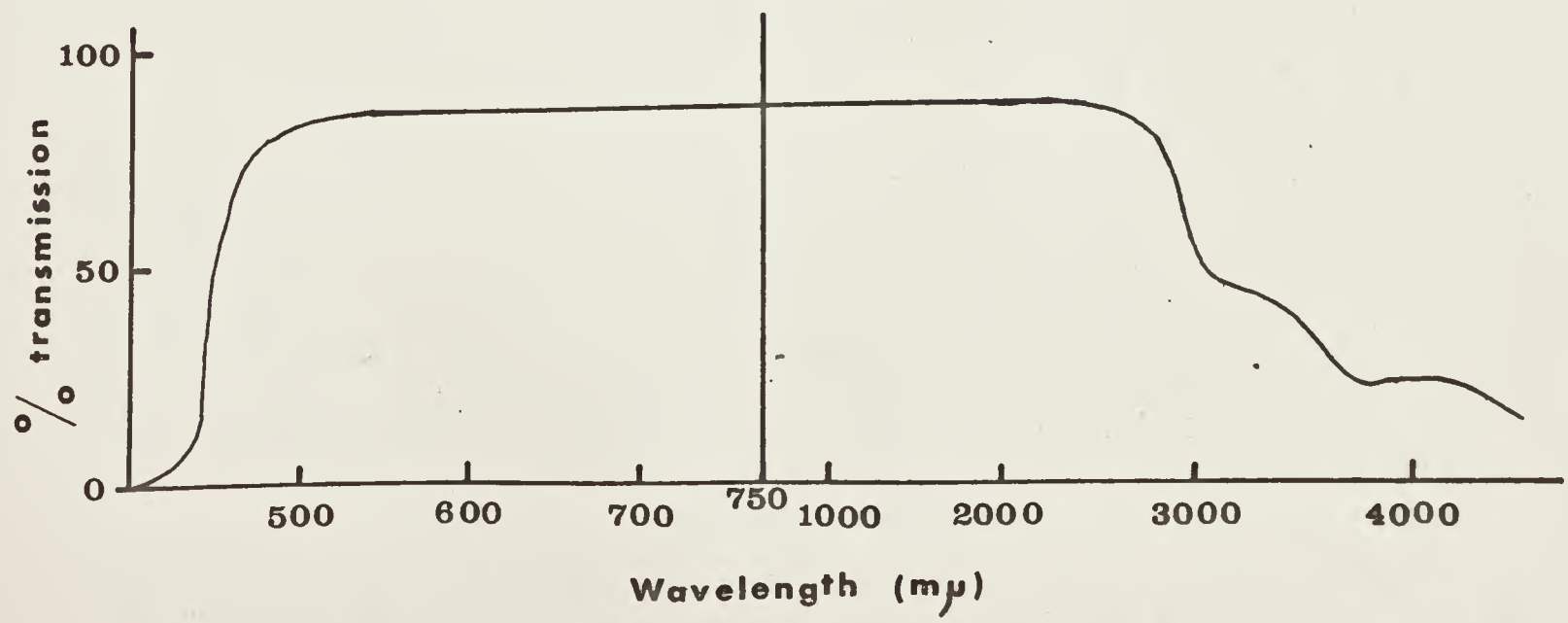
A) Illumination. A 500w tungsten filament projection bulb was used for all illuminations. This was connected with a potentiometer to allow easy adjustments to the lamp intensity. Varying the intensity this way is inappropriate for quantitative measurements because the colour temperature of the lamp is altered, so for quantitative variations neutral density filters were used with the lamp at a constant potential. These filters (supplied by Kodak) had a constant extinction across the visible spectrum.

To avoid unnecessary heating effects and the excitation of simple aromatic substrates as well as the flavin it is best to illuminate with visible light alone. The radiation from the lamp was therefore passed through a water filter to remove infrared wavelengths, and through a cut-off filter (Chance Bros.Ltd., OY 18 - see fig.4-4 for the spectrum) to remove wavelengths shorter than 400 mμ.

The arrangement of the apparatus is shown in figure 4-4; the whole is surrounded by a black cloth to keep out stray light. The lens was glass, with a focal length of 12 cm and a diameter of 5 cm. Focussing the light on the cuvette gives maximum efficiency of sensitization, but if only part of the solution is illuminated the frequency with which the

Fig. 4-4 ; Diagram of the illumination set-up and spectrum of the cut-off filter





mixture is shaken affects the rate of reaction which is measured. The same effect is unavoidable, even under homogeneous illumination with a parallel beam, for solutions with extinctions greater than 0.1 because of attenuation of the light as it passes through the cell. Constant stirring solves this problem, but it is not easy to achieve except in vessels larger than a cuvette. A focussed beam was used in the experiments described in this thesis. To obtain reproducible results it was thus necessary to use a standard method, always taking readings and mixing after the same time intervals.

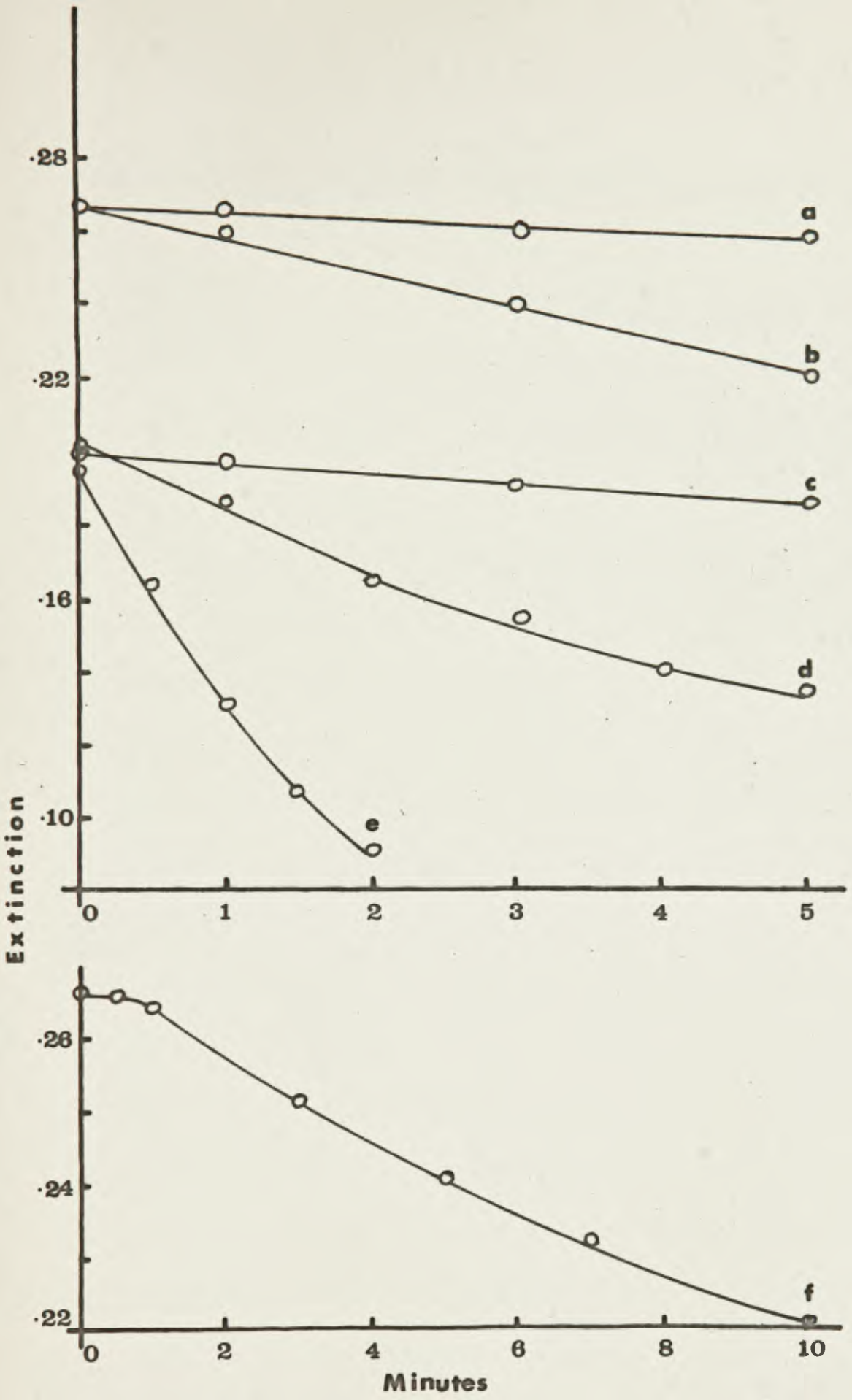
B) Deoxygenation. Deoxygenation was achieved by bubbling oxygen-free nitrogen through the solution to be studied. The nitrogen ('white spot' from British Oxygen Co.) was first passed through a solution of vanadyl sulphate in 2N H_2SO_4 over zinc amalgam. This removes all traces of oxygen (Meites and Meites, 1948). The gas was passed through a second Dreschel bottle containing water to wash out any traces of H_2SO_4 before being bubbled through the reaction mixture.

The vessel containing the solution to be deoxygenated (usually a 1 cm cuvette) was sealed with a rubber bung through which 2 syringe needles were pushed. One was

connected to the gas supply, and the other was left open as an outlet. After deoxygenation, which takes 20 min. for a cuvette, the needles were withdrawn, leaving the cuvette sealed and oxygen-free.

C) Reaction rates. Reaction rates were measured spectrophotometrically by following extinction at the flavin absorption maximum (see table 2-5) where the reduced forms do not absorb significantly (Holmström and Oster, 1961). Reactants were mixed in a 1 cm stoppered cuvette in the concentrations given in the tables of results, and deoxygenated by the process described in B. Extinctions were measured after a series of known time intervals, so the course of reaction could be represented by extinction : time curves (fig.4-5).

Because photochemical reactions are often subject to complex quenching processes and to product inhibition the extinction : time curves are most easily interpreted from their gradients at zero time. The shape of a curve provides a check that deoxygenation has been complete because in the presence of oxygen the reactions show an induction period (fig.4-5). The simple spectrophotometric technique described gives reproducibilities of 2-5%.



a) FAD
b) FAD + EDTA
c) FMN + CMU
d) FMN
e) FMN + EDTA
f) imperfect deoxygenation

Fig. 4-5 ; Sample anaerobic photoreaction curves (runs with EDTA were at lower light intensity than the others)

D) pK_a measurements. pK_a values were measured by titration using a Radiometer automatic titrator type TTT 1b. Amino acids were dissolved in 0.1M NaCl, and titrated with 2N NaOH in CO_2 free water (prepared by boiling doubly distilled water). Amines were titrated with dil.HCl. All runs were made at 25° ± 0.2°. The instrument was calibrated between runs using standard solutions made up from E.I.L. buffer tablets. The baseline (titration of 0.1M NaCl with NaOH or HCl) was subtracted from the curve obtained for an amino acid or an amine to obtain the true titration curve of the compound. The pK_a was taken from the corrected curve as the pH at which half the total volume of base or acid had been added.

Each pK_a was measured as the average of 3 runs. These usually fell within 0.03 of a pK unit of each other. Measurements on tryptophan, whose pK_a is known (pK_a = 9.40 at I = 0.1 - Hermans, Donovan and Scheraga, 1960) gave a value of 9.25. This discrepancy may be caused by small differences in temperature (1° alters the pK of tryptophan by 0.05) and imperfect calibration of the instrument.

E) Product analysis. Benzaldehyde was estimated by vapour phase chromatography using a Perkin Elmer F 11 instrument

with a flame ionisation detector. The 2 m column was packed with diethyleneglycol adipate, pentaerythritol and phosphoric acid in the proportions 25 : 2 : 73 (by weight). The method was as follows. A 40 ml sample of the reaction mixture containing lumiflavin (0.10 - 0.15 mM in different runs) and excess DL-phenylglycine was adjusted to pH 8.3 with Na_2HPO_4 solution, and placed in a vessel thermostatted at 25°. This was deoxygenated thoroughly by bubbling nitrogen for 2 hr, stirring magnetically. The anaerobic mixture was illuminated with visible light until its extinction showed that lumiflavin concentration had fallen below 0.01 mM (typically 2 hr). 1 μl of hexan-1-ol was now added to this solution to act as an internal standard for the chromotography.

The aqueous solution was extracted with one 10 ml portion of diethylether. This extracted virtually all the benzaldehyde as a second extraction did not significantly increase the amount of benzaldehyde in the combined extracts. The extract was dried with anhydrous Na_2SO_4. Samples of this (usually 2 μl) were injected into the instrument with a Hamilton microsyringe. With nitrogen carrier gas at 15 lb/in^2 pressure and a column temperature of 100° the retention times were: hexan-1-ol, 4.1min;

benzaldehyde, 13.0 min. The detector response was calibrated by injecting standard mixtures of benzaldehyde and hexan-1-ol in ether and the extraction procedure was calibrated by starting from known aqueous solutions of the samples (table 4-1).

Peak areas were measured by triangulation. The benzaldehyde : hexan-1-ol area ratios for a given solution and extraction were consistent ± 5%, and for separately prepared and extracted solutions ± 10%. At least two samples of each mixture were analysed and the average was taken. The final values for the quantity of benzaldehyde in a reaction mixture have an accuracy of ± 20% because they are derived from 2 area ratios - that for a standard mixture and that for the reaction mixture.

For estimation of benzoic acid and phenylglyoxylic acid this method was modified. A similar reaction mixture was illuminated, but it was acidified to pH 2 with c.HCl before extraction with ether. 3 10ml aliquots of ether were used in these extractions, and after combining them they were evaporated to a very small volume before injection into the chromatograph. The same column was used but at 190°, when retention times were: benzaldehyde, 1.6 min; benzoic acid, 12.2 min; phenylacetic acid, 19.5 min;

Table 4-1

Calibration of vapour phase chromatography

Operating conditions and retention times are given in the text.

Composition of the sample injected	Peak area of hexan-1-ol / Peak area of benzaldehyde	
1µl hexan-1-ol + 1µl benzaldehyde in 5 ml ether	0.98 1.05	1.015
1µl hexan-1-ol + 1µl benzaldehyde in 10ml ether dried over Na_2SO_4	0.718 0.743	0.730
1µl hexan-1-ol + 1µl benzaldehyde in 25ml Na_2HPO_4 (pH 8.8) extracted with 10ml ether, and dried over Na_2SO_4 (6 different solutions).	1.025 0.983	1.00
	0.812 0.826	0.819
	0.932 0.961	0.947
	0.820 0.891	0.855
	0.836 0.844	0.840
	0.789 0.769	0.779

0.873

phenylglyoxylic acid, 36 min. Blanks showed that this method was capable of detecting 0.05 mM acid in the aqueous solution.

Phenylglyoxylic acid was also estimated by a spectroscopic method (Bruice and Topping, 1963). Phenylglyoxylic acid reacts with o-phenylene diamine to form 3-phenyl-2-oxoquinoxaline (Buraczewski and Marchlewski, 1901). This precipitates quantitatively from aqueous solution. The crystals redissolve in ethanol giving an absorption maximum at 370 mμ .

o-Phenylenediamine hydrochloride was purified by warming with active charcoal and recrystallizing from water to give colourless crystals. 3 ml of the solution to be tested for phenylglyoxylic acid were added to 0.5 ml of saturated o-phenylenediamine hydrochloride solution, and the mixture was left for 48 hr. at 2°. Lower concentrations of phenylglyoxylic acid than 0.5 mM do not lead to the formation of crystals, but the 3-phenyl-2-oxoquinoxaline can be extracted from the aqueous solution into 4 ml of ether, and its spectrum observed in this solvent. Blanks showed that this method can easily detect phenylglyoxylic acid in 0.05 mM aqueous solutions (table 4-2).

CO_2 was determined by acidifying an anaerobic reaction

Table 4-2

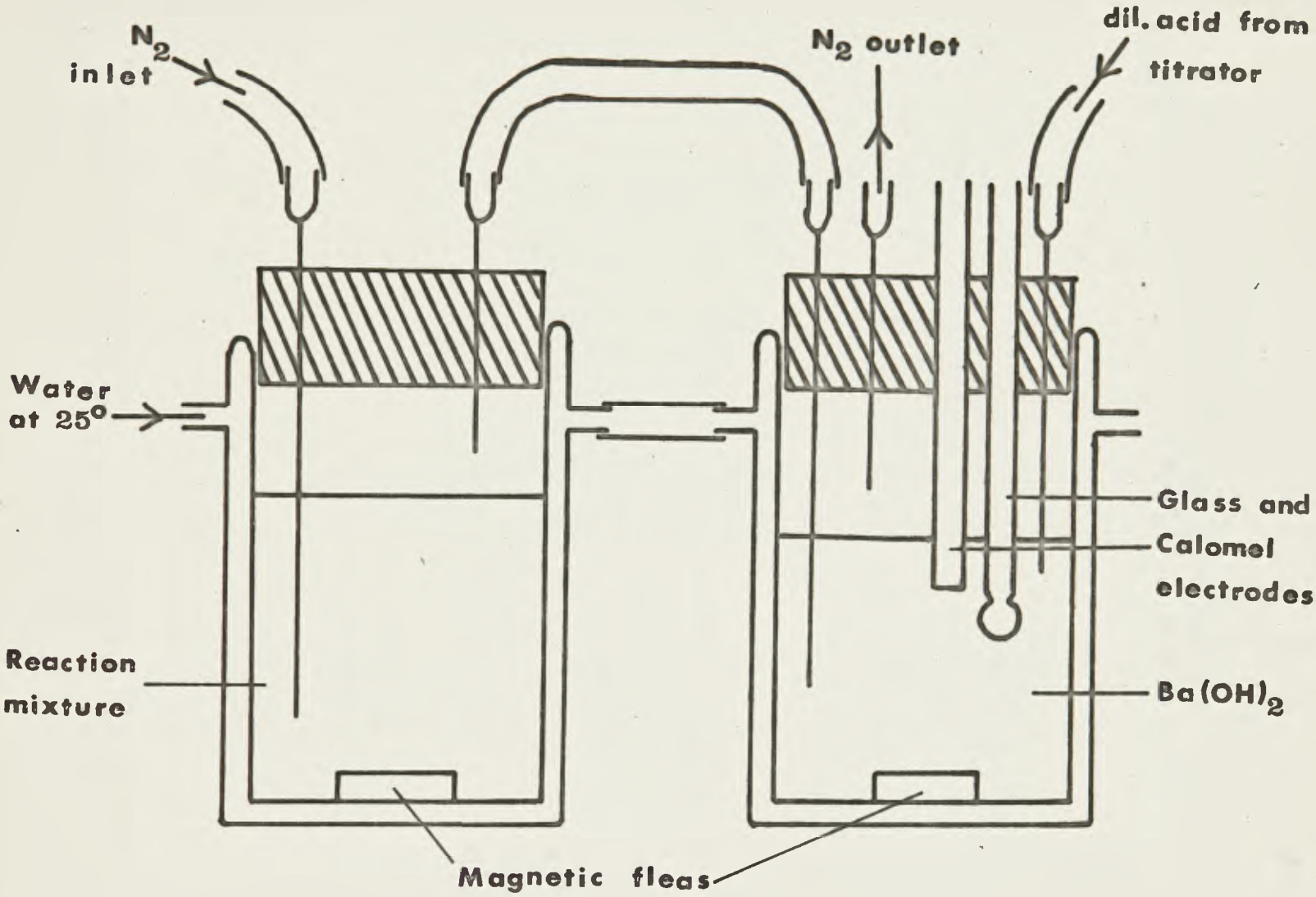
Calibration of the spectrophotometric determination of phenylglyoxylic acid as its 3-phenyl-2-oxoquinoxaline

Concentration of phenylglyoxylic acid (μM)	Extinction at 360 mμ after reaction with o-phenylenediamine and extraction into ether
33	0.105
100	0.38
167	0.64
233	1.00
333	1.05

mixture (identical to those described for the benzaldehyde experiments) with c.HCl. The released CO_2 was then blown by a stream of nitrogen into a 0.5 mM solution of $Ba(OH)_2$ for 1 hr. Both these operations were performed without allowing the reaction mixture to contact the atmosphere. The HCl was injected directly into the reaction vessel through its rubber bung, and the CO_2 was blown into a second deoxygenated vessel containing the $Ba(OH)_2$ by the arrangement of tubes and syringe needles shown in figure 4-6. The $Ba(OH)_2$ was now titrated with standard HCl using the automatic titrator, and the titre was subtracted from that obtained by using an identical procedure on an unilluminated reaction mixture. The quantities of CO_2 involved were insufficient to precipitate $BaCO_3$ (solubility product $=10^{-8}$ - Handbook of Chemistry and Physics, 1964-5) so the reaction occurring was $CO_2 + OH^- \rightleftharpoons HCO_3^-$. Thus the change in concentration of OH^- is equivalent to the amount of CO_2 absorbed.

This method of estimating CO_2 was subject to errors up to 20%. The main reasons were the extreme sensitivity of the method to absorption of atmospheric CO_2, and the irreproducibility of the bubbling procedure which was 70% efficient ($\pm$ 10%) in runs with standard CO_2 solutions.

Fig. 4-6 ; Apparatus for carbon dioxide estimation



A second method to estimate CO_2, 'the CO_2 electrode', was tried. This works on the principle that some membranes are permeable to small uncharged molecules, but not to ions. Polythene, teflon and silicone rubber are typical membranes with this property. It is thus possible to surround a normal glass electrode with a membrane holding a solution at one pH, and to dip the assembly into a solution at a second pH without altering the reading obtained from the electrode. To construct a CO_2-electrode the electrolyte between the membrane and the glass electrode is saturated KCl solution containing 5mM $KHCO_3$ (Snell, 1960).

First a slurry in inert granules (celite) was made in the electrolyte. The electrode was dipped into this, and a teflon membrane was stretched round it while it was still wet. The celite served to keep the layer of electrolyte between the membrane and the glass electrode approximately constant in thickness. The membrane was bound tightly to the neck of the electrode by thread. The assembled electrode was connected to the pH meter unit of the automatic titrator, from which readings were taken directly.

pH is related to pCO_2 (where p has the same significance as in pH) by the expression $pCO_2 = pH - \log [HCO_3^-] + \log K +$

log K_w, where $K = \frac{[HCO_3^-][H^+]}{[CO_2] \cdot K_w}$, and K_w is the ionic product for water. Thus for low pCO_2 values a change in pCO_2 registers as an equal change of pH in the membrane electrolyte. The value of the first dissociation constant of carbonic acid is 4.3×10^{-7} at 20°, and it can be seen from this and the concentration of bicarbonate in the membrane that pCO_2 is about (pH - 4). Even when surrounded by freshly boiled CO_2-free water the pH registered by the electrode did not rise above 7.73 because of the presence of bicarbonate in the membrane electrolyte. Thus a CO_2-electrode is not good for measuring CO_2 concentrations less than millimolar, but it can be used to measure changes in pCO_2 of 0.1 units (i.e. about 100 μM in a very dilute solution).

The CO_2-electrode was thus just sufficiently sensitive for studies of the photoaddition of phenylacetate to flavins, but it could not be used for reactions where another small molecule which could diffuse through the membrane was produced (e.g. ammonia in the reaction of DL-phenylglycine).

Results and Discussion

Some preliminary work on the measurements in this section has been described in a previous thesis (Penzer, 1966).

Acknowledgement has been made where it is reproduced again.

A) Kinetic studies. It is convenient to consider the results in terms of the scheme outlined below (Penzer and Radda, 1968a), which is more detailed than those described before (Radda and Calvin, 1964) or since (Byron and Turnbull, 1968).

$$(1) \quad F + h\nu \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} F^*$$

The rate of production of F^* is proportional to the amount of light absorbed according to the Beer-Lambert law. This may be simplified to $I_o - I = I_o(1 - e^{-\epsilon[F]l}) \approx I_o \epsilon[F]l$ when $\epsilon[F]l$ is less than 0.25 (I_o and I are the light intensities before and after passing through 1 cm of solution of a flavin with extinction coefficient ϵ). Under these conditions k_{+1} is taken as ϵl.

$$(2a) \quad F^* + \text{solv.} \xrightarrow{k_{+2a}} F + \text{solv.}$$

$$(2b) \quad F^* + RH_2 \xrightarrow{k_{+2b}} F + RH_2$$

$$(3a) \quad F^* \xrightarrow{k_{+3a}} F^t$$

$$(3b) \quad F^* + RH_2 \xrightarrow{k_{+3b}} F^t + RH_2$$

$$(3c) \quad F^* + \text{solv.} \xrightarrow{k_{+3c}} F^t + \text{solv.}$$

$$(3d) \quad F^* + FH\cdot \xrightarrow{k_{+3d}} F^t + FH\cdot$$

(4a) $F^t \xrightarrow{k_{+4a}} F$

(4b) $F^t + RH_2 \xrightarrow{k_{+4b}} F + RH_2$

(4c) $F^t + solv. \xrightarrow{k_{+4c}} F + solv.$

(4d) $F^t + FH^\bullet \xrightarrow{k_{+4d}} F + FH^\bullet$

(4e) $F^t + F^t \xrightarrow{k_{+4e}} F + F^*$

(5) $F^t + RH_2 \xrightarrow{k_{+5}} FH^\bullet + RH^\bullet$

or (5') $F^t + RCO_2H \xrightarrow{k_{+5'}} FH\text{-}R + CO_2$

(6) $FH^\bullet + RH^\bullet \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} FH_2 + R$

(7) $2FH^\bullet \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} F + FH_2$

(8) $FH^\bullet + RH_2 \underset{k_{-8}}{\overset{k_{+8}}{\rightleftharpoons}} FH_2 + RH^\bullet$

F, $FH^\bullet$, and FH_2 are oxidised, half-reduced, and fully reduced flavin respectively, and RH_2, $RH^\bullet$, and R are reduced, half-oxidised, and fully oxidised reducing agent. F^* is excited singlet flavin and F^t is the flavin triplet. This scheme supposes that the reactive species in photo-

reduction is the flavin triplet. This has already been shown for photobleaching (Radda and Calvin, 1964), and was confirmed by observing that paramagnetic ions also lower the rate of photoreduction (table 4-3).

The rates of some of the processes listed are negligible. Neither EDTA nor DL-phenylglycine quenches flavin fluorescence so when either of these is the reducing agent k_{+2b} and k_{+3b} are unimportant. Second order triplet deactivation (reaction 4c) may not be unimportant in all cases because the rate of photoreduction of FMN by EDTA does not depend linearly on light intensity (fig.4-7). In contrast, fluorescence intensity is directly proportional to excitation light intensity. By restricting considerations to the initial reaction rates deactivations by the flavin radical (reactions 3d and 4d) can be neglected.

Reaction 8 has been independently studied by investigating the behaviour of the flavin radical produced in solution by catalytic hydrogenation of FMN (Penzer, 1966; Penzer and Radda, 1968a). Following the radical concentration spectrophotometrically at 600 mμ (Gibson, Massey and Atherton, 1962) no reaction with EDTA was observed in the dark, or on illumination by light with wavelengths longer than 550 mμ. Light with wavelengths lower

Table 4-3 *

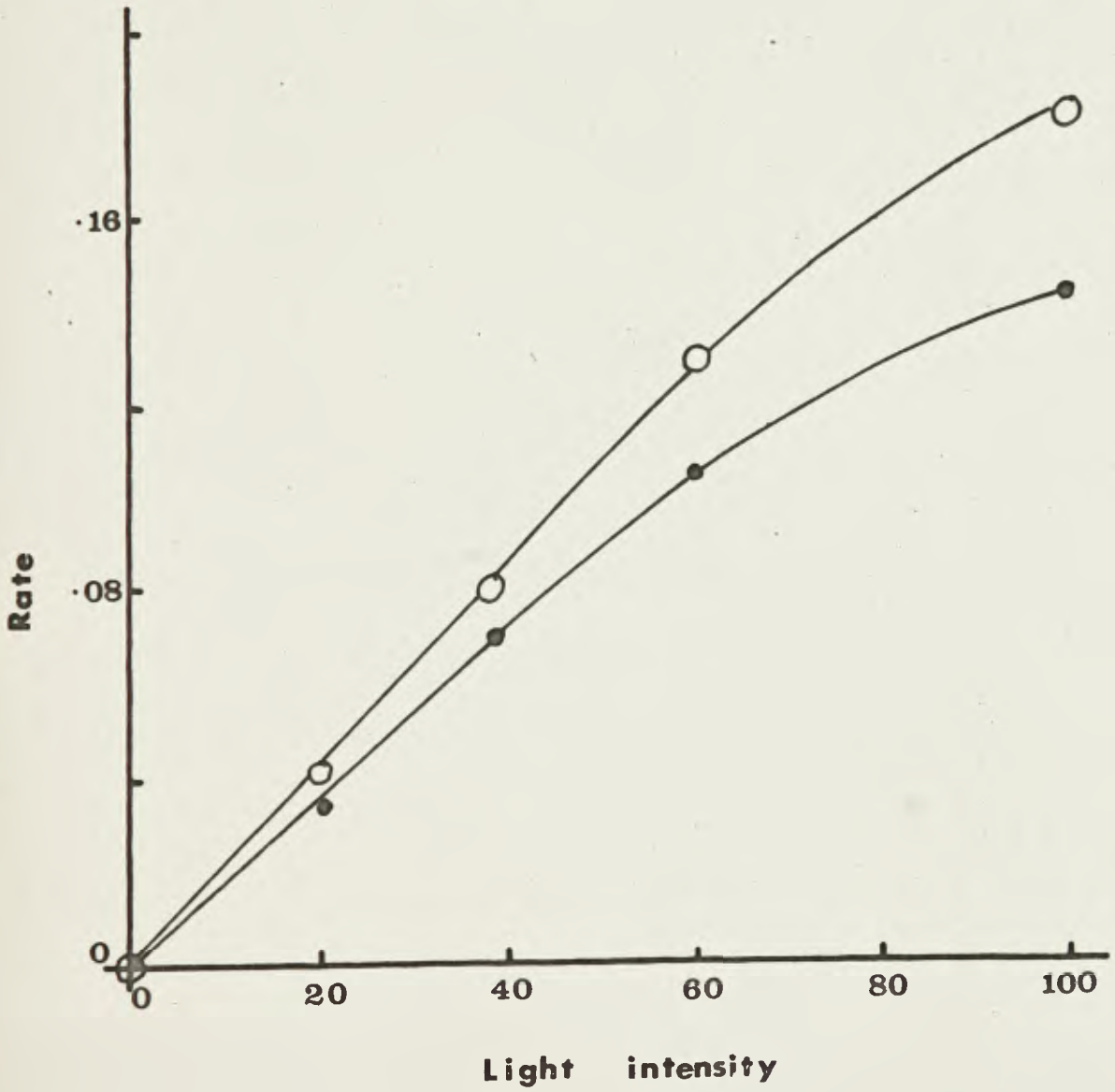
Effect of metal ions on the photoreduction of FMN by EDTA

All measurements were made at 4.0° on solutions containing 0.1 M-NaCl, 0.1 M-EDTA, 0.04 mM-FMN and 1 mM-metal chloride. Under these conditions fluorescence quenching by the metal chlorides was less than 1%.

Metal chloride added	Initial rate (fall in ϵ_{447}/min.)	Percentage inhibition $\left(1-\frac{k_{inhib.}}{k_{uninhib.}}\right) \times 100$
None	0.078	0
$ZnCl_2$	0.079	0
$MgCl_2$	0.080	0
$NiCl_2$	0.067	14
$MnCl_2$	0.055	29

* Taken from Penzer (1966).

Fig. 4-7 ; Effects of temperature and light intensity on the rate of photoreduction of FMN by EDTA.
(Reaction mixtures contained 0.1 M phosphate buffer, pH 7.0, mM EDTA and 0.04mM FMN: O 40^{o}, • 10^{o})



than 500 mμ caused rapid reaction. This was probably a reaction of excited oxidised flavin which caused a change in the radical concentration according to the equilibrium: FMN + $FMNH_2 \rightleftharpoons 2FMNH^{\cdot}$. The radical concentration was maximal at half reaction. These observations suggest that k_{+8} is negligible.

Thus, considering initial rates only, reactions, 2b, 3b, 3d, 4d, 6, 7, and 8 are unimportant. Under these conditions, and assuming that the flavin concentrations are sufficiently low for the rate of excited singlet production to be given by $k_{+1} I_o [F]$, it can be shown that: $- \frac{d[F]}{dt}_{init.} = \left\{ k_{+1}\, k_{+5}\, (k_{+3a} + k_{+3c}[solv])\, I_o [RH_2][F] \right\} \Big/ \left\{ (k_{-1} + (k_{+2a}+k_{+3c})[solv.] + k_{+3a})(k_{+4a}+(k_{+4b}+k_{+5})[RH_2] + k_{+4c}[solv.] + k_{+4e}[F^t]) \right\}$

This expression is obtained when a steady state approximation is applied to the concentrations of the flavin excited singlet and triplet (see appendix 4). It is independent of the relative magnitudes of k_{+6} and k_{+7}, and of whether route 5 or 5' is followed, because it assumes that the rate determining step is the interaction between flavin triplet and the reducing agent. This is consistent with the effects of paramagnetic ions (Radda and Calvin, 1964:

table 4-3).

The scheme accounts for the apparently contradictory reports that the rate of photoreduction is proportional to the concentration of reducing agent (Holmström, 1962) and that the two are independent (Radda and Calvin, 1964). At low reducing agent concentrations the $(k_{+4b} + k_{+5})[RH_2]$ term is unimportant relative to the other quenching terms in the denominator, so linear concentration dependence should be found. At high reducing agent concentrations solvent quenching becomes relatively unimportant as the interactions with reducing agent lower triplet lifetime, and so the $[RH_2]$ terms in the numerator and denominator cancel. The anerobic photoreductions of lumiflavin by EDTA and DL-phenylglycine both show this type of behaviour (fig.4-8). The experimental curves fit those calculated from individual rate constants well (see p. 136).

B) Reaction products. The fall in extinction when any of the flavins listed in table 4-7 is photoreduced by DL-phenylglycine is completely and rapidly reversed by oxygen in the dark. This indicates that reduced flavin is a reaction product. In contrast the reaction with phenylacetic acid is not reversible in this way; the recovery of an oxidised flavin absorption spectrum is only

Fig. 4-8 ; Effects of concentration of a) EDTA and b) DL-phenyl-glycine on the rate of photoreduction of flavins

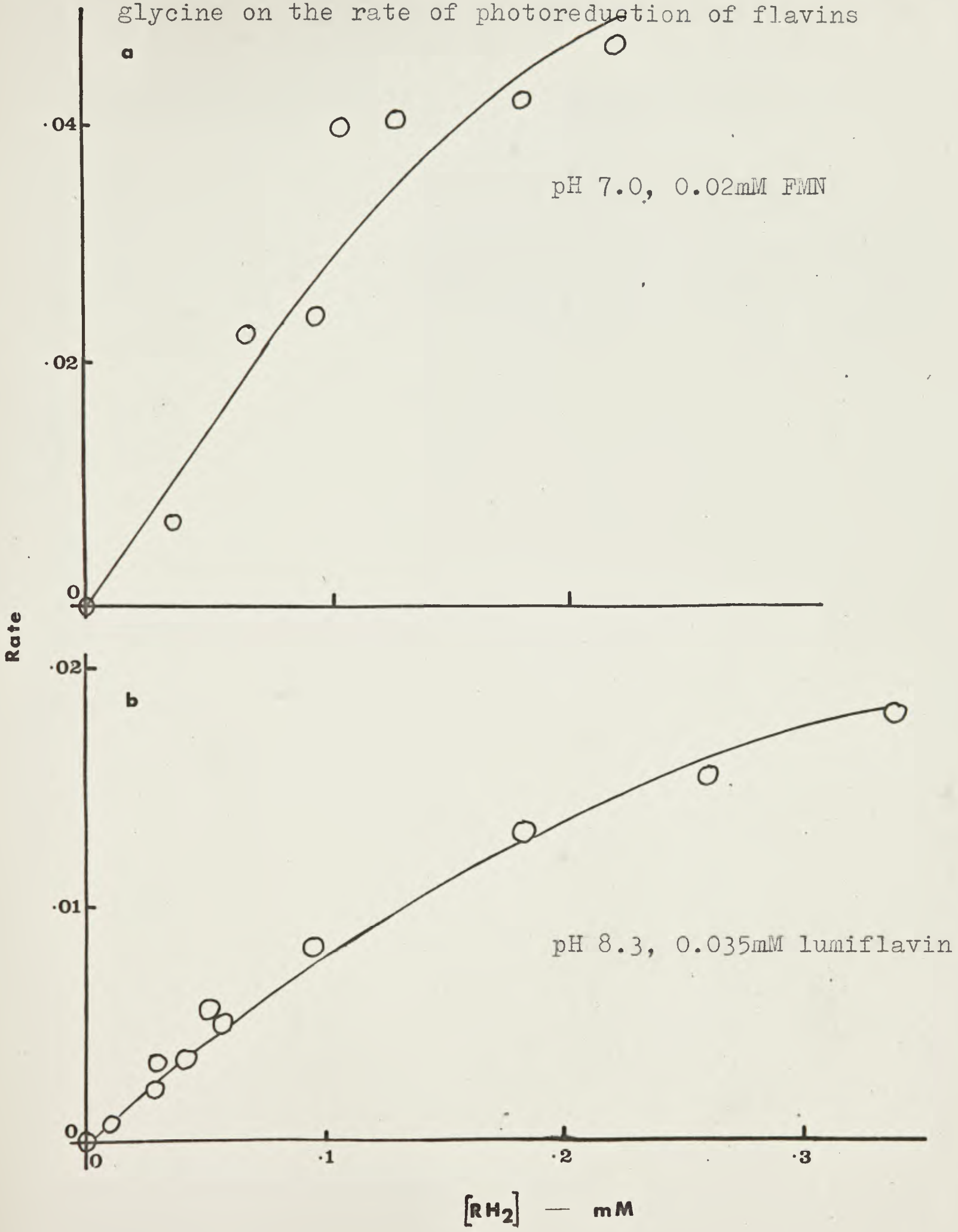


Table 4-4

Reaction products of the photoreduction of of lumiflavin by phenylglycine

All reactions were at 25° and pH 8.4.

Amount of lumiflavin reacting (μ moles)	Amount of benzaldehyde formed (μ moles)	Amount of CO_2 formed (μ moles)
4.8	5.2	
4.8	4.8	
4.8	5.6	
5.2		5.0
5.0		4.8
2.8		3.0

Table 4-5

Photosensitized destruction of phenylglyoxylic acid in the presence of lumiflavin

Phenylglyoxylic acid was estimated by the spectroscopic method using o-phenylenediamine. Lumiflavin concentration was .07 mM. The temperature was 25°.

pH	Initial concentration of phenylglyoxylic acid (mM)	Concentration of phenylglyoxylic acid after 2 hr. illumination (mM)	% destruction
3	0.095	0.072	24
8	0.095	0.084	12

slow and incomplete on readmitting oxygen. This provides a simple experimental criterion for distinguishing between photoreduction and photoaddition, but it will not differentiate between photoreduction and photoaddition to give an unstable adduct which quickly dissociates to reduced flavin.

DL-phenylglycine is oxidised to benzaldehyde and carbon dioxide in a mole-for-mole ratio to lumiflavin reduced (table 4-4). Neither benzoic acid nor phenylglyoxylic acid was detected (see p.95 for details of the analyses). Blanks showed that illuminated lumiflavin sensitizes the decomposition of phenylglyoxylic acid slowly (table 4-5), but the method used should still have detected a yield of 10% or more of the acid.

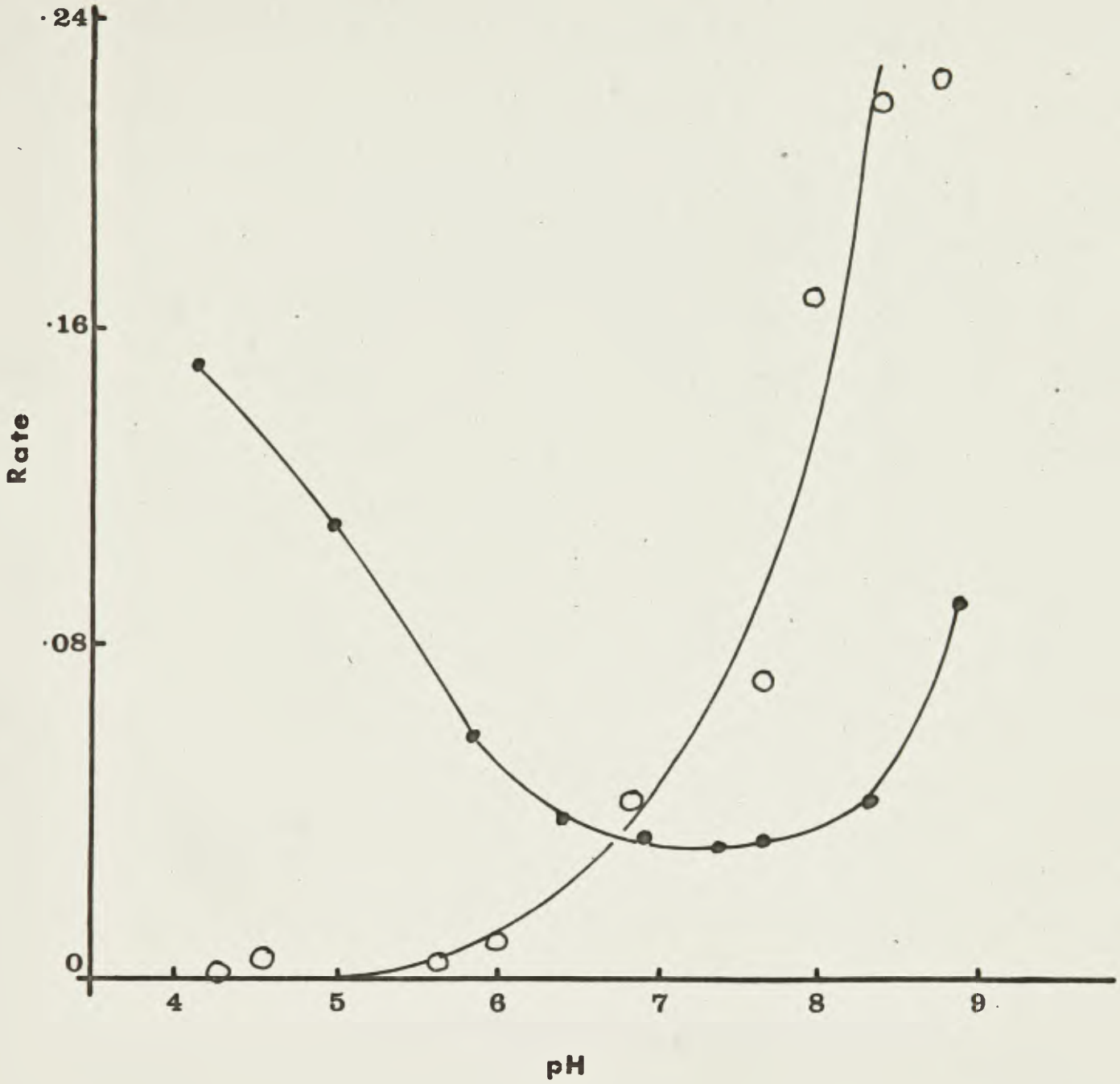
The evolution of CO_2 during photoaddition with phenylacetate has been reported, and this was qualitatively confirmed using the CO_2-electrode. When the photoadduct was decomposed by the action of air and light the main product detected by vapour phase chromatography was benzaldehyde.

c) Effects of reducing agent structure. As benzaldehyde is the major product of reduction by DL-phenylglycine the primary reaction centre involves the α-carbon atom. Thus

the effects of meta and para substituents in the benzene ring on the rate of reaction should indicate the nature of the rate-determining process. The same substituents also alter the dissociation constants of the amino groups, and hence a knowledge of the pK_b values (14.00 - pK_a at 25°) and of the influence of pH on reaction rate is essential for understanding the substituent effects.

The pH-dependences of the reaction rates of a lumiflavin derivative with DL-phenylglycine and phenylacetic acid are shown in figure 4-9. Whereas the phenylacetic acid curve does not correspond to a titration curve of either reactant the DL-phenylglycine result is readily explained if the anion ($Ph.CH(NH_2).CO_2^-$), but not its zwitterion, reacts. This explains the increase in reaction rate as the pH is raised. Above pH 8.5 the rate falls because of acid-dissociation of the flavin nucleus (fig.1-2): fluorescence is quenched in the anion (Beinert, 1960) and so a reduction in photoreactivity is not surprising. A similar fall above pH 8.5 is found for the phenylacetic acid reaction. The increase in rate at low pH is not a result of general acid catalysis (Taylor, 1968), but it may be explained by acid-dissociation of the flavin triplet with a pK_a of 5-6 (see p. 141).

Fig. 4-9 ; pH Dependence of the rates of anaerobic photoreaction between flavins and • phenylacetic acid, O DL-phenylglycine



The flavin used was 9-acetoxyethyl-3-methyl-lumiflavin (.025mM) with 5 mM acid at 25°

These measurements were made in collaboration with Mrs. A.M.Ball

The acid-dissociation constants of DL-phenylglycine and some derivatives were measured by titration. Their rates of photoreduction of lumiflavin at pH 7.0, and the rates corrected for the different pK_a values of the acids are also given in table 4-6. The substituent effects may be analysed in terms of the Hammett equation (Hammett, 1940) - $\log k/k_0 = \sigma\rho$. A ρ-value of -1.1 is obtained for the anerobic photoreduction of lumiflavin, with a correlationcoefficient of 0.94 (fig.4-10). The relatively small ρ-value is consistent with a radical mechanism. Its negative sign suggests a transition state with an electron-deficient α-carbon atom on the amino acid. This is possible if the flavin triplet behaves like an electrophilic radical (the extraction of benzylic hydrogens by bromine atoms may be an analogous reaction; Kooyman, van Helden and Bickel, 1953). In contrast, the photoaddition of substituted phenylacetic acids to lumiflavin gives a very different pattern (Taylor, 1968).

Several effects may have contributed to the comparatively low correlation coefficient of 0.94 apart from inaccuracies in the measurements of reaction rates. The derivatives may have different efficiencies as quenchers of flavin

Table 4-6

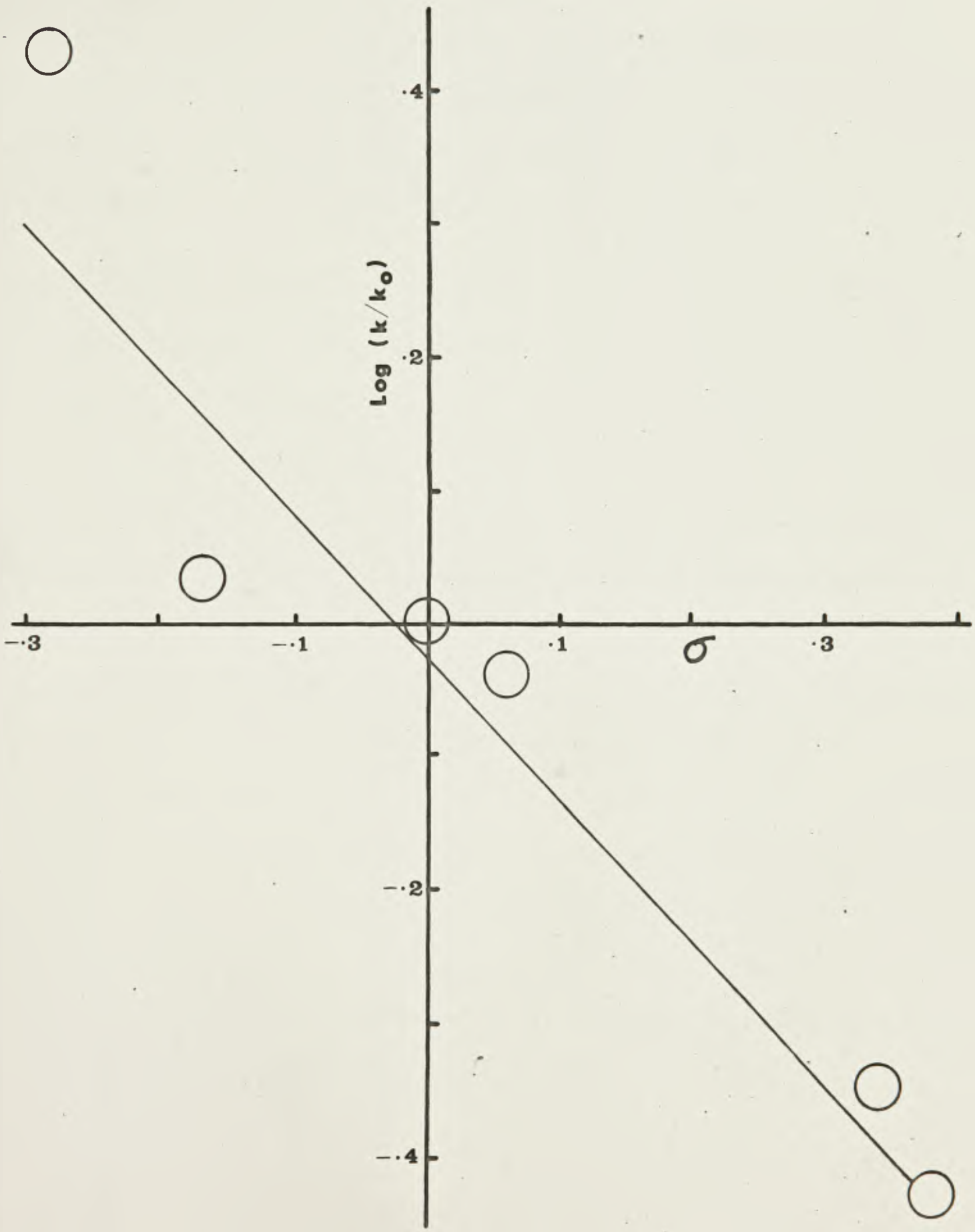
Acid dissociation constants, rates of photoreduction of lumiflavin by substituted phenylglycines, and σ-values.

pK_b values were measured in 0.1M NaCl at 25°. Reaction rates were measured in solutions containing 0.1M phosphate buffer, pH 7.0, 0.015 mM lumiflavin and 0.5 mM phenylglycine at 30°.

Acid	pK_b	Reaction rate (fall in extinction /min)	Corrected rate [measured rate x antilog(pK_b phenylglycine -pK_b substituted acid)] .	
DL-phenylglycine	5.09	0.078	0.078	0
DL-m-fluorophenylglycine	5.45	0.080	0.035	.337
DL-m-chlorophenylglycine	5.52	0.078	0.029	.373
DL-p-methylphenylglycine	5.04	0.076	0.085	-.170
DL-p-fluorophenylglycine	5.16	0.083	0.071	.062
DL-p-methoxyphenylglycine	4.94	0.150	0.210	-.268

σ-values are taken from Wells, 1963.

Fig. 4-10 ; Hammett plot for the photoreduction of lumiflavin by substituted phenylglycines



triplets. This quenching is an important interaction (see p.135), and it is quite likely that a derivative with a polar substituent (like chlorine) has a different quenching constant from unsubstituted DL-phenylglycine. Some of the derivatives (especially DL-p-methoxyphenylglycine) may be able to reduce a flavin at sites other than the α-CH, giving anomalously high reaction rates.

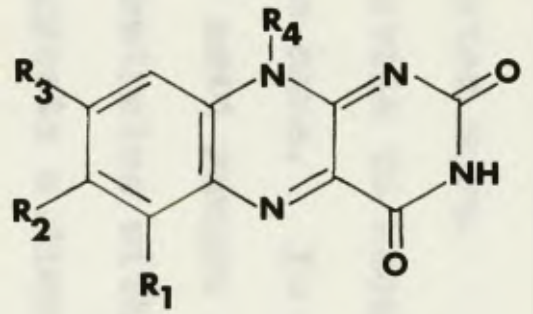
D) Effects of flavin structure. Flavin derivatives are photoreduced by a given reducing agent at different rates. Because of differences in their absorption spectra quantitative comparisons of the derivatives cannot be made directly, but it is still clear that the flavins which are most susceptible to photoreduction are lumiflavin, FMN, 3-methyl lumiflavin, and the 6-chloro-derivative (table 4-7). The uncorrected rates do not correlate with the triplet lifetimes measured in polyacrylamide films at room temperature (see p. 162).

Flavins are photoreduced by a variety of amines (e.g. diethanolamine, benzylamine, triethylamine) in their unprotonated forms. If decarboxylation is the rate determining step for photoaddition of phenylacetic acid (Hemmerich, Massey and Weber, 1967), the amines will not

Table 4-7

Relative rates of photoreduction of flavin derivatives by DL-phenylglycine, phenylacetic acid and benzylamine.

Concentrations of a particular flavin were constant for the three substrates at about 0.02 mM. Solutions contained 5 mM reducing agent in 0.1M phosphate buffer, pH 8.00 (for DL-phenylglycine), pH 7.89 (for phenylacetic acid) or pH 7.93 (for benzylamine).



Flavin				Rate of photoreaction (fall in extinction/min.)			A/B	A/C
R_1	R_2	R_3	R_4	DL-phenylglycine (A)	phenylacetic acid (B)	benzylamine (C)		
H	Me	Me	ribityl phosphate(FMN)	0.132	0.016	0.024	8.25	5.5
H	Me	Me	Me (lumiflavin)	0.140	0.020	0.024	7.0	5.5
H	Me	Me	Me (N^3-Me)	0.192	0.024	0.032	8.0	6.0
Me	Me	H	ribityl (isoriboflavin)	0.064	0.006	0.012	10.0	5.3
H	Cl	MeO	$(CH_2)_2$-N(CH_2-CH_2)(CH_2-CH_2)	0.032	0.012	0.010	2.7	4.0
H	Cl	H	$(CH_2)_3$-NEt_2	0.140	0.040	0.022	3.5	6.4
H	H	H	$(CH_2)_2$-NMe_2	0.001	0.002(5)	0.0002	0.4	5.0

react by the same mechanism. DL-Phenylglycine might, however, giving an unstable adduct which reacts rapidly with water to form reduced flavin, benzaldehyde, and ammonia. A comparison of the relative photoreaction rates of some flavin derivatives with benzylamine, DL-phenylglycine, and phenylacetic acid (table 4-7) shows that this possibility is improbable. All reaction rates were measured by following the change in extinction at the flavin absorption maximum. None of the phenylacetic acid reactions were rapidly reversed on readmitting air, but all those of the other substrates were. The relative reactivities of the different flavins to benzylamine and DL-phenylglycine were all about the same. In contrast, the reactivity of phenylacetic acid ranges from the fastest to the slowest of the three substrates with different derivatives. This behaviour suggests a dissimilarity of mechanism.

E) Inhibition of photoreduction. The interaction of some aromatic amines with excited flavins leads to inhibition of reactivity rather than photoreduction (Radda and Calvin, 1963; Homann and Gaffron, 1964; Radda, 1966). The elucidation of these interactions (cf. step 4b in the scheme) is important for an understanding of the mechanism of photoreduction. The fact that some compounds (e.g.

tryptophan, tyrosine, serotonin) inhibit the photoreduction of other dyes while CMU and DCMU do not has lead to the suggestion that there is a special type of interaction between the ureas and flavins (Homann and Gaffron, 1963). To investigate this possibility the sensitivities of the photoreductions of FMN and methylene blue to inhibition have been compared.

The inhibitions must involve quenching of the flavin triplet because, although some of the inhibitors form ground-state complexes with flavins and are also fluorescence quenchers (Penzer and Radda, 1967a), these effects are too small to explain the magnitude of the effect on reactivity (e.g. CMU quenches fluorescence by less than 1%; for others see Radda, 1966). The triplet may be quenched by either collision or complex-formation with the inhibitor (Radda, 1966). These are potentially distinguishable on the basis of the temperature dependence of reaction rate.

The effects of temperature on the inhibited and uninhibited rates of photobleaching and photoreduction of FMN by EDTA are summarised in table 4-8. The uninhibited reactions both have small positive temperature coefficients, in spite of the increase in collisional quenching. This

Table 4-8

Temperature and light-intensity effects on the rates of inhibited and uninhibited photobleaching and photoreduction of FMN.

Photobleaching: unless stated to the contrary all solutions contained 0.1 M-phosphate buffer, pH 7.0, 0.4 mM-inhibitor and 0.06 mM-FMN.

$$k_{\text{uninhib. at } 38^\circ} / k_{\text{uninhib. at } 17^\circ} = 1.44$$

Photoreduction: unless stated to the contrary all solutions contained 0.1 M-phosphate buffer, pH 7.0, 1 mM-EDTA, 0.5 mM inhibitor and 0.04 mM-FMN:

$$k_{\text{uninhib. at } 40^\circ} / k_{\text{uninhib. at } 10^\circ} = 1.20$$

Photobleaching Inhibitor	Temp.	$\frac{k_{inhib.}}{k_{uninhib.}}$	$\frac{k_{inhib.}}{k_{uninhib.}}$ at 38° / $\frac{k_{inhib.}}{k_{uninhib.}}$ at 17°
CMU	17°	0.18	
CMU	38	0.24	1.33
Tryptophan	17	0.04	
Tryptophan	38	0.08	2.00
serotonin (0.1 mM)	17	0.04*	
serotonin (0.1 mM)	43	0.57*	~14
KI (0.01 mM)	17	0.40	
KI (0.01 mM)	38	0.52	1.30

Photoreduction ≠ Inhibitor	Temp.	Light intensity (relative units)	$\frac{k_{inhib.}}{k_{uninhib.}}$	$\frac{k_{inhib.}}{k_{uninhib.}}$ at 40° / $\frac{k_{inhib.}}{k_{uninhib.}}$ at 10°
CMU	40°	100	0.35	1.00
CMU	10	100	0.35	
CMU	40	60	0.39	1.05
CMU	10	60	0.37	
CMU	40	40	0.40	1.08
CMU	10	40	0.37	
CMU	40	20	0.41	1.11
CMU	10	20	0.37	
Tryptophan	40	100	0.32	0.89
Tryptophan	10	100	0.36	
Tryptophan	40	20	0.32	0.86
Tryptophan	10	20	0.37	
Serotonin (0.1 mM)	40	100	0.29	>1.32
Serotonin (0.1 mM)	20	100	0.22	
CMU (FMN conc.0.08mM)	20	>200	0.55	
Tryptophan (FMN conc.0.08 mM)	20	>200	0.40	

Average 1.06

Average 0.87(5)

* Taken from Radda (1966).

≠ Taken from Penzer (1966).

means that there is a small activation energy for the reaction. Photoreduction of FMN by NADH has a small negative temperature coefficient (Radda and Calvin, 1964), and the coefficient increases in the order of decreasing reducing power (NADH, EDTA, ribityl side-chain).

It is generally assumed that potassium iodide is a collisional quencher, yet its inhibition of photobleaching falls with temperature. Triplet lifetime is decreased because of the increased reactivity at the higher temperature, so that changes in KI quenching are not dominated by changes in collision frequency alone. For the other inhibitors the $k_{inhib.}/k_{uninhib.}$ ratio for the two temperatures is greater than or equal to (for CMU) that found for KI (table 4-8). This suggests that inhibitions by serotonin and tryptophan at least have an element of complex formation.

A given concentration of inhibitor affects photoreduction less than photobleaching. The temperature effects are smaller too. These can be explained as follows. The relative contribution of an inhibition term to the denominator of the rate equation falls as $k_{+5}[RH_2]$ increases. The rate constants for those processes that involve an activation energy increase more with temperature than for simple collision processes. The increase of the k_{+5} term

relative to the quenching term is more important for photobleaching because the activation energy of photobleaching is greater than that for photoreduction.

The rate of photoreduction of FMN by EDTA does not depend linearly on light intensity (fig.4-7). This suggests that second order triplet deactivation (k_{+4e}) becomes significant. The effect is greater for uninhibited reactions at a particular light intensity because of the higher $[F^t]$, but it is not possible to detect significant changes in inhibition over the range of intensities covered quantitatively (table 4-8). At higher intensities the uninhibited reaction rate is too fast for accurate measurement, but approximate results indicate that inhibitions fall under the intense illumination as expected (table 4-8).

Neither DL-phenylglycine nor its derivatives inhibit the photoreduction of FMN by EDTA at pH 7.0 under light intensities where the contribution due to photoreduction by the phenylglycines is negligible. This means that complex formation between the aromatic rings of a flavin and the amino acid is probably insignificant (DL-phenylglycine does not quench the fluorescence of FMN), but it doesn't preclude the possibility of inhibition by the anionic form of the acid only because of its very low concentration at

PH 7.0 (see p. 135).

Photoreduction of methylene blue by EDTA has qualitatively similar temperature and light intensity dependences to the FMN reaction (table 4-9). The temperature coefficient of the uninhibited reaction is higher ($k_{41°} / k_{15°} = 2.4$), and inhibition by a given concentration of inhibitor is less. Tryptophan and serotonin inhibit, but CMU and DCMU do not. Inhibition by tryptophan increases as the temperature is raised, but it falls at very high light intensities.

These differences between the indole and urea derivatives may be a result of the ability of the former to form stronger complexes with the dyes. The methylene blue complexes are generally weaker than flavin ones, so that CMU may fall outside the range of quenching interactions with the thiazine.

F) Effects of urea on inhibitions. Both substituted ureas (Hansch and Deutsch, 1966) and flavins (Weber, 1966) participate in hydrophobic interactions in their ground states. This is shown by the effect of urea and non-aqueous solvents on flavin-aromatic molecule complexes (Weber, 1966; Gascoigne and Radda, 1965a). If similar interactions occur

Table 4-9 *

Temperature and light-intensity effects on the rates of inhibited and uninhibited photoreduction of methylene blue by EDTA.

All solutions contained 0.1 M-phosphate buffer, pH 7.0, 0.5 mM-inhibitor and 0.02 mM-methylene blue: $k_{uninhib.\ at\ 41°} / k'_{uninhib.\ at\ 15°} = 2.4$

Inhibitor	Temp.	Light-intensity (relative units)	$k_{inhib.}/k_{uninhib.}$	$\frac{k_{inhib.}/k_{uninhib.}\ \text{at } 41°}{k_{inhib.}/k_{uninhib.}\ \text{at } 15°}$
Tryptophan	41°	100	0.68	0.79
Tryptophan	15	100	0.86	
Tryptophan	41	60	0.73	0.88
Tryptophan	15	60	0.83	
Tryptophan	41	40	0.72	0.84
Tryptophan	15	40	0.86	
Tryptophan	41	20	0.73	0.97
Tryptophan	15	20	0.75	
CMU	41	100	1.0	
CMU	15	100	1.0	
DCMU	20	100	1.0	
serotonin	20	100	0.05	
Tryptophan	20	>200	0.95-1.0	

* This table is taken from Penzer (1966).

with the flavin triplet they could explain the photo-inhibitions.

Urea slightly inhibits photobleaching, but it accelerates the reaction in the presence of other inhibitors (table 4-10). This suggests that complex formation, partly stabilised by hydrophobic interaction, is a likely contributor to the inhibition (cf. Radda, 1966). The small acceleration which urea induces in the KI quenched reaction may be caused by a change in viscosity. This is also reflected in the slight fall of the fluorescence quenching of FMN by KI in urea. Urea also removes the ability of tryptophan to quench FMN fluorescence, and decreases quenching by serotonin by 60% (table 4-10). The negligible change in the rate of photoreduction of FMN by EDTA in urea solutions corresponds to the lower inhibitions generally observed for photoreduction in accordance with the kinetic scheme.

The low rates of photoreduction and photobleaching of FAD compared to those of FMN (Radda, 1966; McCormick, Koster and Veeger, 1967), have been attributed to the formation of an intramolecular complex in the former (Weber, 1950). When urea is added there is no change in the rate of photobleaching (table 4-11), though there is

Table 4-10

Effects of 6.5 M-urea on the rates of inhibited and uninhibited photobleaching, photoreduction and fluorescence of FMN

Solutions contained 0.1 M-phosphate buffer, pH 7.0, and 0.015 mM-FMN.

Reactants	Reaction rate in absence of urea / Reaction rate of FMN alone	Reaction rate in 6.5 M-urea / Reaction rate in absence of urea	Fluorescence intensity / Fluorescence intensity of FMN alone	Fluorescence intensity in 6.5 M-urea / Fluorescence intensity in absence of urea
FMN	1.00	0.84	1.00	0.98
FMN + KI (0.01mM)	0.34	1.16	-	-
FMN + KI (20 mM)	-	-	0.67	1.07
FMN+tryptophan(0.5mM)	0.06	2.00	-	-
FMN+tryptophan(2 mM)	-	-	0.89	1.07
FMN+CMU (0.5 mM)	0.18	1.52	-	-
FMN+serotonin (2 mM)	-	-	0.59	1.41
FMN+EDTA (1 mM)	-	1.01	-	-

Table 4-11

Effect of urea on the rates of photobleaching and photoreduction of FAD by EDTA

All solutions contained 0.1 M-phosphate buffer, pH 7.0, 0.025 mM-FAD and 1 mM-EDTA.

Reactants	Conc. of urea (M)	$\frac{\text{Reaction rate in urea}}{\text{Reaction rate in absence of urea}}$
FAD	6.5	1.00
FAD + EDTA	1.6	1.04
FAD + EDTA	3.2	1.15
FAD + EDTA	4.9	1.24
FAD + EDTA	6.5	1.30

a large increase in fluorescence, indicating unfolding (Gascoigne and Radda, 1965a). With the inhibitory effect of urea on the photobleaching of FMN being taken into account, unfolding must cause a faster rate. The effect is more clearly shown for photoreduction by EDTA, where inhibition by urea is less important. Even so, the increase in reactivity (1.30-fold) is less than the increase in fluorescence (2.13-fold).

The dark reactions of FMN and FAD with NADH (cf. Radda and Calvin, 1964) are not significantly altered on unfolding. This has been shown by comparing the rates of reaction of FMN and FAD in solvents containing urea or dimethyl sulphoxide (Penzer and Radda, 1968a). The relative fluorescence intensities change markedly under these conditions. This means that in the dark reaction steric hindrance is about the same for the folded and unfolded forms. This should apply equally to photoreactions, so the increase in rate observed in this case is probably because of decreased intramolecular triplet quenching (cf. the effect of urea on the intermolecular inhibitions).

CHAPTER FIVE

Aerobic Flavin Photochemistry

Introduction

In the presence of oxygen flavins can act as photosensitizers, but riboflavin is photobleached as well (more slowly than under anaerobic conditions). In alkaline solution the main product of photobleaching is lumiflavin but below pH 7 it is lumichrome (Halwer, 1951). The reaction is slowest at neutral pH, and the rates of both the aerobic and anaerobic reactions increase as the ionic strength of the buffer is raised (Halwer, 1951; Yang and McCormick, 1965). The effects are not compatible with general acid-base catalysis, as was suggested initially.

Flavins, in common with many aromatic dyes, can sensitize the photo-oxidation of amines, amino acids, alcohols, and some metal ions (Taylor and Radda, in the press). They can also cause the polymerisation of olefins (Oster, 1954) and deiodination of aromatic iodo compounds (Lissitzky, Benevent and Rogues, 1961). The products and mechanisms of these reactions have rarely been characterised, but one exception is the photosensitized oxidation of sarcosine by FMN (Frisell, Chung and Mackenzie, 1959). C^{14} labelling of the amino acid shows that it breaks up in

the following way to give an amine, an aldehyde and carbon dioxide: $\overset{\delta}{R}NH_2\text{-}\overset{+}{C}H_2\text{-}\overset{*}{C}O_2H \rightarrow \overset{\delta}{R}NH_2 + \overset{+}{C}H_2O + \overset{*}{C}O_2$. This is compatible with a mechanism in which the flavin and amino acid react as in anaerobic photoreduction, but because oxygen is present the reduced flavin is immediately reoxidised with production of hydrogen peroxide. The reaction with aromatic amino acids cannot be as simple because they quench photoreduction, and photo-oxidation of tryptophan gives many products in which the aromatic nucleus has reacted (P.S.Song, personal communication to G.K.Radda).

Both lumichrome and riboflavin photosensitize destruction of the biological activity in DNA by destroying the guanine bases. Several products are formed, but C^{14} labelling shows that about 50% of the observed reaction can be explained by: guanine → guanidine + parabanic acid (Sussenbach and Berends, 1963). The reaction is inhibited by paramagnetic ions, and the peroxides which are produced are not intermediates (Knowles and Roe, 1968). It has been suggested that triplet flavin interacts with oxygen to give singlet oxygen and ground-state flavin because the excited oxygen is known to degrade guanine (Berends, Posthuma, Sussenbach and Mager, 1966).

There is evidence for the participation of singlet oxygen in only one flavin photosensitized reaction, the

photo-oxygenation of cholest-4-en-3β-ol (Kearns, Hollins, Khan, Chambers and Radlick, 1967), though it has been implicated in several others (Taylor and Radda, in the press). The reaction of cholest-4-en-3β-ol, which can be promoted by many aromatic dyes, gives only two products, cholest-4-en-3-one and 4α,5-epoxy-5α-cholestan-3-one (Kearns, Hollins, Khan, Chambers and Radlick, 1967). The ratio of epoxy ketone to en-one depends on the triplet energy of the sensitizer. On theoretical grounds it has been shown that for sensitizers with triplet energies above 50 kcal/mole about 10 $^1\Sigma_g^+$ singlet oxygen molecules are generated for each $^1\Delta_g$ molecule. When the sensitizer energy drops below 38 kcal/mole only $^1\Delta_g$ oxygen is generated. The product ratios which occur with different sensitizers can be explained if $^1\Delta_g$ oxygen gives an enone: epoxy ketone ratio of 1:5, while the 10:1 mixture of singlet oxygens gives a product ratio of 3:1. Riboflavin is the only sensitizer which causes product ratios different from those predicted theoretically. This is attributed to competition from a reaction between the flavin semiquinone (produced by photobleaching) and the sterol. On reducing the sterol concentration the enone:epoxy ketone ratio falls from 30:1 to 1:5, which suggests that of the three species

which could react with cholest-4-en-3β-ol (flavin radical, $^1\Sigma_g^+$oxygen, and $^1\Delta_g$ oxygen) $^1\Delta_g$ oxygen has the longest life (Kearns, Hollins, Khan and Padlick, 1967).

Flavins photosensitize the destruction of indoleacetic acid (Galston, 1950; Nathanson, Brody, Brody and Broyde, 1967). A quantum yield of about 0.7 was found for this reaction, suggesting an efficient reaction between flavin triplets and the acid. The products include CO_2 in mole for mole ratio to the indoleacetic acid destroyed (Nathanson, Brody, Brody and Broyde, 1967).

In the absence of added proteins FMN photosensitized deiodination of thyroxine yields iodide. In the presence of many proteins the reaction is faster, and the iodine liberated is detected as some compound with the protein (Jacobar, Rodriguez, John and del Rey, 1963). In contrast to anaerobic photoreduction, tryptophan accelerates this reaction.

Many dyes, including flavins, photosensitize the destruction of proteins and nucleic acids, though their specificities for the individual residues vary (Simon, 1967; Ray, 1967). These reactions can thus be used to induce specific modifications in the polymers if the individual reactivities of the residues are known. Model studies on the behaviour of individual amino acids have been done for

some sensitizers (e.g. methylene blue - Weil, Gordon and Buchert, 1951; proflavin - Sluyterman, 1962), but flavins are not among them. The results of a recent attempt to compare the reactivity of riboflavin towards several amino acids are hard to interpret because tris buffer was used (Bellin and Yankus, 1968). Being a tertiary amine, this buffer itself reduces flavin triplets.

Three general types of mechanism have been proposed for sensitized photo-oxidations, in all of which the reactive state of the dye is its triplet. The first is photoreduction (as under anaerobic conditions - see chapter 4) with rapid reoxidation of the reduced dye by oxygen. The second mechanism is production of singlet oxygen which subsequently reacts with the substrate. The third mechanism, formation of a dye-oxygen intermediate, is hard to distinguish from the second because their kinetics are the same.

Examples of flavin reactions which probably involve the first two mechanisms have already been discussed. The pattern of aromatic hydroxylation produced by flavins illuminated in the presence of hydrogen peroxide suggests the formation of a dye-oxygen intermediate (Young, 1966) and this is also implicated in the autoxidation of reduced flavins by oxygen (Mager and Berends, 1966). It is thus possible that some photosensitized oxidations may involve

a similar species, but there is no direct experimental evidence for this.

The results in this chapter mainly concern the sensitized photo-oxidation of amino acids and a consideration of their different reactivities, but various other compounds (including some enzymes) have also been tested for their susceptibility to aerobic photoreaction.

Measurements and Accuracy

A) Oxygen concentration. Oxygen concentrations were measured using an oxygen electrode (Clark, 1956). The model used was made by E.I.L. (SOH 33), and consists of a platinum electrode covered by a thin polythene membrane. A silver/silver chloride electrode is incorporated for reference. The electrolyte inside the cell connecting its two electrodes was a phosphate buffer (equal amounts of 0.25 M KH_2PO_4 and 0.25 M Na_2HPO_4). The stability and sensitivity of this assembly depends critically on the way the membrane is fixed. If it is not stretched smoothly over the platinum electrode the sensitivity is reduced, and any flaws in the membrane which allow leakage mean that the electrode will be rapidly poisoned.

When a constant potential is applied across the

electrodes (Pt cathode) the current flowing measures the oxygen concentration outside the membrane. The electrode assembly was thus connected to a polarograph (Radiometer PO 4b) which could apply a steady potential of -06v and continuously record currents from 0.01 μ amp upwards. The current in the electrode is directly proportional to the partial pressure of oxygen in the solution outside the membrane providing that there is efficient stirring. In an unstirred solution an oxygen concentration gradient is rapidly set up as the electrode reduces the oxygen close to it, causing inaccurate measurements.

The reaction vessel used in oxygen uptake experiments was a glass cylinder with a capacity of about 45 ml, surrounded by a water jacket. The electrode fitted tightly into a rubber bung which fitted the top of the reaction vessel. Reaction solutions were stirred magnetically. The procedure was to fill the vessel to its brim with the reaction mixture and then carefully push on the bung so that no air bubbles were allowed to remain in the vessel. The exclusion of air is vital because even a small bubble contains as much oxygen as is in the entire solution. The vessel was thermostatted at 25° by circulating water from a regulated water bath through the water jacket. This was

essential because the electrode response depends critically on the temperature, and a change of less than 0.1° can be significant. It is also important that the solutions are thermostatted at 25° before being enclosed in the reaction vessel because of the different coefficients of solubility of oxygen at different temperatures.

To avoid the effects of stray light the outside of the reaction vessel was covered with black tape except for a small window through which the exciting beam could pass. Care was taken to arrange the position of the electrode so that the light did not shine directly on to it.

The membrane sensitivity is not constant over long periods, nor is it possible to fix all membranes in an identical way so that they have the same response. Thus regular calibration of the method is important for quantitative work. Only two fixed points are necessary for calibration because of the linear dependence of current on the oxygen activity. One suitable standard solution is air saturated water at 25° ($[O_2]$ = 0.257 mM at standard barometric pressure - Dixon and Kleppe, 1965). The current passed by the electrode at zero oxygen concentration is close, but not necessarily equal, to zero, and it varies slightly for different membranes. Its value can easily be

found by illuminating an aerobic mixture of FMN and excess EDTA until the colour of the flavin begins to fade. Reduced flavin is only stable in the complete absence of oxygen, and so the electrode reading under these conditions gives the true zero. In all cases the zero was less than 1% of the reading from an air saturated solution (which was itself 2.5 μamp $\pm$ 1 μamp).

The stability of the electrode over a period of hours was good. After observing a given solution for 30 min. the reading fell by less than 3% due to consumption of oxygen by the electrode. The readings obtained for air saturated water remained constant $\pm$ 5% in a day's use of the instrument. The response time of the polythene membrane was quoted as 2-4 min. by the suppliers (E.I.L.), and this was about the time taken for the electrode to give a steady reading after a large change in oxygen concentration. When following a reaction, however, in which the oxygen concentration changed steadily the electrode response was only a few seconds (fig.5-1). The noise level on the recorder trace was less than 1/4%.

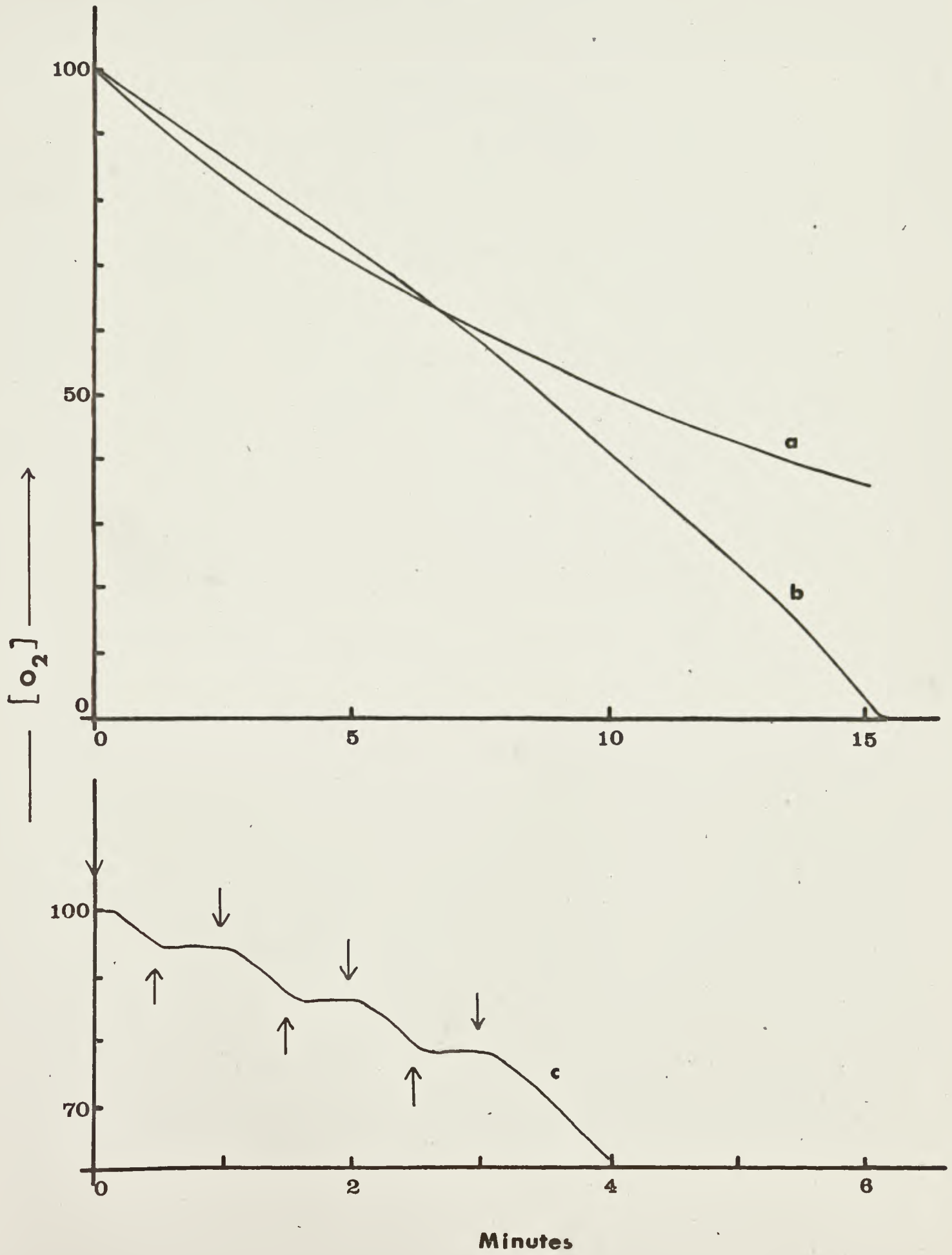
B) Enzyme preparations and assays. Beef liver glutamate dehydrogenase (GDH) was obtained as an ammonium sulphate suspension. Before use it was dialysed against pH 7.8

Fig. 5-1 ; FMN photosensitised oxygen uptake

a) Type II - [FMN] = .1mM + tryptophan (mM) at pH 8 and 25°

b) Type I - [FMN] = .2mM + EDTA (5mM) at pH 7 and 25°

c) Response time of the electrode - FMN (1·0 mM) and triethanolamine (10 mM)



phosphate buffer for 24 hr. at 2° during which time it dissolved to give a clear solution.

The prosthetic group of GDH is NADH, and the enzyme was assayed by following the fall in extinction of NADH at 340mμ as it reduced α-oxoglutarate. The enzyme concentrations necessary for this assay are about $5x10^{-5}$mg/ml, and the extinction changes are about 0.01 units. The instrument used for the assays was a Cary 14 recording spectrophotometer with a 0.1 full scale slide wire. The standard procedure for assay was as follows: 2.8 ml of a stock solution of α-oxoglutarate, ammonium chloride and EDTA in phosphate buffer (pH 7.7, I 0.1) were mixed with 0.1 ml of a freshly made solution of NADH in tris in a 1 cm cuvette so that the final concentrations in the mixture were

5mM α-oxoglutarate

50mM NH_4Cl

50 μM EDTA

100 μM NADH.

The stock solution was preincubated at 25° and the NADH was stored at 0° Samples of the GDH to be assayed (usually 0.02 ml) were pipetted into the cuvette and mixed rapidly with the rest of its contents. The change in extinction at 340 mμ was now followed. Linear rates were obtained for about the first minute of reaction, and the activity of

the enzyme was taken from this portion of the reaction curve.

Inaccuracies in this assay come mainly from pipetting errors and optical deviations caused by the slightest loss of clarity in the assay mixture. Identical runs were found to agree to ± 5%.

The benzoate complex of hog kidney D-amino acid oxidase was prepared from fresh kidneys by the method of Yagi, Naoi, Harada, Okamura, Hidaka, Ozawa and Kotaki (1967), except that the second calcium phosphate gel column filtration was omitted. The crystalline complex was stored at 0° as an ammonium sulphate suspension. There was no loss of activity over several weeks. To redissolve the enzyme the ammonium sulphate suspension was dialysed against M/60 sodium pyrophosphate (pH 8.3) at 2° for at least 12 hr.

D-Amino acid oxidase was assayed spectrophotometrically by following the increase in extinction at 252 mμ as phenylglyoxylic acid is formed when D-phenylglycine is the oxidised substrate (Fonda and Anderson, 1967). L-Phenylglycine does not interfere with this assay (Fonda and Anderson, 1967), so DL-phenylglycine solutions were used throughout. Measurements were made on a Hilger-Gilford recording spectrophotometer, with full scale deflection of 0.1 units and backing off of 0.5 - 0.8 units. A sample of the enzyme to be assayed (usually 0.05 ml) was added to 2 ml of

mM DL-phenylglycine in 50 mM sodium pyrophosphate adjusted to PH 7.9 with HCl in a cuvette. The increase in extinction at 252 mμ was recorded, and the reaction curve showed a linear increase for about 10 min. The gradient of this line was measured to find the activity of the enzyme. The reproducibility of the rates measured this way was about ± 7%.

Results and Discussion

When a flavin sensitized photo-oxidation is studied by measuring the rate of oxygen consumption two forms of kinetic behaviour are observed. In the first (type I) the rate of oxygen uptake increases as the reaction proceeds, while in the second (type II) it decreases. Various photo-oxidisable substrates have been studied and distinguished according to the shape of the oxygen uptake curve (table 5-1). Those compounds whose reaction character is described as intermediate have a sigmoidal uptake curve, type I at high oxygen concentrations and type II at low ones.

substrates which have type I behaviour are those which photoreduce flavins under anaerobic conditions. No type I substrate failed to give the anaerobic reaction at a similar pH, and no photoreducing agent failed to give type I

Table 5-1

FMN photosensitized oxygen uptake in the presence of different substrates

All reactions were at 25° starting with solutions in equilibrium with the atmosphere. Substrate concentrations were about 5 mM.

substrate	pH	Type of kinetic behaviour	Approximate initial relative rate
-	7	I	+
uracil	7	intermediate	++++
thymine	7	I	+
cytosine	7	I	+
guanosine	8	II	++
adenosine	8	II	+
benzoic acid	7	I	+
N,N-dimethylaniline	7	II	+
benzaldehyde	7	I	+
p-toluidine`	7	II	+++
p-toluidine	8	II	++++
m-toluidine	8	II	++
p-cresol	8	II	++
m-cresol	8	II	++
o-cresol	8	II	++
p-aminobenzoic acid	8	I	+
phenylacetic acid	7	intermediate	++

EDTA	7	I	++++
DL-phenylglycine	8	I	++
ethanolamine	7	I	+
diethanolamine	7	I	++
triethanolamine	7	I	++++
CMU	8	II	+++
L-tryptophan	8	II	++++
L-tyrosine	8	II	+++
L-alanine	8	I	+
L-phenylalanine	8	I	+
L-cysteine	8	I	++
L-methionine	8	I	++
L-histidine	8	I	+++
ethyleneglycol	7	I	+
ethylamine	7	I	+
diethylamine	7	I	+
triethylamine	7	I	+
benzylamine	8	I	++
serotonin	8	II	++
phenol	8	I	+
nicotinamide	8	I	+

behaviour in the aerobic reaction. This suggests that type I kinetics are typical of the reduction-reoxidation mechanism. This has been confirmed by observing the effects of varying pH, substituents in the substrate, and oxygen quenching.

A) Type I reactions - pH effects. The effects of pH on the initial rate of oxygen uptake when aqueous solutions of FMN and ethanolamine, and FMN and benzylamine are illuminated are shown in figure 5-2. In both cases the rate of reaction is greatest at about pH 9.5. These pH profiles mean that reaction only occurs between the unprotonated form of the amine (pK_a ethanolamine = 9.49, pK_a benzylamine = 9.37 - Handbook of Biochemistry, 1968), and an excited, unprotonated isoalloxazine nucleus (pK_a about 10 - Hemmerich, Veeger and Wood, 1965). The pH dependence of anaerobic photoreduction is similar (chapter 4). The relative rates of oxygen uptake of the unprotonated forms of ethanolamine, diethanolamine, and triethanolamine are in the order tertiary amine > secondary amine > primary amine (table 5-2). This agrees with the predicted ease of abstraction of hydrogen from a carbon α to the amino group.

When a solution of FMN is illuminated oxygen is

Table 5-2

Rates of oxygen uptake with mono-, di-, and triethanolamine as substrates

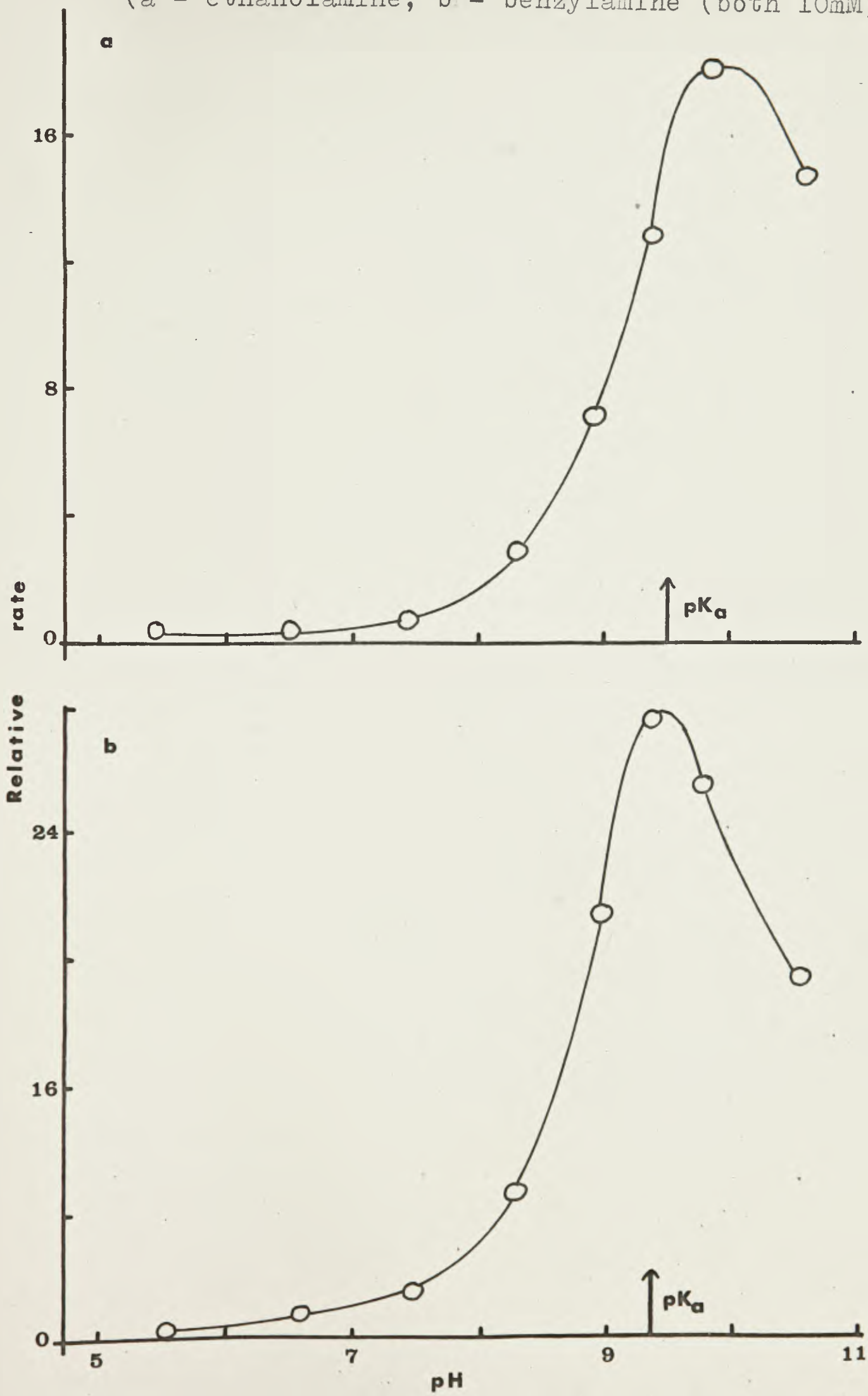
All reactions were at 25° with 0.4 mM FMN and 10 mM amine. Initial rates were measured for solutions in equilibrium with air.

Amine	pKa′	pH of reaction	Relative rate of oxygen uptake	Relative rates corrected for pKa *
Ethanolamine	9.56	7.02	1.4	40
Diethanolamine	9.13	7.08	8.8	82
Triethanolamine	8.10	7.02	100	100
FMN alone	-	7.00	0.4	

* Assuming that rate $\propto [RNH_2]$. Whilst not strictly true (see p. 132) this is a reasonable approximation at the high oxygen and low reducing agent concentrations involved.

′ Measured by titration in 0.1M NaCl.

Fig. 5-2 ; Dependence of rate of sensitized oxygen uptake on pH (a - ethanolamine, b - benzylamine (both 10mM))



consumed more slowly than in the presence of an unprotonated amine (anaerobic photobleaching is also slower than photo-reduction). The reaction shows a small increase in rate at pH values less than 5, and a rather larger enhancement above pH 8 (fig.5-3). This agrees with the previous observations (Halwer, 1951; Yang and McCormick, 1965).

B) Type I reactions - oxygen quenching. The rate of oxygen uptake in the FMN sensitized photo-oxidation of EDTA increases as the concentration of oxygen falls (table 5-3). Initial rates at different oxygen concentrations are the same as the rates at those concentrations during a single reaction. This means that product inhibition is unimportant. When hydrogen peroxide (a likely product) is added to the solution (10^{-4}M) it has no effect on the rate of oxygen uptake (table 5-3). Thus the effect of a whole range of oxygen concentrations on the rate of reaction can be followed during a single kinetic run.

Consider the following kinetic scheme, which resembles the one used for discussion of anaerobic photoreduction. The difference is that the present scheme includes terms for oxygen quenching.

(1) $F + h\nu \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} F^*$

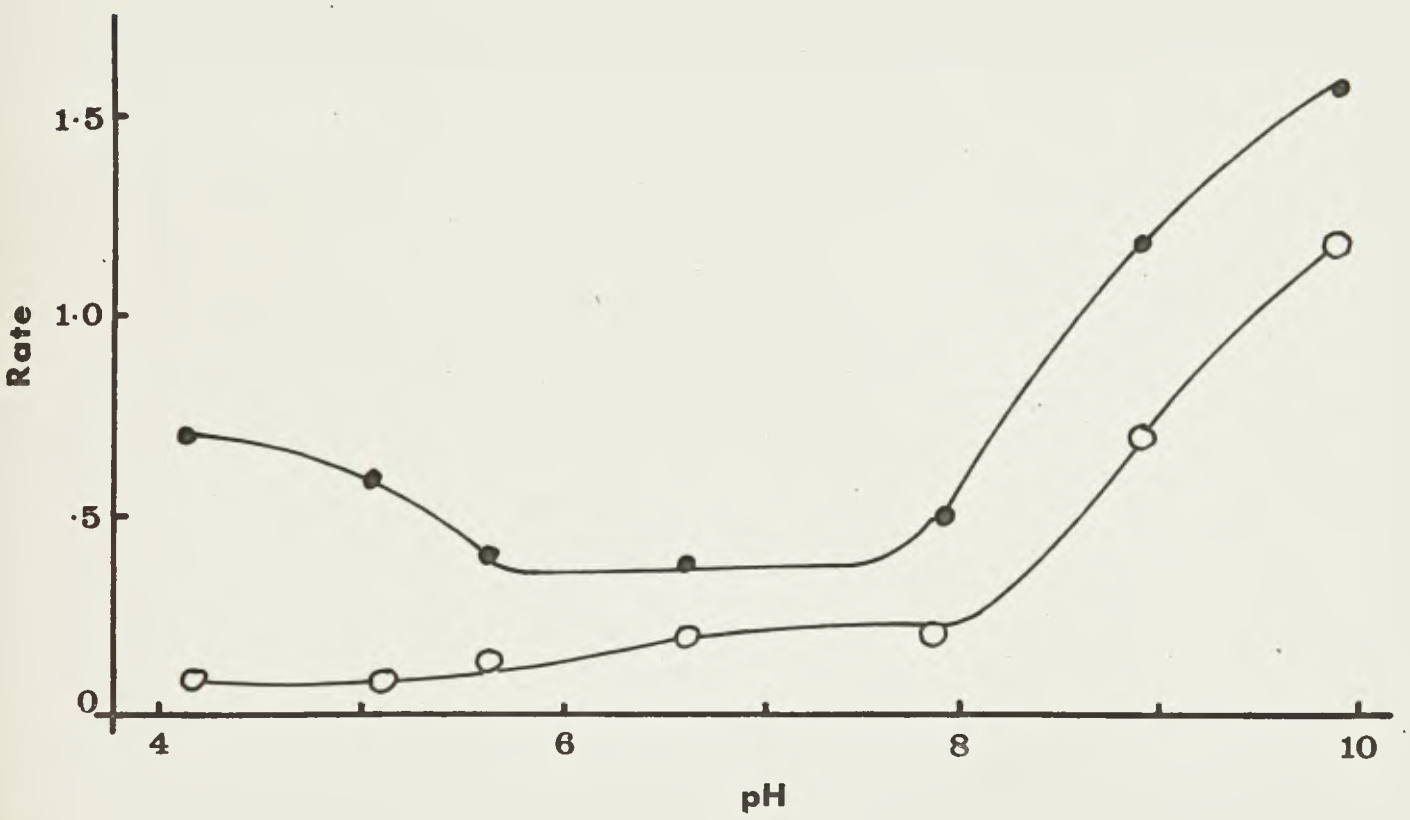
Table 5-3

Rates of oxygen uptake by EDTA and illuminated FMN at different oxygen and hydrogen peroxide concentrations

All reactions were at pH 7.0 and 25°. FMN concentrations were 0.2 mM and EDTA concentrations were 5 mM.

Relative oxygen concentration (100 is for buffer in equilibrium with air)	Initial rate of oxygen uptake (units/min)	Rate during the run starting at $[O_2]$ = 167
45	6.2	6.5
50	5.8	6.1
73	5.1	5.1
98	4.8	4.5
100	4.6	4.5
148	3.4	3.1
167	3.1	3.1
100 (no hydrogen peroxide)	9.8	
100 (+ 10^{-4}M hydrogen peroxide)	9.9	

Fig. 5-3 ; Rate of oxygen uptake by illuminated FMN solutions (.1mM) at different pH-values and temperatures (• 46°, O 25°)



(2a) $F^* + \text{solv.} \xrightarrow{k_{+2a}} F + \text{solv.}$

(2b) $F^* + RH_2 \xrightarrow{k_{+2b}} F + RH_2$

(2c) $F^* + O_2 \xrightarrow{k_{+2c}} F + O_2$

(3a) $F^* \xrightarrow{k_{+3a}} F^t$

(3b) $F^* + RH_2 \xrightarrow{k_{+3b}} F^t + RH_2$

(3c) $F^* + \text{solv.} \xrightarrow{k_{+3c}} F^t + \text{solv.}$

(3d) $F^* + FH\cdot \xrightarrow{k_{+3d}} F^t + FH\cdot$

(3e) $F^* + O_2 \xrightarrow{k_{+3e}} F^t + O_2$

(4a) $F^t \xrightarrow{k_{+4a}} F$

(4b) $F^t + RH_2 \xrightarrow{k_{+4b}} F + RH_2$

(4c) $F^t + \text{solv} \xrightarrow{k_{+4c}} F + \text{solv.}$

(4d) $F^t + FH\cdot \xrightarrow{k_{+4d}} F + FH\cdot$

(4e) $F^t + F^t \xrightarrow{k_{+4e}} F + F^*$

(4f) $F^t + O_2 \xrightarrow{k_{+4f}} F + O_2$

(5) $F^t + RH_2 \xrightarrow{k_{+5}} FH\cdot + RH\cdot$

(6) $FH\cdot + RH\cdot \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} FH_2 + R$

(7) $2FH\cdot \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} FH_2 + F$

(8) $FH\cdot + RH_2 \underset{k_{-8}}{\overset{k_{+8}}{\rightleftharpoons}} FH_2 + RH\cdot$

(9) $FH_2 + O_2 \xrightarrow{k_{+9}} F + H_2O_2$

The meanings of the symbols are the same as those given on p. 101. A type I reaction is one in which the rate determining step is number 5. At low enough oxygen concentration step 9 must become the slowest, but in many cases this does not occur until over 99% of the oxygen has reacted. As before, k_{+2b}, k_{+3b}, and k_{+8} can be neglected for most type I reducing agents. The fluorescence of FMN is not quenched in an aerobic solution, so k_{+2c} and k_{+3e} can also be neglected. As long as step 5 determines the rate no large concentration of reduced flavin can build up, and so even when considering rates other than initial ones steps 3d and 4d should be unimportant. Step 4e must be insignificant compared to step +1 because no delayed fluorescence can be detected from aerobic solutions of flavins.

When reaction 9 is very fast compared to reaction 5 the rate of oxygen consumption (R) = $k_{+5}[F^t][RH_2]$. When a steady state treatment is applied to $[F^*]$ and $[F^t]$, R is

given by:

$$\left\{k_{+1}k_{+5}(k_{+3a} + k_{+3c}[\text{solv.}]\,)\, I_o[RH_2][F]\right\} \Big/ \left\{(k_{-1} + (k_{+2a}+k_{+3c})[\text{solv.}]\right.$$

$$\left. +k_{+3a})(k_{+4a} + (k_{+4b} + k_{+5})[RH_2] + k_{+4c}[\text{solv.}] + k_{+4e}[F^t] + k_{+4f}[O_2]\,)\right\}$$

(see appendix 4 for details).

Let A represent $\dfrac{k_{+1}(k_{+3a} + k_{+3c}[\text{solv.}]\,)I_o}{(k_{-1} + (k_{+2a} + k_{+3c}[\text{solv.}]\,) + k_{+3a})}$.

This is constant for a given intensity of illumination.

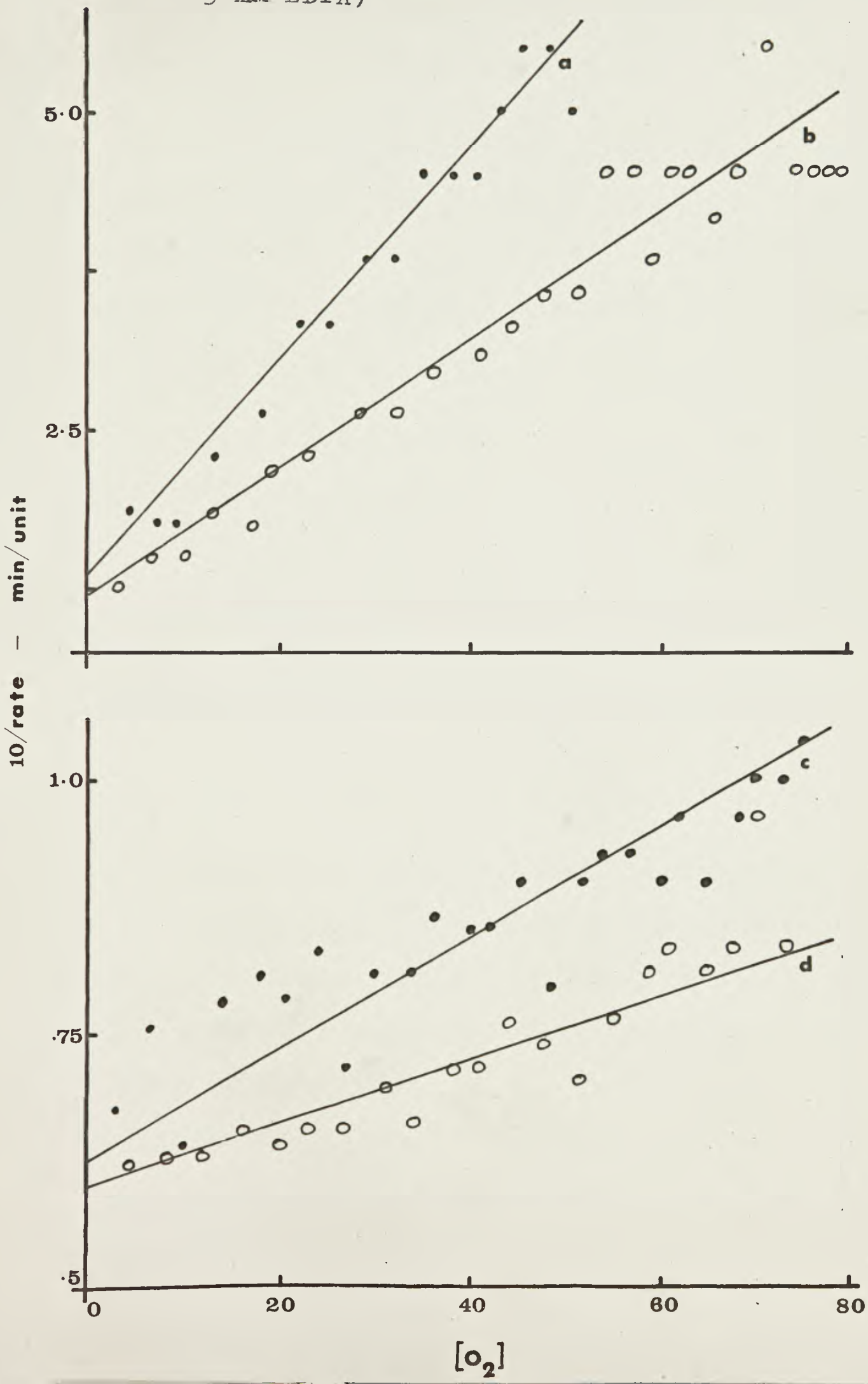
Let B represent $(k_{+4a} + k_{+4c}[\text{solv.}] + k_{+4e}[F^t])$. This is not constant because $[F^t]$ varies with $[O_2]$. In general, however, $k_{+4e}[F^t]$ will be small compared to other triplet deactivation processes, so to a first approximation the variations in B can be neglected. Taking the reciprocal of R, and dividing up the terms,

$$\frac{1}{R} = \frac{B}{k_{+5}[RH_2][F]A} + \frac{(k_{+4b} + k_{+5})}{k_{+5}[F]A} + \frac{k_{+4f}[O_2]}{k_{+5}[RH_2][F]A}.$$

The behaviour of several type I reducing substrates in photo-oxidation can be fitted well to this kinetic expression.

When $\frac{1}{R}$ is plotted against $[O_2]$ reasonable straight lines are obtained for reactions with EDTA, DL-phenylglycine, and diethanolamine (fig.5-4). The gradients of these plots (G_1) vary inversely with reducing agent concentration (when reducing agent is always in large excess over both oxygen and flavin). In some cases the intercept of $\frac{1}{R} : [O_2]$ plots

Fig. 5-4 ; Sample 1/R : $[O_2]$ plots (a - 0.9 mM phenylglycine, b - 1.8 mM phenylglycine, c - 11.5 mM EDTA, d - 23 mM EDTA)



at zero $[O_2]$ was independent of $[RH_2]$ over the range studied (e.g. for EDTA), but in some cases a graph of this intercept (J_1) against $\frac{1}{[RH_2]}$ gives a straight line with a positive slope (e.g. for DL-phenylglycine). The results for EDTA, DL-phenylglycine, and diethanolamine are given in figures 5-5 and 5-6.

Inspection of the kinetic scheme shows that the gradients (G_2) of the $G_1 : \frac{1}{[RH_2]}$ plots are given by $k_{+4f}/k_{+5}[F]A$. Under constant illumination conditions, and at a constant flavin concentration, k_{+5} is the only term in this expression which varies when the substrate is changed. Thus the ratios of $1/G_2$ for the different substrates gives the ratios of the k_{+5} values for these compounds (table 5-4).

The gradient (G_3) of a $J_1 : \frac{1}{[RH_2]}$ plot is given by $B/k_{+5}[F]A$, and its intercept at zero $\frac{1}{[RH_2]}$ (J_2) is given by $(k_{+4b} + k_{+5})/k_{+5}[F]A$. Thus the ratio $G_2:G_3:J_2$ gives $k_{+4f} : B : (k_{+4b} + k_{+5})$ for a given reducing substrate. k_{+4f} and B are independent of the nature of the substrate so it is possible to deduce the ratio of $(k_{+4b} + k_{+5})$ terms for different substrates.

In all cases k_{+4f} is about 30 times greater than

Table 5-4

Composite rate constants for the photoreduction of FMN by EDTA, DL-phenylglycine and diethanolamine

All reactions were at 25º under the same light intensity. The meanings of symbols are given in the text.

a) EDTA - reactions at pH 7.3

EDTA concentration (mM)	G_1 (l^2min/mole^2)	J_1 (l.min/mole)
23	$2.04.10^8$	$6.9.10^4$
11.5	$3.44.10^8$	$7.1.10^4$
9.2	$3.80.10^8$	$7.1.10^4$
6.9	$3.68.10^8$	$7.1.10^4$
4.6	$5.22.10^8$	$6.9.10^4$
2.3	$12.30.10^8$	$7.1.10^4$

$G_2 = 2.68.10^6$, so $\frac{1}{G_2} = 3.6.10^{-7}$ mole/l.min.

G_3 cannot be measured

$J_2 = 7.0.10^4$ lmin/mole

b) DL-phenylglycine - reactions at pH 7.8

phenylglycine anion concentration (mM)	G_1 (l^2.min/mole^2)	J_1 (l.min/mole)
1.8	$3.3.10^9$	$1.37.10^5$
0.9	$6.1.10^9$	$1.57.10^5$
0.36	$13.1.10^9$	$2.51.10^5$
0.18	$29.4.10^9$	$3.72.10^5$

$G_2 = 5.04.10^6$, so $\frac{1}{G_2} = 2.10^{-7}$ mole/l.min

$G_3 = 50$ min.

$J_2 = 1.07.10^5$ l.min/mole

c) diethanolamine - reactions at pH 7.8

concentration of uncharged diethanolamine (mM)	G_1 (l^2min/mole^2)	J_1 (l min/mole)
1.27	$2.70.10^9$	$8.6.10^4$
0.64	$4.08.10^9$	$7.1.10^4$
0.25	$9.00.10^9$	$7.1.10^4$
0.13	$14.80.10^9$	$11.4.10^4$

$G_2 = 2.10^6$, so $\frac{1}{G_2} = 5.10^{-7}$ mole/l.min.

G_3 = about 5 min.

J_2 = about 7.10^4 l min/mole

Table 5-5

Summary of rate constants for photoreduction

Substrate	k_{+4f} (M^{-1} sec^{-1})	B (M^{-1} sec^{-1})	k_{+5} ($M^{-1}sec^{-1}$)	k_{+4b} ($M^{-1}sec^{-1}$)
EDTA	$6.6.10^9$		$1.38.10^7$	$1.60.10^8$
DL-phenylglycine	$6.6.10^9$	$6.6.10^4$	$0.73.10^7$	$1.25.10^8$
diethanolamine	$6.6.10^9$	$(1.6.10^4)$	$1.83.10^7$	$2.05.10^8$

The half life of the FMN triplet is buffered solution with no reducing agent present = $\frac{1}{B}$ = 15 μsec.

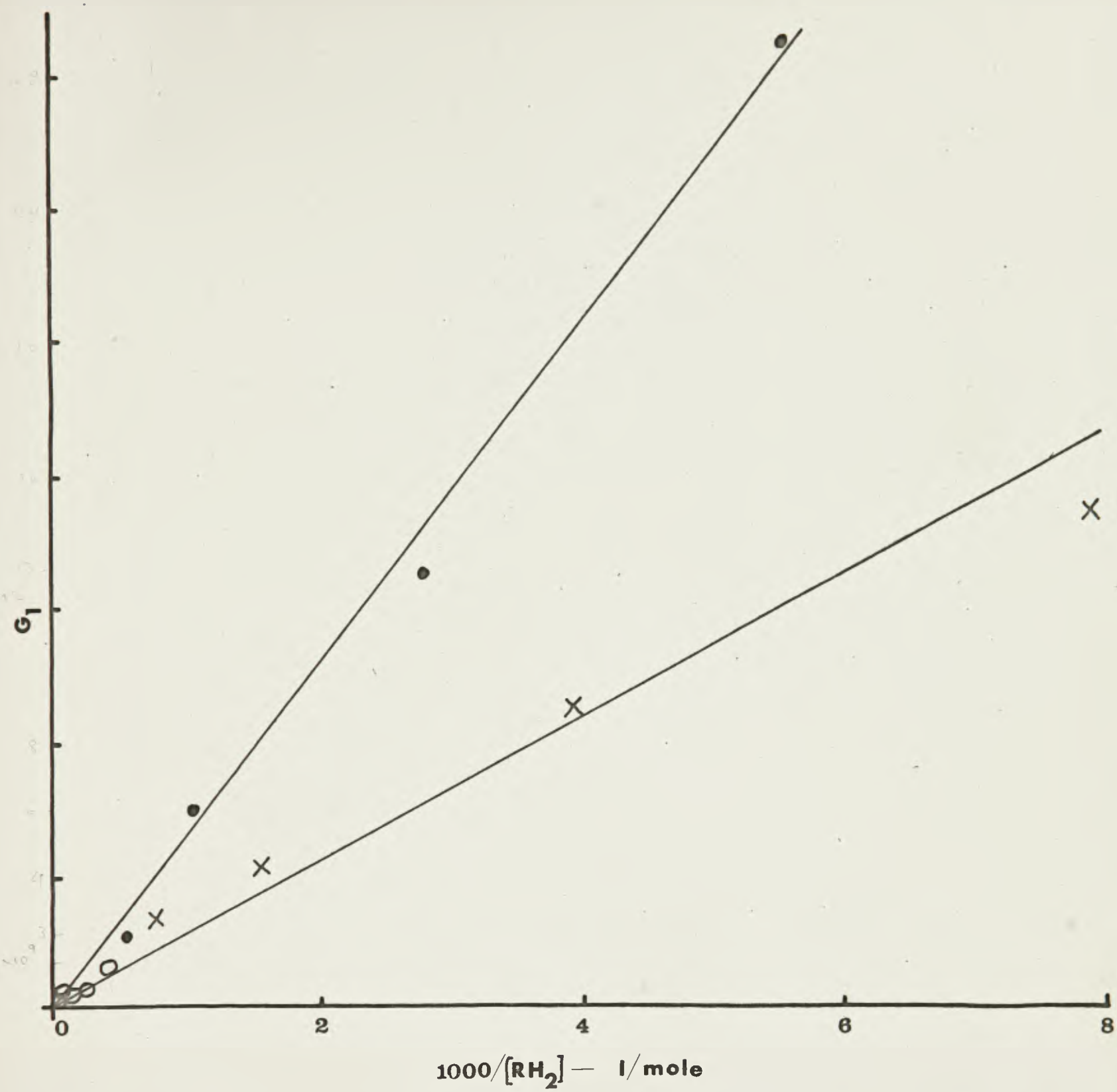


Fig. 5-5 ; G_1 : $1000/[RH_2]$ for • DL-phenylglycine, O EDTA and X diethanolamine (conditions are given in table 5-4)

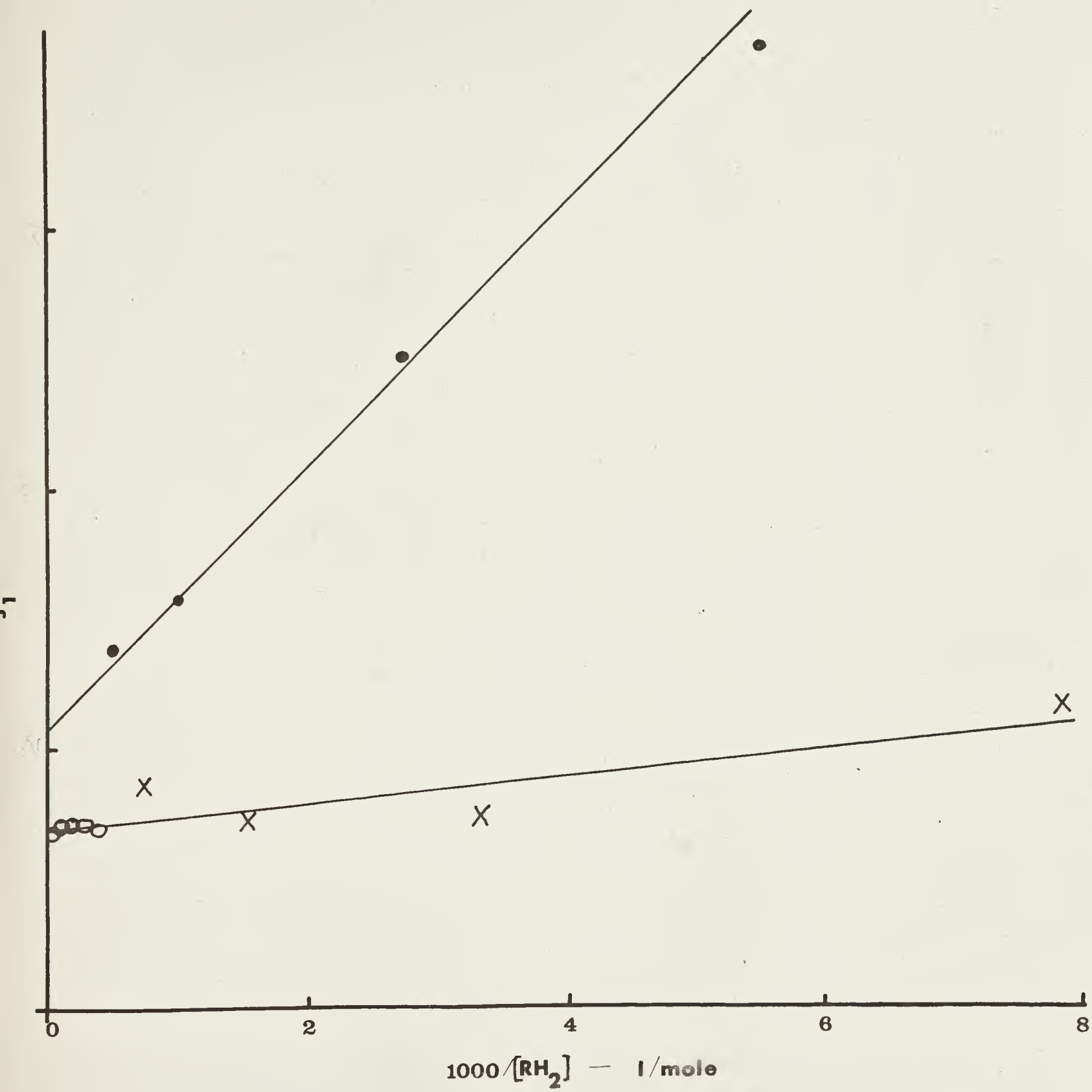


Fig. 5-6 ; J_1 : $1000/[RH_2]$ for • DL-phenylglycine, O EDTA and X diethanolamine (conditions are given in table 5-4)

($k_{+4b} + k_{+5}$). It has been found that triplet quenching by oxygen is often a diffusion controlled reaction (Chien, 1967), and so on this assumption the actual values of ($k_{+4b} + k_{+5}$) and B can be calculated. The rate constant of a diffusion controlled reaction is given by $\frac{8RT}{3.10^3\eta}$ $M^{-1}sec^{-1}$ (Umberger and LaMer, 1945), which gives a value of $6.6x10^9$ $M^{-1}sec^{-1}$ for an aqueous solution at 25°.

It is now possible to calculate k_{+4b} and k_{+5} separately using the known quantum yield (Q) of anaerobic photoreduction of FMN by EDTA (Radda and Calvin, 1964). Q equals 0.06 at high EDTA concentration where the rate is independent of $[RH_2]$. In this situation $Q = \frac{Q_o \cdot k_{+5}}{k_{+4b} + k_{+5}}$, where Q_o is the quantum yield of triplet production. Because of the probable inefficiency of radiationless deactivation of excited singlets direct to the ground state (steps -1 and 2, see chapter 3) a reasonable value of Q_o is $1 - Q_f$, where Q_f is the quantum yield of fluorescence (0.26 for riboflavin - Weber and Teale, 1957). Taking the ($k_{+4b} + k_{+5}$) values calculated from comparison with k_{+4f}, k_{+4b} and k_{+5} have been calculated separately (table 5-5).

Consider the individual and composite rate constants given in table 5-5. 1/B gives the half-life of the flavin

triplet in solution in the absence of reducing agent or oxygen, and the value of 15μsec seems reasonable. The three reducing agents all have quenching rate constants (k_{+4b}) at least 10 times greater than their photoreduction rate constants (k_{+5}).

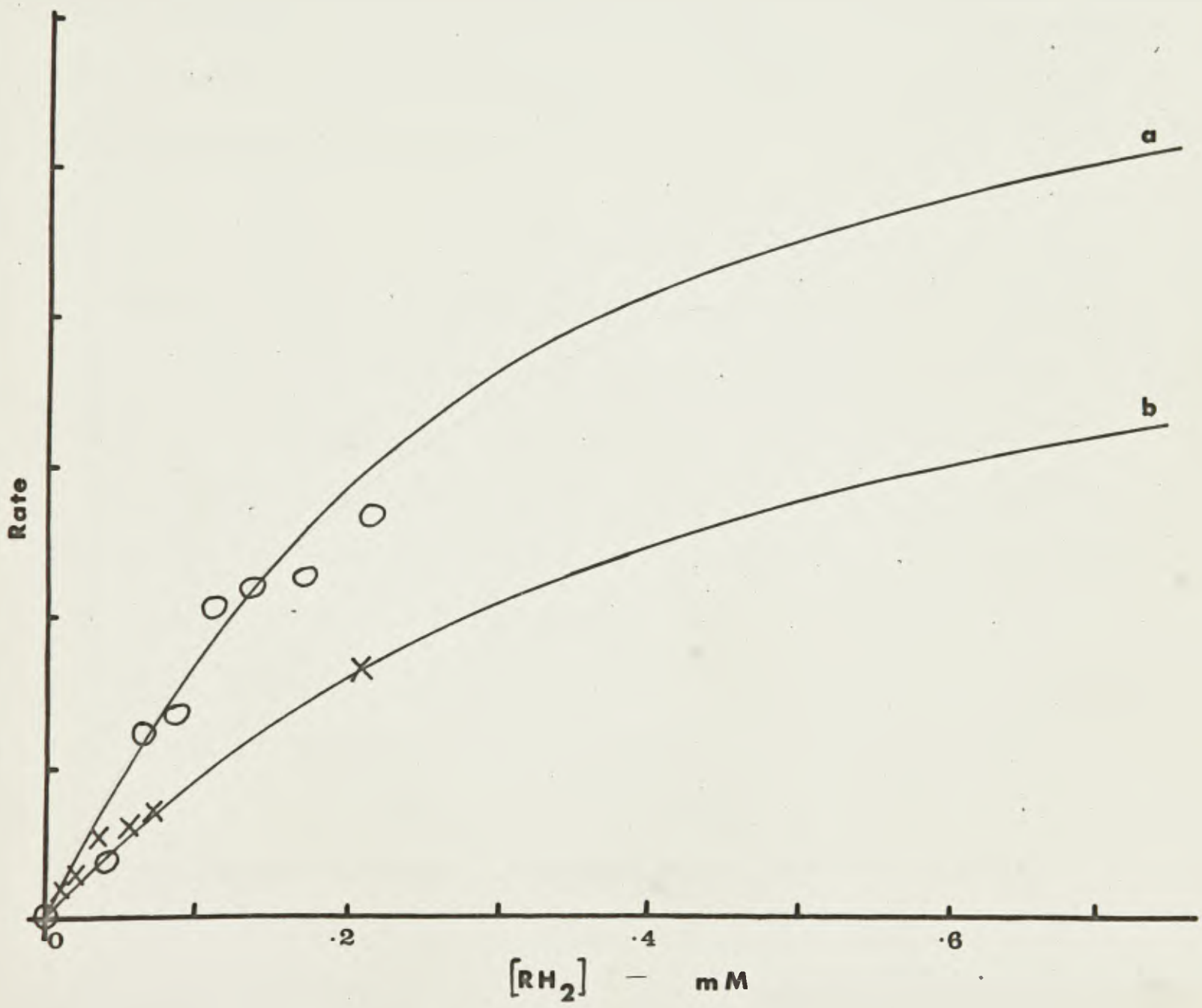
Three mechanisms may be envisaged for the quenching; i) collisional quenching, ii) quenching by forming a complex with the excited state, iii) quenching by a rapidly reversed electron transfer (i.e. $F^t + RH_2 \rightarrow FH^\bullet + RH^\bullet \rightarrow F + RH_2$). Good quenchers by process i) are ions with large diffuse electron clouds (e.g. I^-). None of the reducing agents fits this category. quenching by process ii) may be by the formation of hydrophobic complexes between the chromophore and an aromatic quencher (chapter 4). DL-Phenylglycine could quench by this mechanism, in which case the neutral PH form would probably have an effect as well as the anionic form. This was not observed for the reduction of FMN by EDTA under anaerobic conditions (chapter 4), All three reducing agents might quench by process iii), and this seems the most likely of the three possibilities. The quenching rate constants increase in the same order as the k_{+5} terms for the different substrates, indicating that the better electron donors are also the better quenchers. This agrees

with previous work on quenching by non-reducing agents in anaerobic photoreduction (Radda, 1966).

It is possible to use the rate constants in table 5-5 to calculate the variation in the rate of anaerobic photoreduction with substrate concentration. By inspection of the kinetic expression on p. 132 it is clear that at constant flavin concentration and illumination intensity the rate of reaction varies as $k_{+5}\,[RH_2]/(B + (k_{+4b} + k_{+5})\,[RH_2])$. This function has been plotted against $[RH_2]$ using the rate constants for EDTA and for DL-phenylglycine (fig. 5-7). The experimental points (taken from figure 4-8) fall close to the theoretical curve.

All the rate constants deduced in this section have been found from measurements of the rate of oxygen uptake on the assumptions i) that the rate determining step of the aerobic reaction is the same as that for anaerobic photoreduction by the same substrate, and ii) that the rate of triplet quenching by oxygen is diffusion controlled. The coherence of the results, and their correlation with the anaerobic photochemistry, further justify the validity of the assumptions.

Fig. 5-7 ; Theoretical curves for the concentration dependence of anaerobic photoreduction by a) EDTA (O experiment) and b) DL-phenylglycine (X experiment)



C) Type I reactions - dependence on FMN concentration. The effect of varying FMN concentration on the rate of oxygen uptake with diethanolamine was measured. The rate of reaction was proportional to the amount of light absorbed (calculated from the extinctions of the reaction mixtures - fig.5-8), up to 2.10^{-4}M FMN. At higher flavin concentrations dimer formation becomes more important (Radda and Calvin, 1964), and under these conditions a fall in the rate of oxygen uptake was observed.

D) Type I reactions - dependence on substrate. A good comparison of the rate-determining steps of oxygen uptake and anaerobic photoreduction may be obtained from the Hammett plots for the two reactions with substituted phenylglycines as substrates. The aerobic rates must be corrected for the pK_a values of the acids in the same way as the anaerobic ones (table 5-6).

The ρ-values and correlation coefficients (C) for the two types of reaction are : anaerobic photoreduction, $\rho = -1.1$, C = 0.94, (fig.4-10) aerobic oxygen uptake, $\rho = -1.2$, C = 0.83 ($\rho = -0.6$, C = 0.98 excluding p-methoxy-DL-phenylglycine) (fig.5-9). The same limitations apply to the correlations for the aerobic reaction as were discussed for anaerobic photoreduction

Table 5-6

Uncorrected and corrected rates of oxygen uptake with substituted DL-phenylglycines

All reactions were at 25° ± pH 7.61. The concentrations of the acids were 5 mM, and that of FMN was 0.2 mM.

Acid	pK_b	Measured rate (relative to DL-phenylglycine=100)	Corrected rate (k) (relative to DL-phenylglycine=100(k_o))	$\log \frac{k}{k_o}$
DL-phenylglycine	5.09	100	100	2.000
DL-m-fluorophenylglycine	5.45	133	58	1.7634
DL-m-chlorophenylglycine	5.52	176	65	1.8129
DL-p-methylphenylglycine	5.04	108	121	2.0828
DL-p-fluorophenylglycine	5.16	104	88	1.9445
DL-p-methoxyphenylglycine	4.94	432	609	2.7846

Fig. 5-8 ; Dependence of the rate of sensitized oxygen uptake on the amount of light absorbed by FMN (substrate diethanolamine (10 mM) at 25°)

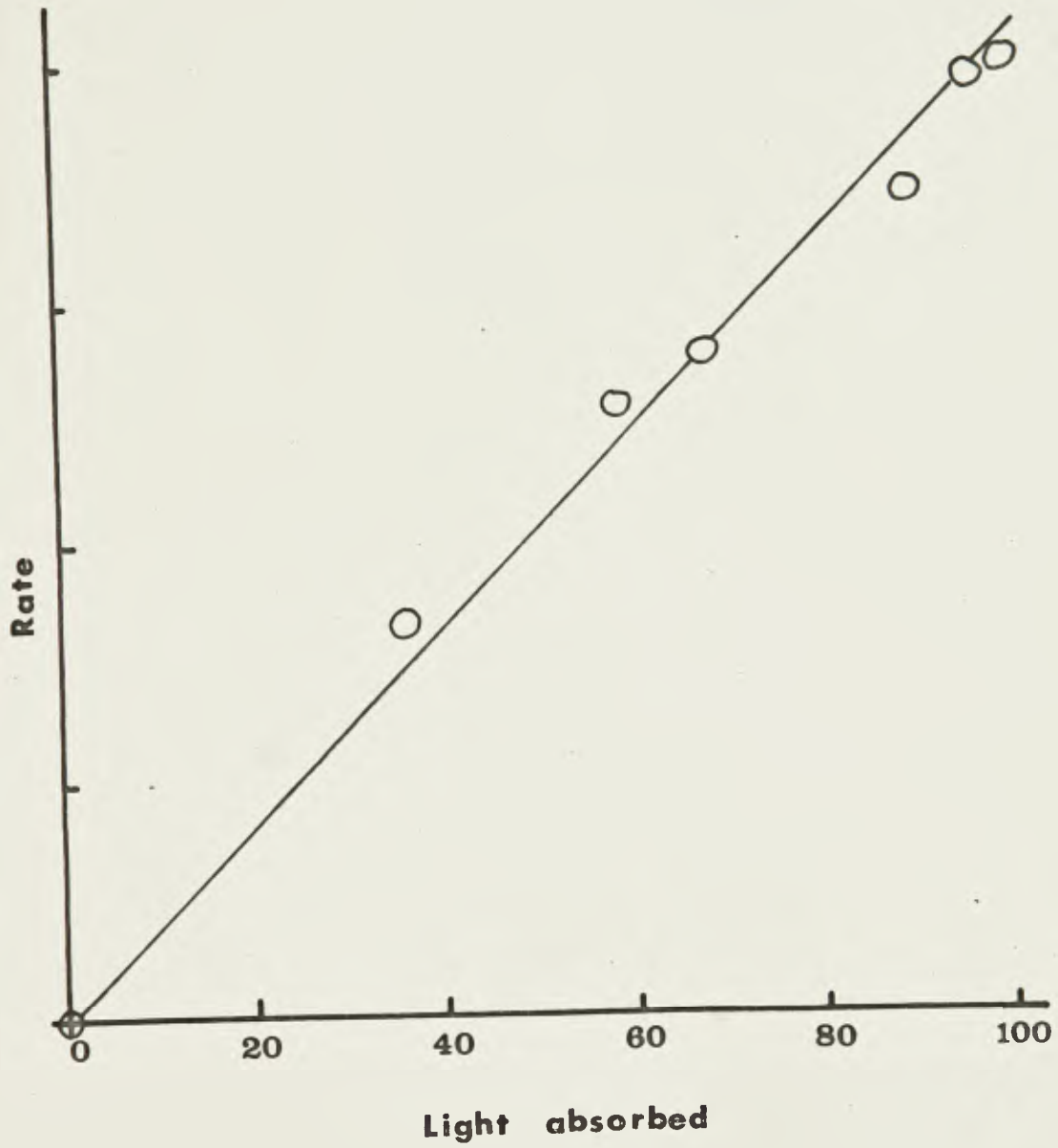
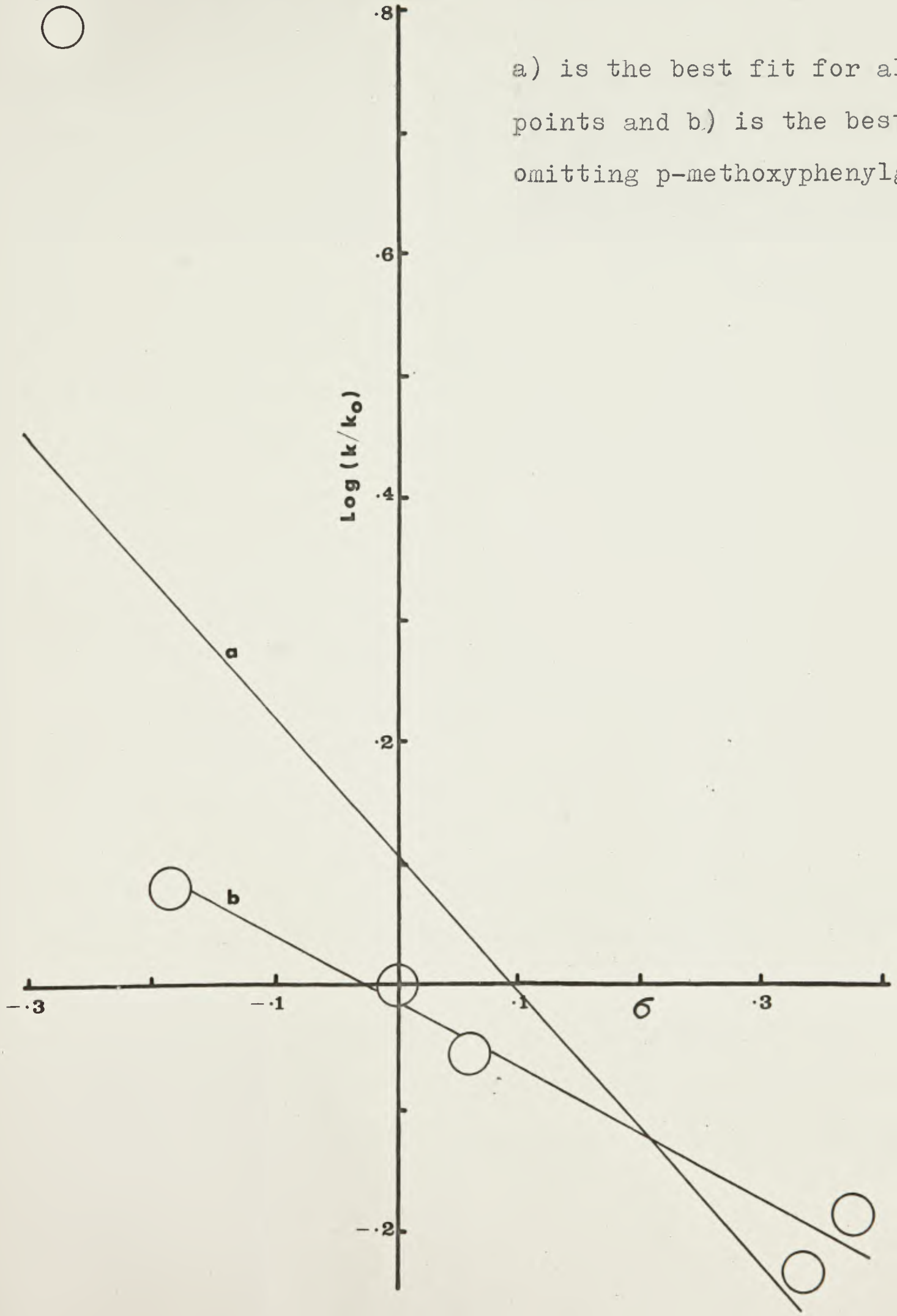


Fig. 5-9 ; Hammett plot for FMN photosensitized oxygen uptake with substituted phenylglycines



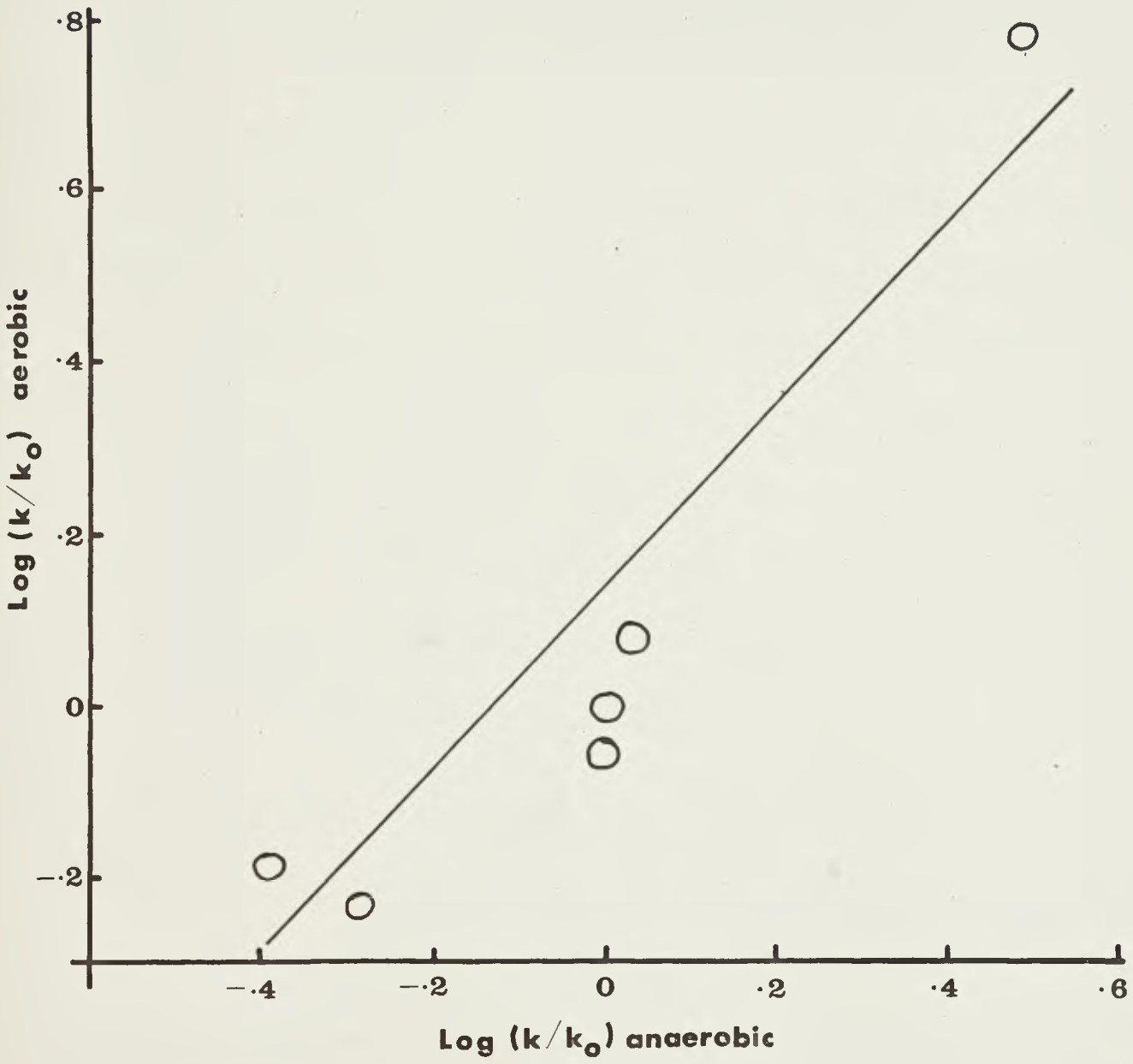
a) is the best fit for all the points and b) is the best fit omitting p-methoxyphenylglycine.

(p. 107). When the two reactions are compared directly (fig.5-10) C is 0.89 and the gradient is 1.1. It should be remembered that the rates for the two reactions are proportional to $k_{+5}[RH_2]/(B+(k_{+4b}+k_{+5})[RH_2]$ for photoreduction, and $k_{+5}[RH_2]/(B+(k_{+4b}+k_{+5})[RH_2]+k_{+4f}[O_2])$ for oxygen uptake. Comparisons are thus most meaningful when the $(k_{+4b} + k_{+5})[RH_2]$ term is negligible compared to the rest of the denominator. In both the cases considered here the concentration of the acid anion was chosen to be low so that $(k_{+4b} + k_{+5})[RH_2]$ was less than 0.25B (anaerobic photoreduction), and less than $0.25 (B + k_{+4f}[O_2])$ (aerobic photoreaction). Thus the corrected rates used in the Hammett plots are reasonable, though not perfect, measures of k_{+5}.

Considering the errors discussed above, the ρ-values found for the aerobic and anaerobic reactions are close enough to support the view that the reactions have the same rate-determining step.

E) Type II reactions. Substrates which typically undergo type II photo-oxidation usually contain easily oxidisable aromatic residues (table 5-1). Two kinds of mechanism can be envisaged, i) production of singlet oxygen which reacts with the substrate, and ii) formation of an active adduct 'moloxide' between flavin and oxygen. The rate expressions

Fig. 5-10 ; Direct comparison of phenylglycine substituent effects on anaerobic photoreduction and aerobic oxygen uptake



obtained by applying a steady state treatment to the concentration of either singlet oxygen or the moloxide are the same, so kinetic measurements alone cannot, even in principle, distinguish between the mechanisms. In practice the photo-oxidation of tryptophan sensitized by FMN does not even fit the simple rate expression.

It seems, therefore, that type II photo-oxidation is not a clean reaction, but that oxygen uptake can occur by various routes. This interpretation is consistent with the finding that 4 moles of oxygen are consumed for each mole of tryptophan destroyed in the methylene blue sensitized reaction (Weil, Gordon and Buchert, 1951). At least 7 products are found in the FMN sensitized reaction (P.S.Song, personal communication to G.K.Radda). The reaction is also subject to product inhibition (table 5-7).

F) Intermediate reactions. Some compounds have sigmoidal oxygen uptake curves, type I at high oxygen concentrations and type II at lower ones. The reasons for this behaviour probably vary, but in the case of phenylacetic acid at least it is probably a result of the formation of a photoaddition product. This does not react rapidly with oxygen, though the reaction is faster in the light (Hemmerich, Massey and

Table 5-7

Rates of oxygen uptake by tryptophan and illuminated FMN at different oxygen concentrations

All reactions were at pH 6.9 and 25°. FMN concentrations were 0.2 mM, tryptophan concentrations were 10 mM

Relative oxygen concentration (100 is for buffer in equilibrium with air)	Initial rate of oxygen uptake (units/min.)	Rate of oxygen uptake after 3 min. reaction (units/min)
71	6.1	3.6
79	6.4	3.6
87	6.3	3.6
100	6.5	4.0
107	6.6	4.2
125	6.7	4.3
181	8.2	5.6
204	8.0	-

Weber, 1967). sigmoidal oxygen uptake curves would also be expected for slow type I reactions in which step 9 became rate-determining at comparatively high oxygen concentrations.

G) Sensitized photo-oxidation of natural amino acids. Sensitized photo-oxidation has been used as a way to modify both nucleic acids and proteins selectively (Simon, 1967). The behaviour of FMN in the model reactions described above suggests that it too may prove a useful sensitizer. In addition there is the possibility that bound flavin sensitizes the destruction of a nearby residue in those proteins for which FMN or FAD is the prosthetic group, thus helping to identify the environment of the binding site.

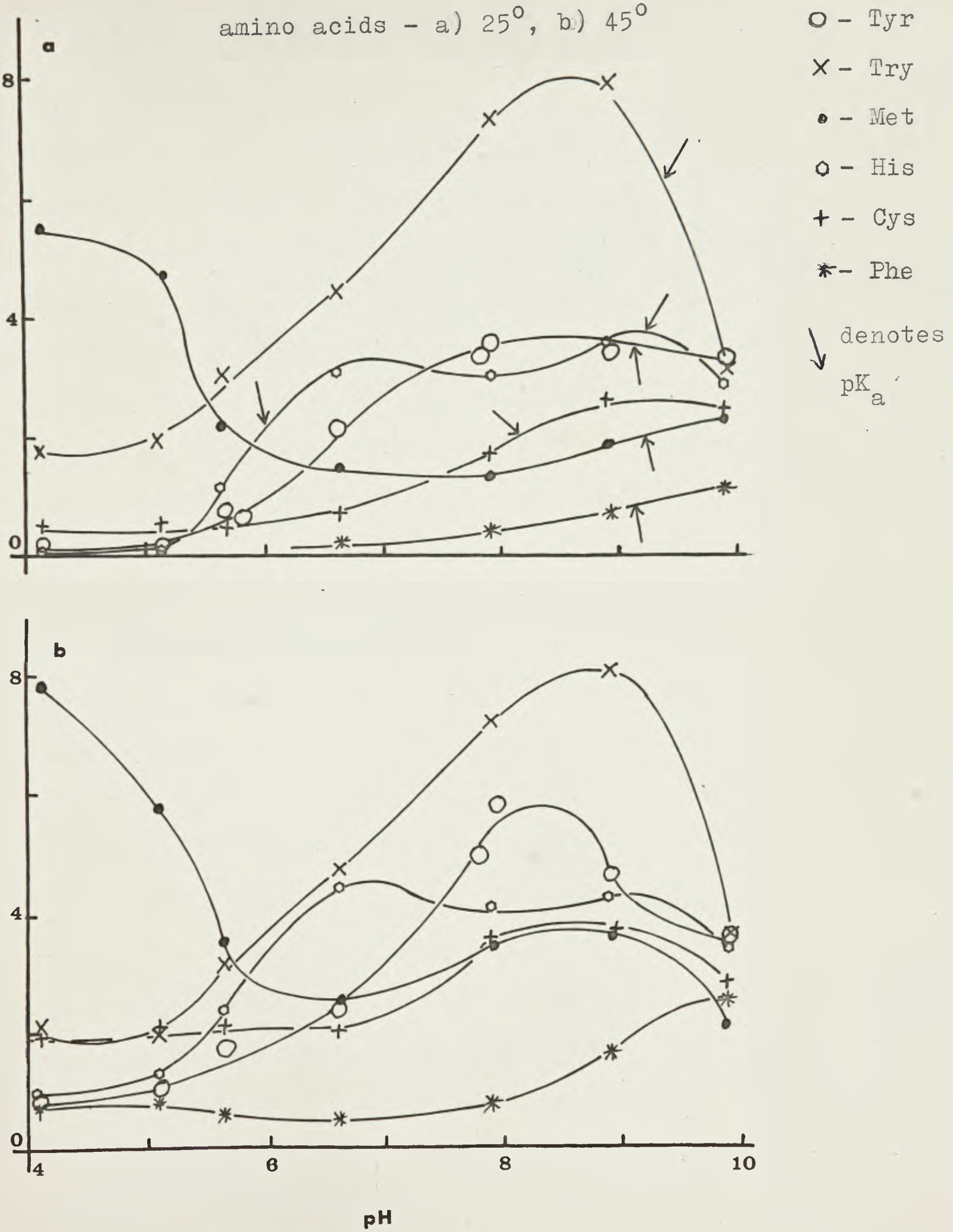
Two sets of experiments were undertaken to investigate the possible applications of flavins as photosensitizers. A variety of compounds was tested to see whether they could act as substrates for photo-oxidation (table 5-1). This survey was necessary to find the kinds of compound which might interfere with the sensitized photo-oxidation of a macromolecule. Second, the reactions with amino acids were studied in greater detail, measuring the rate of oxygen uptake at two temperatures over a range of pH. The

purpose of this was to see whether the pH profiles for different amino acids were sufficiently distinct to permit the identification of which residue was reacting in a macromolecule.

The survey of different substrates has already been mentioned (see p.127). It does not contain many results which are not explicable by the typical behaviour of type I and type II substrates. Considerable selectivity is shown amongst the nucleotides, uracil being the fastest of the pyrimidines by a factor of 10, and guanosine being 3 times faster than adenosine.

The pH profiles for the reactivity of some amino acids are given in figure 5-11. They do not correspond to the titration curve of the acid for tryptophan, tyrosine or methionine, with the largest discrepancies between the titration and reactivity curves around neutral pH. The behaviour of tryptophan and tyrosine might be expected to be complex because they are type II substrates, but all the other acids (including methionine) react in the type I mode. The methionine reactivity pH profile resembles that found for the anaerobic photoaddition of phenylacetic acid (fig.4-9). The increase in reactivity at low pH in both cases may correspond to protonation of the flavin triplet (pK_a about 5.5)

Fig. 5-11 ; Rate of oxygen uptake by FMN illuminated with mM amino acids - a) 25°, b) 45°



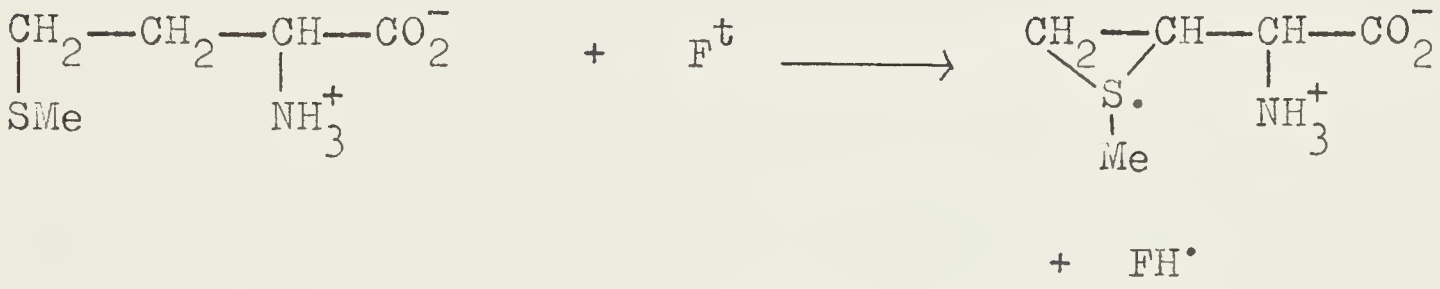
[FMN] = .1mM; rates are in the same units as in fig.5-3

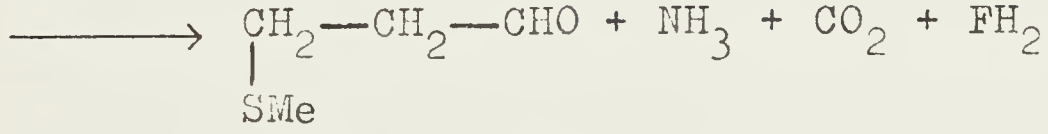
to give a better electron acceptor than the unprotonated ring. A lower value of the pK_a of the triplet has been measured by spin resonance and phosphorescence studies of flavins in glasses at 77°K, but this cannot be compared directly with solution measurements (Lhoste, Haug and Hemmerich, 1966). The low pH rate enhancement is not found for most amino acids (like phenylglycine) which only act as reducing agents in their anionic forms. The SMe group in methionine, however, means that this compound may reduce by hydrogen abstraction from one of the carbons adjacent to the sulphur or (by neighbouring group participation) from the carbon β to sulphur. The role of thio substituents is confirmed by the pH dependence of the reactivity of cysteine (pK_a values 8.33 and 10.78 probably for SH and NH_3^+ respectively). Assuming this assignment of the pK_a values to the different dissociations, cysteine reacts appreciably even while the amino groups are entirely protonated, but it does not have the same rate enhancement at low pH as methionine. This implies that the cause (for methionine) was a neighbouring group effect (fig.5-12). The corresponding intermediate for cysteine is less stable than its methionine analogue because of the proximity of positive charges.

The reactions with histidine and phenylalanine, and

Fig. 5 - 12

Photoreduction by methionine





the reaction of FMN alone all show type I behaviour, and the pH profiles for these amino acids correspond to their titration curves. The fall in rate at high pH is due to the flavin dissociation under these conditions (see also p. 106). Alanine does not react significantly faster than the FMN blank reaction.

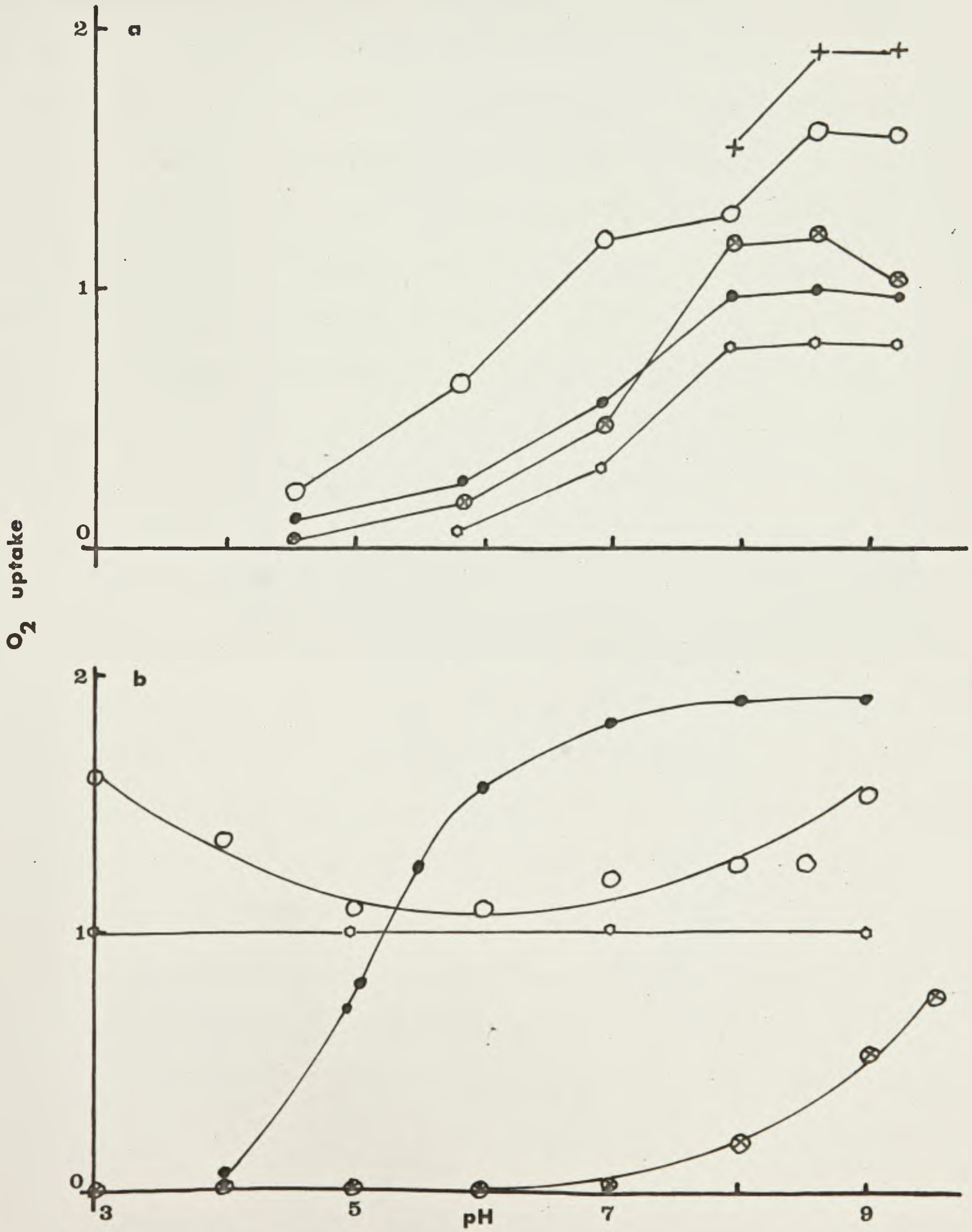
As the pH profiles for the photo-oxidation of the amino acids are all different, selective photodestruction is a possibility. The profiles also provide a way to compare the effects of different sensitizers (figs.5-11, 5-13). The effects of FMN, methylene blue and proflavin on the acids are different, and so by using different sensitizers as well as different reaction conditions with the same sensitizer many combinations of reactivity can be observed.

H) Sensitized photo-oxidation of proteins. Preliminary observations on two proteins have been made to see whether FMN can sensitize the photodestruction of amino acids in a polymer as well as in the free state. The enzymes chosen were GDH and D-amino acid oxidase. GDH was studied because it is commercially available in a pure form and because it is easily assayed spectrophotometrically. D-Amino acid oxidase was studied because it is one of the most easily prepared and purified flavoproteins.

Fig. 5-13 ; a) Methylene blue and b) proflavin photosensitized oxidation of amino acids (+- Cys, ⊗- Tyr, O- Try, •- His, o- Met)

a from Weil, Gordon & Buchert, 1951

b from Sluyterman, 1962



FMN photosensitizes the loss of GDH activity (fig. 5-14). The rate of loss of activity increases with enzyme concentration, but the rate of percentage loss of activity is greatest for the lowest concentrations (table 5-8).

α-Oxoglutarate, one of the substrates of GDH, apparently exerts a small protective effect (10%) against this inactivation (table 5-8). This could be a result of genuine protection of the enzyme, or simply the inactivation of the flavin or oxygen excited state.

No photosensitized inactivation of D-amino acid oxidase was detected in the absence of added flavin. There are two possible reasons; either the bound coenzyme is capable of activating oxygen in the same way as free flavin, but because there is no oxidisable residue close to it no destruction occurs, or bound flavin does not activate oxygen at all. This could be either because the excitation energy is rapidly degraded by quenching, or because the position of the bound flavin is sterically unfavourable to its role as a sensitizer. The second of the possibilities probably applies to the benzoate complex of D-amino acid oxidase which was used for these experiments. The enzyme is certainly susceptible to photoinactivation in the presence of added FMN. The rate of inactivation increases with light intensity (table 5-9).

Table 5-8

photoinactivation of GDH

All reactions were at 25° in pH 7.8 phosphate buffer

Reaction mixture	Relative activity before illumination	$-\frac{d(\log \text{activity})}{dt}$ (min^{-1})
30mls GDH stock soln.+ FMN	0.99 *	0.0102
	1.08 *	0.0080
	0.90	0.0130
30mls GDH stock soln.+ FMN in 10mM α-oxoglutarate	1.00 *	0.0078
	1.18 *	0.0080
	0.89	0.0115
20mls GDH stock soln.+ 10ml buffer + FMN	0.75	0.0138
10mls GDH stock soln.+ 20ml buffer + FMN	0.32	0.0191
5mls GDH stock soln.+ 25ml buffer + FMN	0.16	0.0270

* These solutions were not illuminated under identical conditions to the others.

Table 5-9

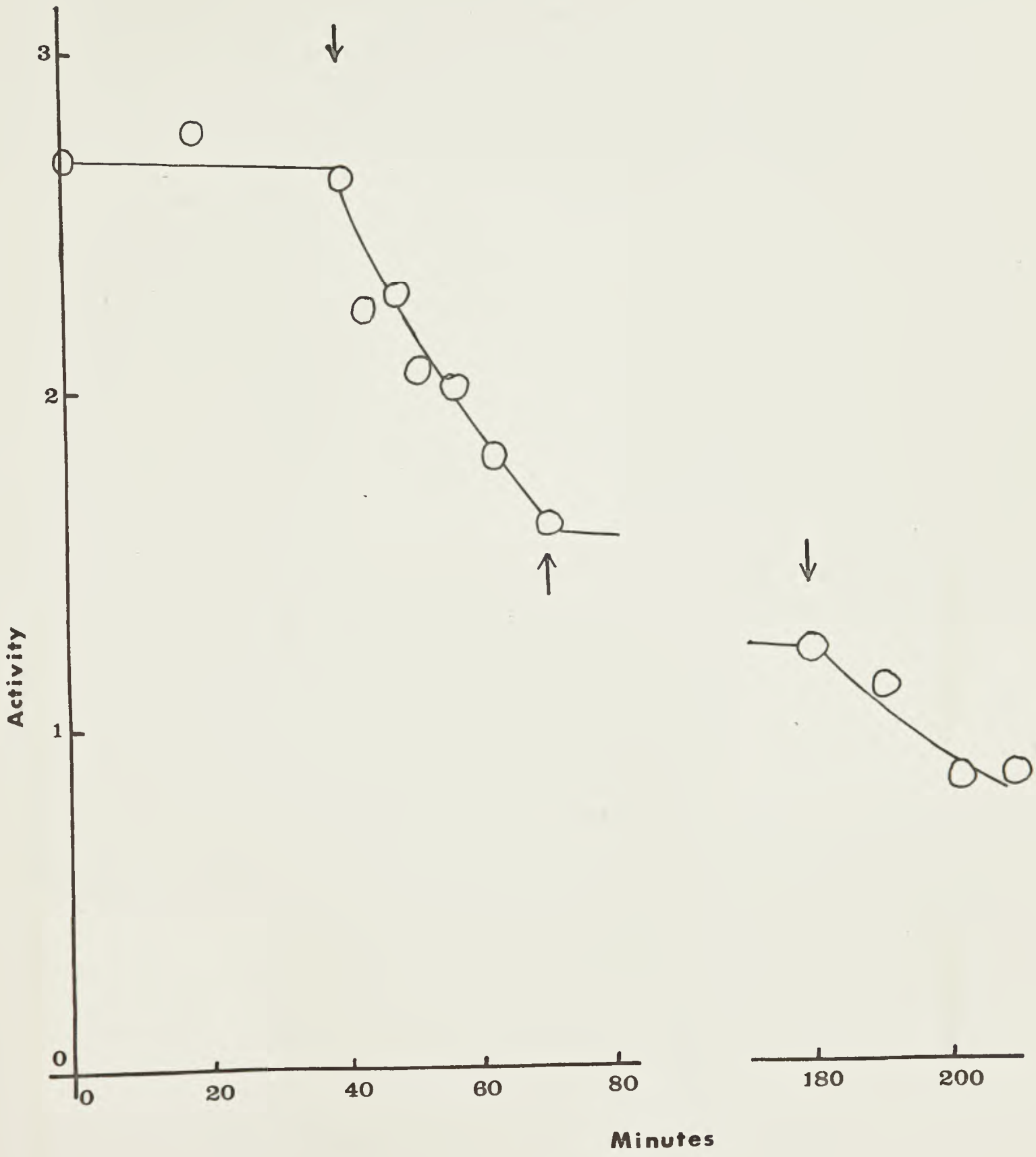
Photoinactivation of D-amino acid oxidase

All reactions were at 25° in pH 8.3 sodium pyrophosphate buffer containing M/60 benzoic acid

Reaction mixture	Relativity activity					
	0 min.	10min.	15 min.	30min.	45min.	60min.
Enzyme alone	1.00	-	0.95	1.20	-	0.96
Enzyme + FAD	1.00	-	1.00	1.00	-	0.97
Enzyme + FMN	1.00	0.65	-	0.49	-	0.37
Enzyme + FMN	1.00	-	0.71	0.60	-	0.41
Enzyme + FMN (light intensity about 650 lumens/ sq.ft.)	1.00	0.89	0.87	0.81	0.74	0.67
Enzyme + FMN (light intensity about 1000 lumens/ sq.ft.).	1.00	0.87	0.81	0.81	0.67	0.62

Fig. 5-14 ; Photoinactivation of GDH by FMN (.1mM)

(pH = 7.6 and I = .1 ; ↓ light on, ↑ light off)



These preliminary experiments indicate that more detailed investigations of the photosensitized inactivation of enzymes by flavins could be fruitful. In particular, experiments with flavoproteins might give information about the coenzyme binding site.

CHAPTER SIX

Reduction of flavins by illuminated chloroplasts

Introduction

In photosynthesis the light energy is used to force the reactants up a redox potential energy gradient. The overall change is the splitting of water to oxygen and a reducing equivalent (as NADPH). In the preparation of isolated chloroplasts some of the essential linking compounds (e.g. ferredoxin) are lost, and so when the extract is illuminated there is negligible reaction. There are many compounds which restore the ability to evolve oxygen (the Hill reaction) when added to isolated chloroplasts. These compounds act as acceptors for the reducing equivalents generated photochemically in the absence of ferredoxin, and are known as Hill oxidants. FMN acts as a Hill oxidant in restoring oxygen evolution (Stern and Vennesland, 1962) and as a cofactor for cyclic photophosphorylation (Whatley, Allen and Arnon, 1959).

There is one well characterised flavoprotein in the electron transport chain of photosynthesis, ferredoxin-NADP reductase. FAD is the prosthetic group, and the enzyme catalyses the reduction of $NADP^+$ by reduced ferredoxin. The enzyme is tightly held by the chloroplast because it is not washed out by treatment which removes ferredoxin (Shin, Tagawa and Arnon, 1963).

The participation of flavins close to the oxygen evolution step of photosynthesis has also been implicated, though there is no good supporting evidence for this. Flavins can reverse the innibition of photosynthesis by the herbicides CMU and DCMU (Sweetser, 1963) which act by blocking electron transport close to oxygen evolution (Bishop, 1958). This effect is probably just photosensitized destruction of the herbicide (table 5-1 and chapter 5 E) rather than any direct effect on photosynthetic electron transport. There is good evidence that manganese has a role in the oxygen evolution mechanism (Cheniae and Martin, 1967), and illuminated flavin solutions can lead to the oxidation of manganous to manganic ions (Andreae, 1955). The redox potentials of flavins make it most unlikely that they participate in this kind of couple in their ground states. If excited flavins were important for oxygen evolution it would be reflected in the action spectrum of photosymthesis, but this is not the case. The action spectrum for the different process of light dependent oxygen uptake by chlorella, however, does resemble a flavin absorption spectrum (Pickett and French, 1967).

Although there have been several studies of the effect of FMN on the electron transport chain (oxygen evolution,

photophosphorylation, etc.) little interest has been shown in what happens to FMN itself. It is possible to follow the fluorescence of flavins added to chloroplast suspensions (Vennesland, Gattung and Birkicht, 1963). This method is more sensitive than absorption spectroscopy, and is specially useful for studying opaque solutions. It has been used for the experiments described in this chapter which were designed to elucidate how a flavin behaves when it acts as a Hill oxidant. In enzymology the nature of a relatively unspecific process is often clarified when the reactions of a series of structurally related compounds are compared. A similar approach has been used here in comparing the reactivities of a number of flavin derivatives to photoreduction by illuminated chloroplasts.

Methods and Accuracy

A) Preparation of chloroplast suspensions. To prepare chloroplasts 100 gm of deribbed annual spinach was washed and then homogenised for 2 min. at 0° in 500 ml of 0.01 M phosphate buffer (pH 7.0) containing 0.35 M NaCl, in a Waring blender. The brine was strained through cheese-cloth; the resulting suspension was centrifuged at 2000g to remove all debris, and then at 20,000g when the chloroplasts were spun down. The supernatant was discarded, and the

chloroplasts were resuspended in half the volume of buffer and again spun down at 20,000g. The precipitate was resuspended in the same buffer, and aliquots of this mixture were used in the experiments described below. The chlorophyll content of the suspensions was determined spectrophotometrically after extraction into acetone by the method of Vernon and Zaugg (1960). The chlorophyll content of the stock suspensions was around 300 mg/l.

B) Measurement of reaction rates. Solutions were illuminated in the apparatus described on p. 90. The exciting light was filtered to remove wavelengths shorter than 500 mμ. Oxygen was removed by passing nitrogen through the cuvette, but with the tip of the inlet needle just above the surface of the solution. Deoxygenation by this method prevented the frothing which occurred if direct bubbling was attempted. It required frequent shaking of the cuvette, and took 1-2 hr. to reach completion.

Fluorescence measurements were made with an Eppendorf fluorimeter. This is suited to front face observation, the emitted light being observed at 45° to the exciting beam. The 366 mμ line from a mercury lamp, isolated by use of a filter, was used for excitation. Before

detection the emitted light passed through a band pass filter with maximal transition at 520 mμ . No fluorescence could be detected from a solution containing chloroplasts alone under these conditions.

Reaction rates were measured by observing the initial fall in fluorescence when anaerobic mixture was illuminated for different periods of time. As the cuvette was transferred from the illumination set-up to the fluorimeter it was shaken. In between each illumination the solution was left until the fluorescence had increased to a steady reading close to the initial value (see next section). Each rate was measured on at least two samples, giving a reproducibility of about ± 10%. Inaccuracies resulted from the lag between removing a sample from illumination and reading its fluorescence, slight variations in the efficiency of deoxygenation, and changes in the activity of the chloroplast preparation from day to day.

Results and Discussion

When an anaerobic reaction mixture of FMN (10^{-6} M) containing suspended spinach chloroplasts (chlorophyll concentration about 60mg/l) was illuminated with light of wavelengths greater than 500 mμ (where FMN does not absorb) there is a fall in the fluorescence of the flavin

(fig.6-1). The fluorescence is slowly regained in the dark (cf. Vennesland, Gattung and Birkicht, 1963). The extent of the initial fall depends on the period of illumination, and the recovery in the dark is nearly complete, though there is a slow irreversible decrease as well. The regain in fluorescence is almost instantaneous when air is admitted to the cuvette.

The reaction is not specific to FMN, and the relative rates of reduction for ten flavin derivatives are summarised in table 6-1. These rates were measured by following the extent of reduction as a function of illumination time, taking fluorescence readings as soon as the exciting light was switched off. The inaccuracies introduced by different rates of dark reoxidation were thus minimised.

The initial rates of reduction are independent of the flavin concentration (fig.6-2), but the rate of reaction increases with chloroplast concentration (fig.6-3). On increasing light intensity the rate increases to a saturation level (fig.6-4).

The order of reactivity of the different flavins in this reaction are best considered in relation to other chemical properties of the same flavins (table 6-1). The redox potentials and the rates on nonenzymic reduction by

Table 6-1

The reactivities of some flavin derivatives in different reactions

In the chloroplast experiments flavin concentrations were about 10 μM. The solvent was 0.35 M NaCl solution containing 0.01 M phosphate buffer (pH 7.0).

* These results are from Gascoigne and Radda, 1967.

Derivative	Half-wave potential (volts)	Reaction rate				Partition coefficient ($\frac{[F]_{water}}{[F]_{butan-1-ol}}$)
		Reduct.by dihydrol-ipoic acid	Reduct.by NADH	Photoreduct.by phenylglycine	Reduction by illuminated chloroplasts	
FMN	-0.248	100*	100*	100	100	24
Riboflavin	-0.218*	169*	221*		100	1.8
Isoriboflavin	-0.208			50	60	0.88
Isoperacetyl-riboflavin					60	0.18
Lumiflavin	-0.218*	158*	294*	106	30	0.42
9-(NN-dimethyl-amino-ethyl)-isoalloxazine	-0.143*	2580*	914*	<1	80	10

6-chloro-9-(NN-diethylaminopropyl) isoalloxazine	-0.059*	65700*	2210*	106	70	2.6
6-methyl-9-(NN-dietnylaminoethyl) isoalloxazine	-0.018	3210*	1960*		70	3.1
3-methyl lumiflavin				145	20-30	0.26
3,6,7-trimethyl-9-acetoxyethyl isoalloxazine					20-30	

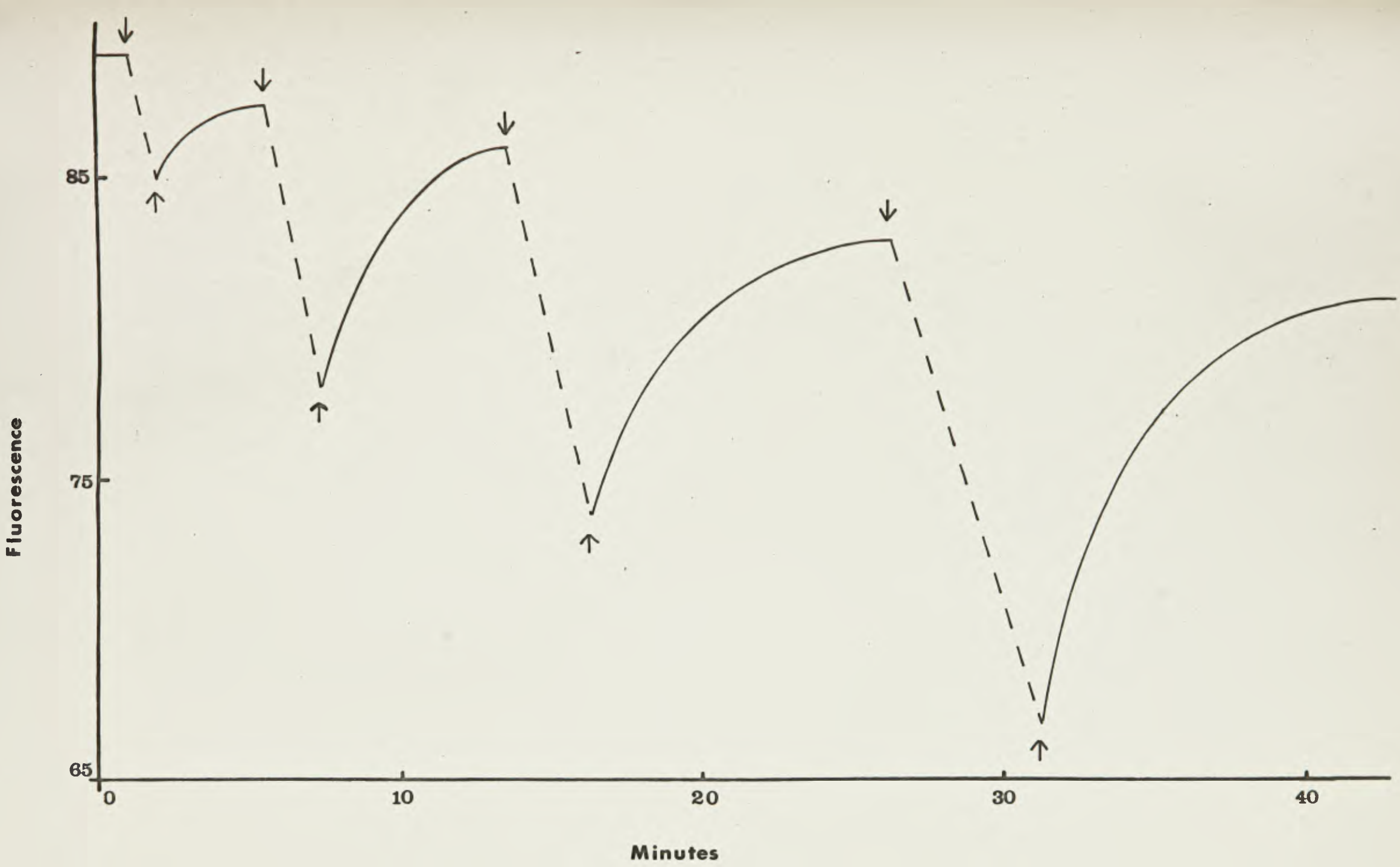
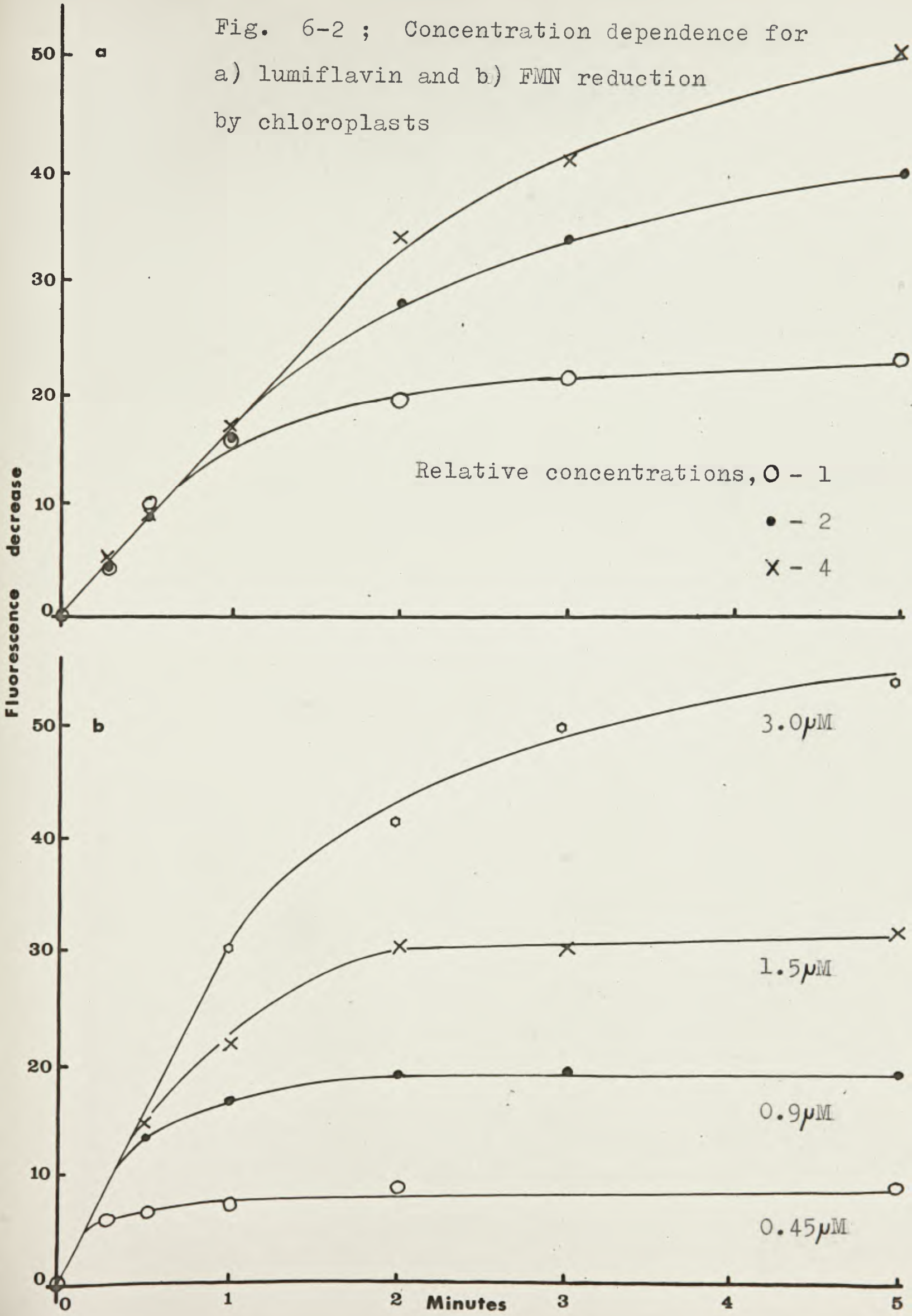


Fig. 6_1 ; Fluorescence changes on illuminating FMN with isolated chloroplasts (conditions are given in the text; ↓ light on, ↑ light off)

Fig. 6-2 ; Concentration dependence for a) lumiflavin and b) FMN reduction by chloroplasts



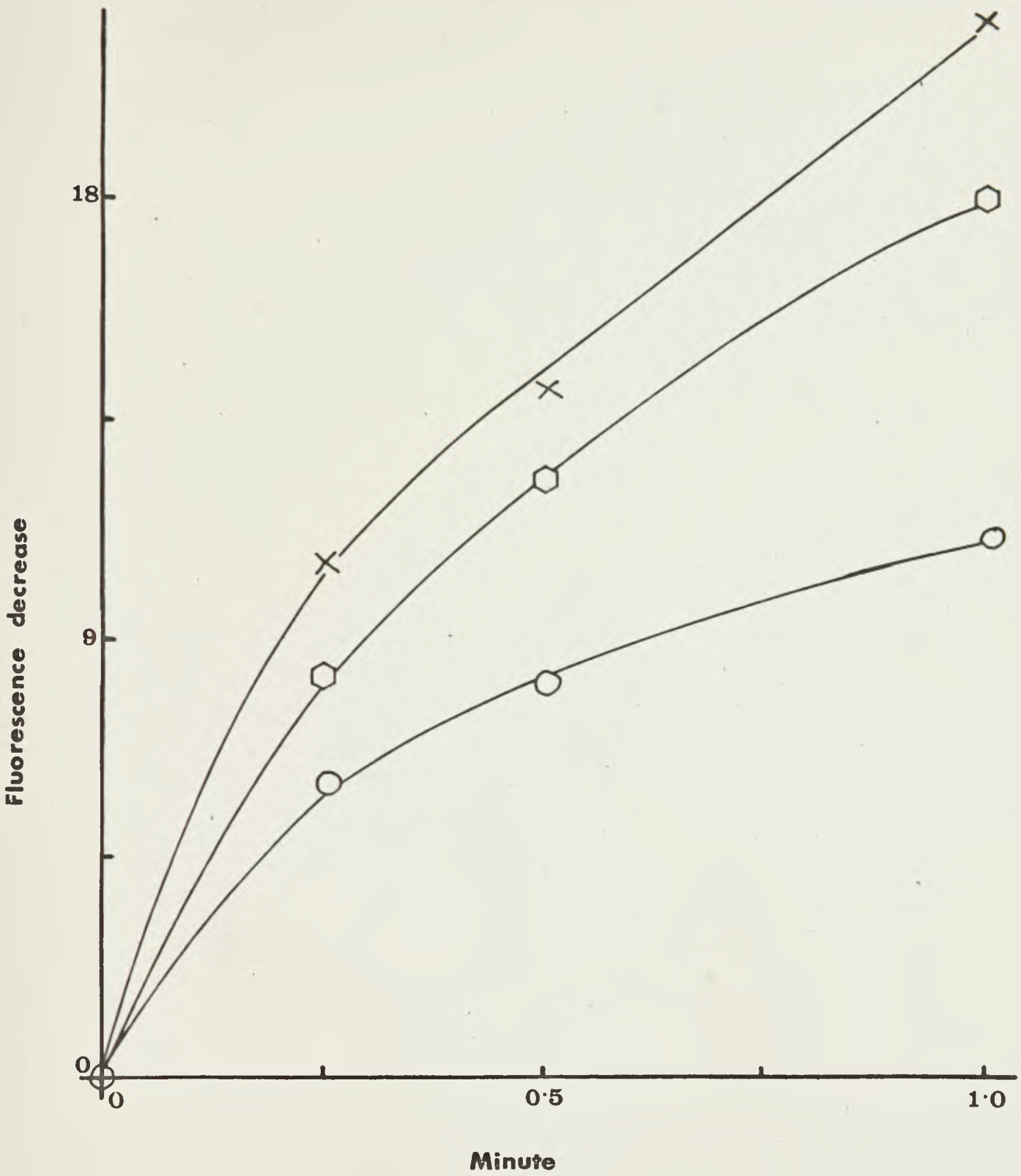


Fig. 6-3 ; Dependence of the rate of reduction of FMN on chloroplast concentration

([FMN] = 10μM ; Relative chloroplast concentrations O - 1, O - 2, X - 4)



Fig. 6-4 ; Variation in the rate of reduction of FMN by chloroplasts with light intensity ([FMN] = 10μM ; intensity was varied with neutral density filters)

both NADH and dihydrolipoic acid all follow the same trend (Gascoigne and Radda, 1967) while reduction by the chloroplast system has a different order of reactivity. This shows that the rate-determining step of the reaction is not just chemical reduction by a simple reducing agent like NADH. If the triplet state of the flavin were responsible for reaction there might be some similarities between the order of photochemical reactivity (table 4-7; Radda, 1966) and the reactions with chloroplasts. This is not the case (table 6-1).

The action of inhibitors suggests that the slow overall reaction is a reaction of the flavin triplet (which may be formed by direct excitation due to scattered light, or by energy transfer from excited chlorophyll). CMU (0.13 mM) nearly stops the reversible reaction because of its herbicidal activity, but it has a smaller effect on the slow overall decrease. Tryptophan (0.36 mM) and serotonin (2 mM) do not effect the fast light reaction, but they strongly inhibit the slow irreversible change (cf. chapter 4 E).

The fact that rates, whilst independent of flavin concentration (fig.6-2) vary with the nature of the flavin suggests that the reaction involves a small number of active binding sites which are saturated under all the

conditions studied here. The chlorophyll:flavin ratios of the solutions were such that flavin was in excess over chloroplast reducing sites if one reducing site per 400 chlorophylls is assumed (Kok and Hoch, 1961). This means that the measured rates represent the reactivity of the complex between flavin and reducing centre, in analogy with the v_{max} of enzymic reactions. Thus the rate increases with chloroplast concentration and light intensity because of the larger number of 'active sites'. This interpretation implies that at lower flavin to active centre ratios the rate comes to depend on flavin concentration. problems of light scattering prevent a study of the reaction under these conditions using the technique described above.

The behaviour of flavin derivatives in a fourth system, partition between water and butan-1-ol, has been investigated by measuring the extinctions of the flavin in the two phases. The relative partition coefficients (table 6-1) change in much the same order as the reactivities with chloroplasts. The major discrepancies are the behaviour of riboflavin and isoperacetylriboflavin. It has been shown that there is a similar order of partition coefficients for a given series of solutes in a wide variety of organic solvents (Hansch, Quinlan and Lawrence, 1968),

and so it is not too unreasonable to take butan-1-ol as a model for the hydrophobic regions of a chloroplast. Thus it can tentatively be concluded that the reactivity of the flavins is affected by binding or partition in addition to their electronic properties. This is analogous to the relationship found between v_{max} and binding constant in some enzymic systems (Knowles, 1965). The fair correspondence between flavin reactivities and the partition coefficients suggests that the more hydrophilic flavins react faster.

MATERIALS

FMN was purchased from C.F.Boehringer und Sohne G.m.b.H. (Mannheim, Germany), FAD from Sigma Chemical Co. (St.Louis, Mo.,U.S.A.), and riboflavin from British Drug Houses Ltd., (Poole, Dorset). Lumiflavin was synthesised by the method of Hemmerich, Fallab and Erlenmeyer (1956). The other isoalloxazine derivatives were gifts from the following : 3-methyl lumiflavin, 2- and 4-morpholino lumiflavins, isoriboflavin and isoperacetylriboflavin, Professor P.Hemmerich; 9-(N,N-dimethylaminoethyl)-isoalloxazine and 6-chloro, 7-methoxy, 9-(ethyl-2'N-pyrrolidino)-isoalloxazine, Professor T.Wagner-Jauregg; 6-chloro,9-(N,N-dimethylaminopropyl)-isoalloxazine and 6-methyl, 9(N,N-diethylaminoethyl)-isoalloxazine Dr.R.M.Acheson. Lumichrome was a gift from Dr.D.E.Metzler.

DL-Phenylglycine and phenylglyoxylic acid were purchased from L.Light and Co., Colnbrook, England. Substituted phenylglycines were given by Dr.J.H.C.Nayler. CMU and DCMU were gifts from E.I.Du Pont de Nemours and Co.Inc. (Wilmington, Del., U.S.A.).

o-Phenylenediamine hydrochloride was prepared from the free base and recrystallized from water.

GDH and lipoyldehydrogenase were purchased as ammonium sulphate suspensions from Boehringer.

Buffers and all other reagents were commercial products in their purest forms.

Fresh annual spinach was bought in the market and pig kidneys were collected from the slaughter house within an hour of the animal being killed.

Appendix One

Optical Rotatory Dispersion and Circular Dichroism of Flavins

Optical rotatory dispersion (ORD) spectra measured on a Cary spectropolarimeter (Kistowsky, Englard, Betheil and Seifert, 1966; Simpson and Vallee, 1966, Wellner, 1966) differ from those found with the Bendix-Ericsson instrument (Gascoigne and Radda, 1965). A positive Cotton effect associated with the visible absorptions of FMN and FAD was found with the second instrument only. Circular dichroism (CD) spectra have been measured on an adapted JASCO instrument (Kakagi, Aki, Isemura and Yamani, 1966), on a Cary 60 fitted with a model 6001 CD attachment (Miles and Urry, 1968), and on a non-commercial instrument (Tollin, 1968). The most detailed work (Tollin, 1968) shows that the CD spectra have different shapes in different buffers, but all CD studies give peaks at the wavelength of the visible absorption.

Artifacts associated with non-optically active absorption bands have been reported for the Bendix polarimeter (Resnik and Yamoaka, 1966). They are most noticeable for solutions with high extinctions. The results reported here were obtained with a Bendix instrument modified by the makers to try to remove the detection of such artifacts.

FMN and FAD contain asymmetric side-chains, and so may

have Cotton effects associated with the flavin chromophore. Lumiflavin has the same chromophore, but there is no asymmetry in the molecule. The ORD curves for FMN and lumiflavin are given in figure A-1. The apparent Cotton effect for FMN is small and there is a similar anomaly for lumiflavin. If the lumiflavin curve is subtracted from that obtained for FAD a result similar to that found with the Cary instrument is obtained (fig.A-2). It thus seems that the Cary results are correct and that the long wavelength Cotton effects found on the Bendix were artifacts.

The rotatory dispersion curves found for flavins in films involve far stronger rotations than the solutions (fig.3-10). Comparison with identical lumiflavin films is not possible because of the low solubility of lumiflavin in water, so it cannot be said whether an artifact is involved here.

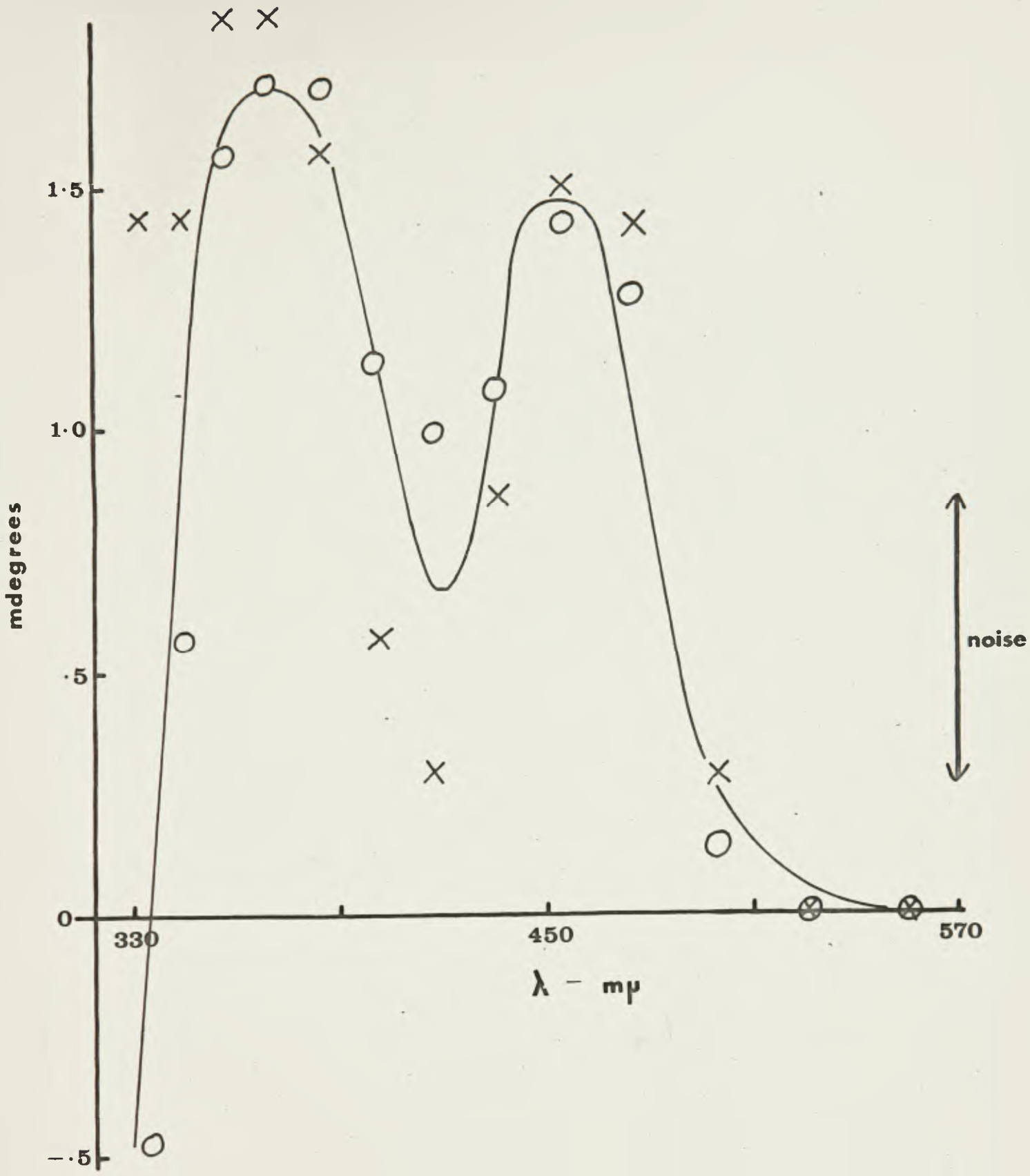


Fig. A-1 ; ORD artefact for FMN (O) and lumiflavin (×) (both flavins were in aqueous solution with extinction 1.0)

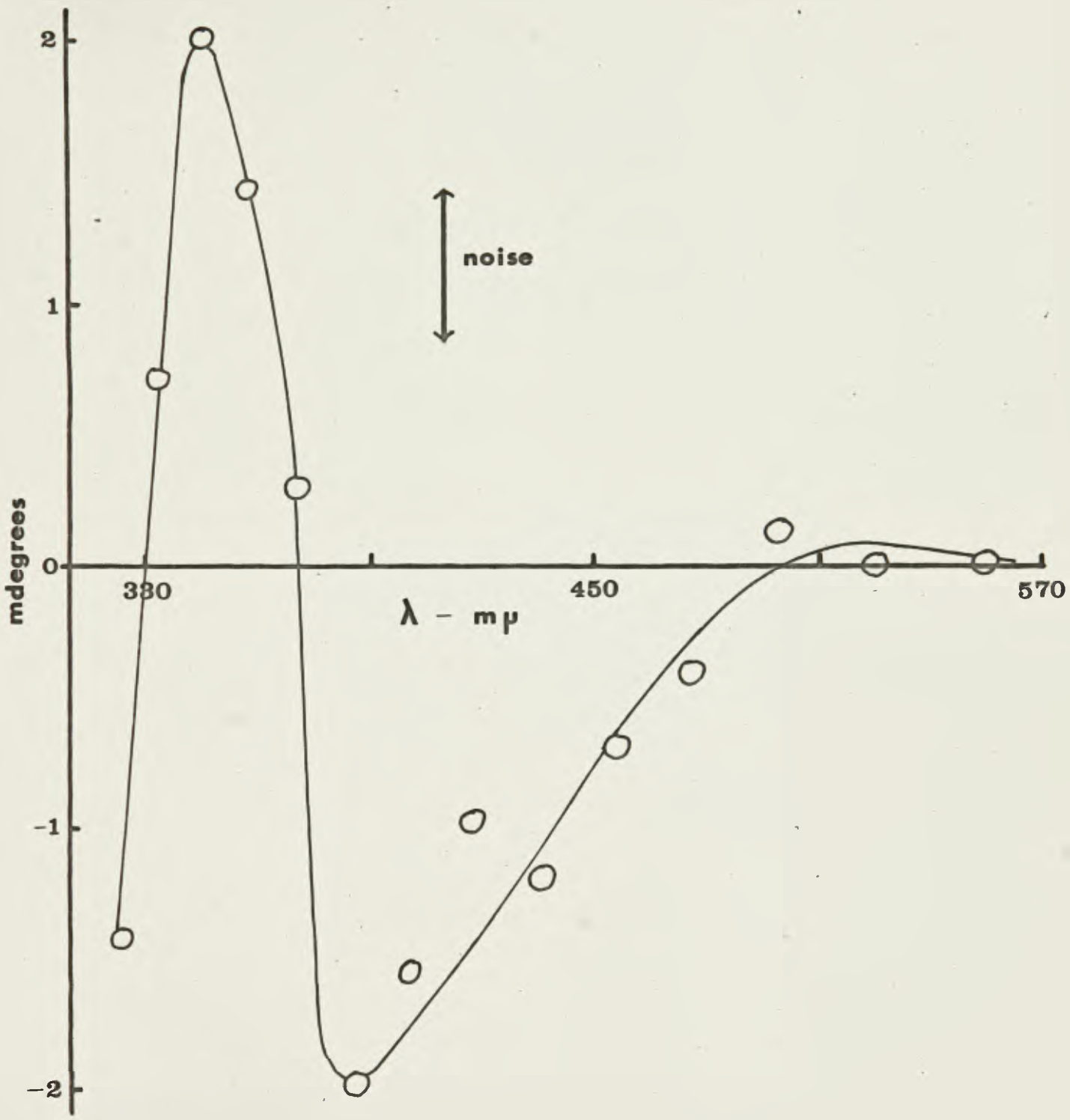


Fig. A - 2 ; ORD curve of FAD minus that of lumiflavin (both flavins were in aqueous solution with extinction 1.0)

Appendix Two

Details of Dichroic Bleaching Theory

To find the mean (cosine)^2 (M) of the angle between the 375 mμ absorption oscillator (O) and the direction of polarised light (P), let O be at an angle Θ from the 447 mμ oscillator which is at an angle α from P in the plane perpendicular to the plane of propagation of light (fig.A-3). Consider now all possible orientations of O about A, and of O about an axis perpendicular to A.

1) All orientations around A

$$M_1 = \frac{1}{2\pi} \int_0^{2\pi} \cos^2(\alpha + y) \frac{\cos^2\Theta}{\cos^2 y} . \, dx$$

where $\cos x = \cot\Theta . \tan y$, and all values of x between $0 = 2\pi$ have equal weight.

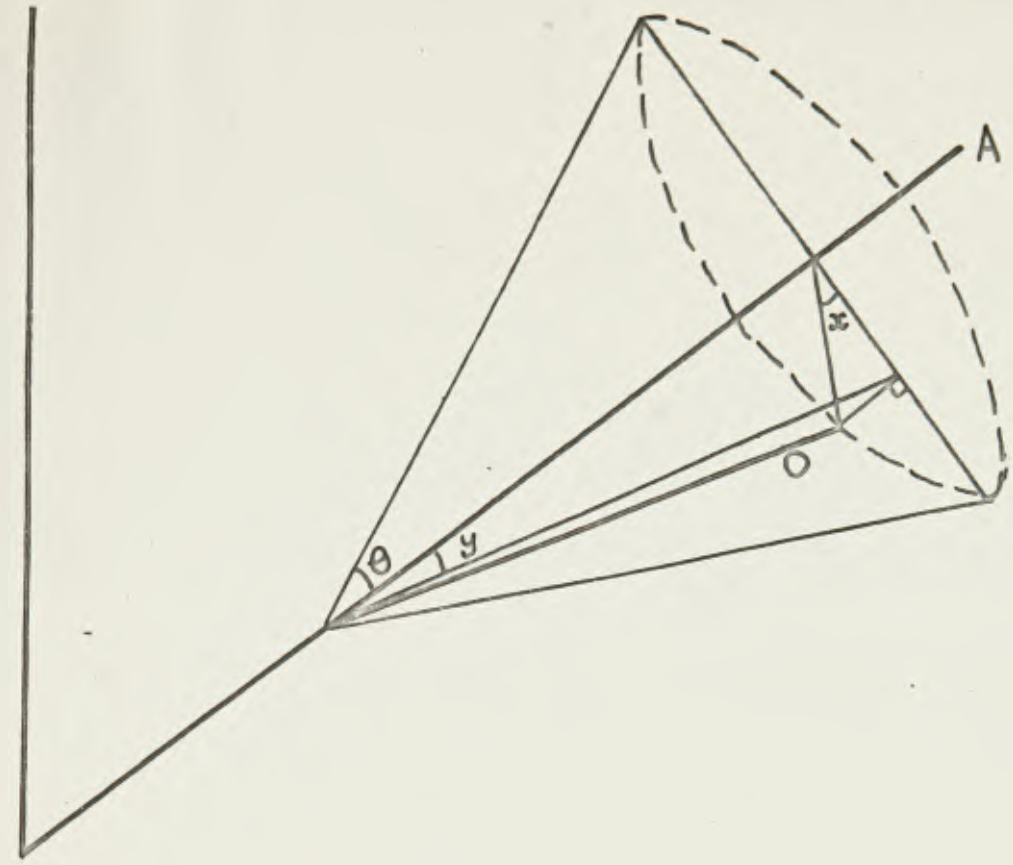
$$2\pi M_1 = \int_0^{2\pi} (\cos\alpha \cos y - \sin\alpha \sin y)^2 . \frac{\cos^2\Theta}{\cos^2 y} . \, dx$$

$$= \int_0^{2\pi} (\cos^2\alpha - \sin^2\alpha + \sin^2\alpha \sec^2 y - \sin 2\alpha . \tan y) . \cos^2\Theta . \, dx$$

$$= \int_0^{2\pi} (\cos^2\alpha + \sin^2\alpha \cos^2 x \tan^2\Theta - \sin 2\alpha \cos x \tan\Theta) . \cos^2\Theta . \, dx$$

$$= \left[\int_0^{2\pi} (\cos^2\alpha + \tfrac{1}{2}\sin^2\alpha \tan^2\Theta) \cos^2\Theta . x \right] = 2\pi (\cos^2\alpha \cos^2\Theta + \tfrac{1}{2}\sin^2\alpha \sin^2\Theta)$$

2) All orientations about an axis perpendicular to A



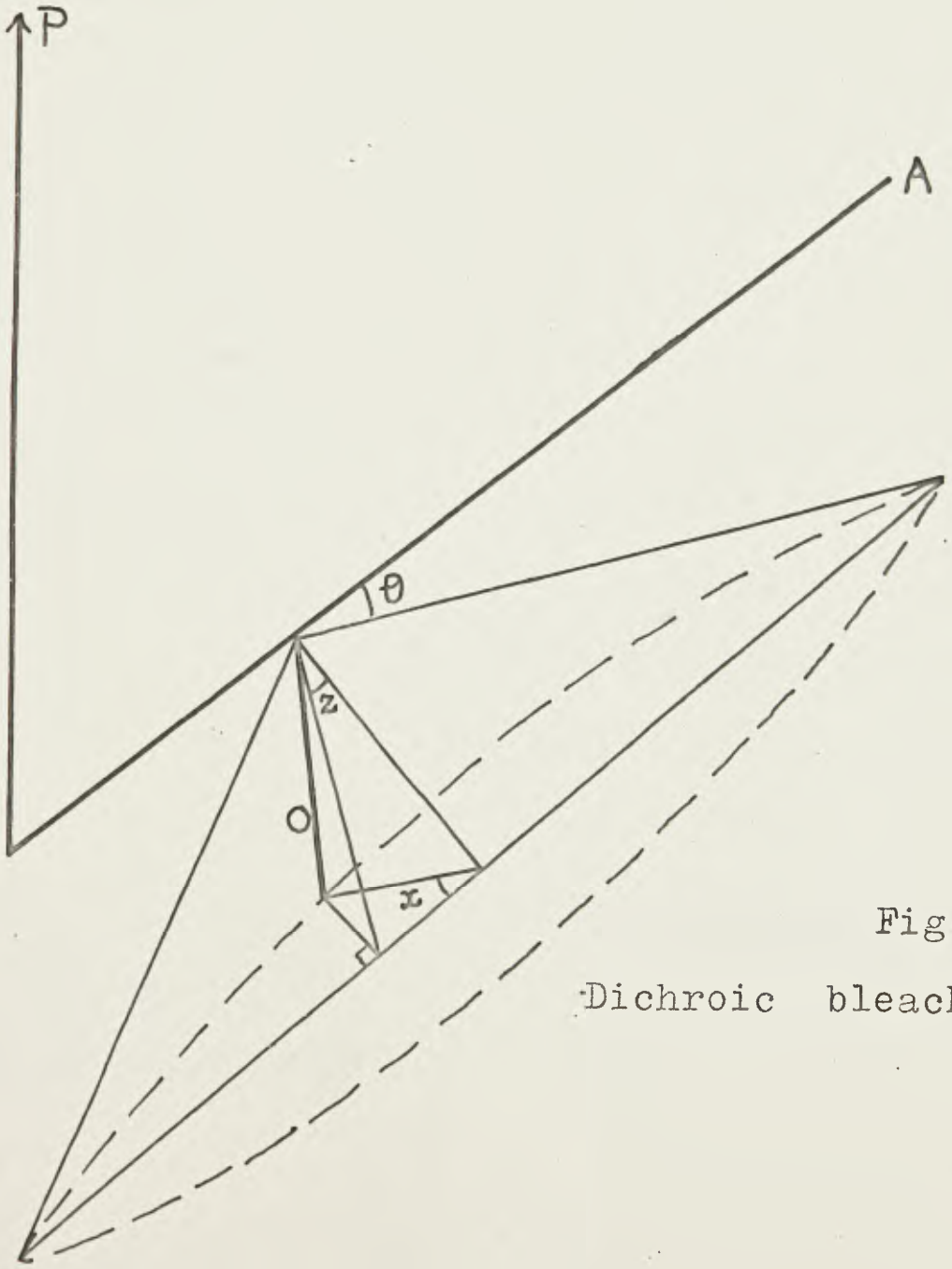


Fig. A - 3

Dichroic bleaching theory

$$M_2 = \frac{1}{2\pi}\int_0^{2\pi} \sin^2(\alpha + z).\ \frac{\sin^2\theta}{\cos^2 z}\ dx$$

where $\cos x = \tan\theta . \tan z$, and all values of x between $0-2\pi$ have equal weight

$$2\pi M_2 = \int_0^{2\pi} (\sin^2\alpha\cos^2 z + \cos^2\alpha\sin^2 z + \tfrac{1}{2}\sin 2\alpha . \sin 2z)\frac{\sin^2\theta . dx}{\cos^2 z}$$

$$= \int_0^{2\pi} (\sin^2\alpha + \cos^2\alpha\tan^2 z + \sin 2\alpha . \tan z)\sin^2\theta . dx$$

$$= \int_0^{2\pi} (\sin^2\alpha + \cos^2\alpha\cot^2\theta\cos^2 x + \sin 2\alpha\cos x\cot\theta)\sin^2\theta . dx$$

$$= \left[\int_0^{2\pi} (\sin^2\alpha + \tfrac{1}{2}\cos^2\alpha\cot^2\theta)\sin^2\theta . x\right]$$

$$= 2\pi(\sin^2\alpha\sin^2\theta + \tfrac{1}{2}\cos^2\alpha\cos^2\theta)$$

when all orientations covered by these two operations are equally likely, the overall value of M is given by

$$M = \frac{3}{4}(\sin^2\alpha\sin^2\theta + \cos^2\alpha\cos^2\theta)$$

$$= \frac{3}{4}(\tfrac{1}{2}\cos^2(\alpha+\theta) + \tfrac{1}{2}\cos^2(\alpha-\theta))$$

Thus the extinction ratios calculated on the simple 2 dimensional theory considered in chapter 2 are the same as those given by this more rigorous treatment. The calculation given in 2, however, is not strictly correct because not all values of x are equally likely for bleached molecules. To correct for this the expression must be weighted by the $(\text{cosine})^2$ of the angle between A and P thus allowing for the changing probability of absorption by 447 mμ oscillators

as they are directed further out of the plane perpendicular to the plane of propagation of the light.

3) All orientations about an axis perpendicular to A, weighted to allow for the angle A makes with P.

$$M_3 = \frac{1}{\pi}\int_0^{2\pi} \sin^2(\alpha + z).\frac{\sin^2\theta.\cos^2 x}{\cos^2 z}.dx$$

where $\cos x = \tan\theta.\tan z$, and all values of x between $0-2\pi$ have equal weight.

$$\pi M_3 = \int_0^{2\pi} \sin^2\alpha + \cos^2\alpha.\cot^2\theta.\cos^2 x + \sin 2\alpha \cos x \cot\theta)\ \sin^2\theta \cos^2 x.dx$$

$$= \int_0^{2\pi} (\tfrac{1}{2}\sin^2\alpha(1+\cos 2x) + \tfrac{1}{4}\cos^2\alpha\cot^2\theta(1+\cos 2x)^2 + \sin 2\alpha \cot\theta\cos^3 x)\ \sin^2\theta.dx$$

$$= \left[\left(\tfrac{1}{2}\sin^2\alpha + \tfrac{3}{8}.\cos^2\alpha\cot^2\theta\right)\sin^2\theta.x.\right]_0^{2\pi}$$

$$= \pi(\sin^2\alpha\sin^2\theta + \tfrac{3}{4}\cos^2\alpha\cos^2\theta)$$

When all orientations covered by operations 1 and 3 are equally likely, the overall value of M is given by

$$M = \frac{3}{4}\sin^2\alpha\sin^2\theta + \frac{7}{8}\cos^2\alpha\cos^2\theta$$

Using this expression to calculate optical density ratios after dichroic bleaching the ratio at 447 mμ is unchanged from the value given in chapter 2, $\frac{4C-3a}{4C-3a}$. For the 375 mμ absorption, however, the corrected extinction

ratio is

$$\frac{56C - 27a - 15a.\cos 2\theta}{56C - 25a + 11a.\cos 2\theta} \text{ (rather than } \frac{4C - 2a - a\cos 2\theta}{4C - 2a + a\cos 2\theta}\text{).}$$

For values of θ around 30° (such as those considered in chapter 2) use of the detailed theory leads to calculation of angles about 3° larger than those obtained from the simplified expression.

Appendix Three

Delayed Emission Results

Two series of delayed fluorescence experiments were done for each compound. The results were not in perfect agreement, series A generally giving higher activation energies. This is attributed to inaccurate measurement of temperature because of poor thermal contact between the thermocouple and film during series A. This was corrected during series B, and the phosphorescence measurements.

FMN delayed fluorescence (λmax=525 mμ)

Series A		Series B		
Temperature (°K)	Half-life (m.sec.)	Temperature (°K)	Half-life (m.sec.)	Relative intensity of delayed emissims
289.0	50	275.9	62	0.61
293.0	44	284.4	50	0.90
294.0	50	291.3	52	1.15
305.5	28	298.1	47	1.65
310.5	30	302.8	40	1.71
313.7	28	307.5	38	1.85
315.0	29	313.1	36	2.25
317.7	28			

Activation energy A = 3.9 kcal/mole
B (half-life) = 2.6 kcal/mole
B (intensity) = 5.9 kcal/mole

Riboflavin delayed fluorescence (λ max = 525 mμ)

Series A		Series B		
Temperature (°K)	Half-life (m.sec.)	Temperature (°K)	Half-life (m.sec.)	Relative intensity of delayed emission
278.8	51	279.1	47	1.15
285.4	49	287.6	38	1.35
288.6	49	292.8	40	1.65
292.7	38	297.3	42	1.70
292.7	41	300.7	35	2.0
300.1	35	305.5	37	2.25
316.5	30	308.8	37	2.40

Activation energy A = 3.3 kcal/mole
B (half-life) = 1.6 kcal/mole
B (intensity) = 4.3 kcal/mole

Lumiflavin delayed fluorescence (λ max = 525 mμ)

Series A		Series B		
Temperature (°K)	Half-life (m.sec.)	Temperature (°K)	Half-life (m.sec.)	Relative intensity of delayed emission
278.8	59	277.0	60	3.13
286.2	54	286.2	60	3.63
293.5	49	290.4	58	5.13
305.0	35	297.0	50	6.75
309.1	32	302.8	46	7.75
316.5	24	306.8	50	9.0
		310.8	42	10.5

Activation energy A = 4.6 kcal/mole
B (half-life) = 2.4 kcal/mole
B (intensity) = 5.0 kcal/mole

3-Methyl lumiflavin delayed fluorescence (λ max=527μ) and phosphorescence (λ=max 597μ).

Series A		Series B			Phosphorescence		
Temp. (°K)	Half-life (m.sec.)	Temp. (°K)	Half-life (m.sec.)	Relat. inten.	Temp. (°K)	Half-life (m.sec.)	Relative intens.
268.1	86				77	151	1.45
278.0	65	277.2	60	4.5	77	155	1.43
287.0	65	285.4	60	6.5	196	115	.62
292.7	54	293.5	64	10.25	196	115	.51
300.1	49	301.8	50	12.25	226	45	.20
307.5	49	307.3	48	12.60	253	76	.60
304.0	38	310.0	47	13.50	269	94	.56
					286	60	.53

Activation energy from A = 2.7 kcal/mole
B (half-lives) = 2.2 kcal/mole
B (intensity) = 5.5 kcal/mole
Phosphorescence (half-lives) = 65 cal/mole

9-(N,N-dimethylaminoethyl)-isoalloxazine delayed fluorescence (λ max = 513 mμ)

Series A		Series B		
Temperature (°K)	Half-life (m.sec.)	Temperature (°K)	Half-life (m.sec.)	Relative intensity of delayed emission
267.0	58	277.0	50	1.55
276.0	43	284.9	47	1.60
283.0	50	289.2	44	1.75
294.0	33	293.9	40	1.75
299.0	27	300.5	37	2.10
308.0	21	305.3	35	2.35
317.0	14	310.0	33	2.60

Activation energy from A = 5.1 kcal/mole
B (half-lives) = 2.5 kcal/mole
B (intensity) = 3.1 kcal/mole

6-chloro, 7-methoxy, 9-(ethyl-2'N-pyrrolidino)-isoalloxazine delayed fluorescence (λ max = 510 mμ) and phosphorescence (λ max = 600 mμ).

Series A		Series B			Phosphorescence		
Temp. (°K)	Half-life (m.sec.)	Temp. (°K)	Half-life (m.sec.)	Relat. inten.	Temp. (°K)	Half-life (m.sec.)	Relative inten.
285.3	81	279.4	80	1.55	77	188	2.80
288.6	70	285.2	80	1.70	77	193	2.45
296.8	70	289.1	89	1.80	196	185	1.60
300.9	49	295.2	73	1.90	196	130	1.40
307.5	54	299.4	75	2.80	226	125	1.08
313.2	46	304.7	68	3.0	248	115	1.05
		310.2	73	3.5	272	105	.80

Activation energy from A = 3.8 kcal/mole
B (half-lives) = 1.8 kcal/mole
B (intensity) = 6.3 kcal/mole
Phosphorescence (half-lives)=120 cals/mole
Phosphorescence (intensity)=-145 cals/mole

2-morpholino-lumiflavin delayed fluorescence (λmax = 533 mμ)

Series A		Series B		
Temperature (°K)	Half-life (m.sec.)	Temperature (°K)	Half-life (m.sec.)	Relative intensity of delayed emission
282.0	50	276.7	62	1.7
288.0	51	284.1	57	2.3
296.0	38	289.7	47	2.65
301.0	33	295.2	42	2.50
310.0	22	299.5	38	3.50
314.0	19	304.7	34	4.25
		309.0	33	4.75

Activation energy from A = 8.3 kcal/mole
B (half-lives) = 3.6 kcal/mole
B (intensity) = 5.6 kcal/mole

4-morpholino-lumiflavin delayed fluorescence (λ max = 540 mμ) and phosphorescence (λ max = 605 mμ)

Series A		Series B			Phosphorescence		
Temp. (°K)	Half-life (m.sec.)	Temp. (°K)	Half-life (m.sec.)	Relat. inten.	Temp. (°K)	Half-life (m.sec.)	Relative inten.
275.0	22	278.0	20	1.05	77	55	4.90
283.0	22	284.6	19	1.20	77	62	4.40
290.0	22	291.2	18	1.34	196	42	.83
296	22	297.5	19	1.65	241	44	
301	16	303.7	18	1.95	269	31	.83
306	19	306.0	16	1.98	284	29	.24
311	11	310.0	15	2.20	284	29	.21

Activation energy from A = 4.1 kcals/mole
B (half-lives) = 1.6 kcals/mole
B (intensity) = 4.4 kcals/mole
Phosphorescence (half-lives)=50 cals/mole
Phosphorescence (intensity)=-250 cals/mole

Isoriboflavin delayed fluorescence (λ max = 527 mμ) and phosphorescence (λ max = 590 mμ)

Series A		Series B			Phosphorescence		
Temp. (°K)	Half-life (m.sec.)	Temp. (°K)	Half-life (m.sec.)	Relat. inten.	Temp. (°K)	Half-life (m.sec.)	Relative inten.
285.0	45	277.5	37	.95	77	81	2.45
287.0	28	284.9	31	1.10	77	92	2.50
289.0	39	290.2	24	1.10	77	78	1.65
295.0	27	294.4	30	1.20	77	83	2.22
304.0	25	297.0	23	1.40	77	83	2.20
310.0	16	300.7	21	1.30	196	52	1.60
318.0	15	305.7	27	1.40	196	52	1.20
		310.0	20	1.80	196	49	1.20
					225	47	.97
					226	50	.85
					226	47	.95
					250	42	.97
					254	37	.90
					272	34	.71
					289	30	.60

Activation energy from A = 6.7 kcals/mole
B (half-lives) = 3.4 kcals/mole
B (intensity) = 3.1 kcals/mole
Phosphorescence (half-lives)=120 cals/mole
Phosphorescence (intensity)=-145 cals/mole

Isoperacetylriboflavin delayed fluorescence (λ max = 531mμ)

Series A		Series B		
Temperature (°K)	Half-life (m.sec.)	Temperature (°K)	Half-life (m.sec.)	Relative intensity of delayed emission
272.0	55	275.1	39	2.60
279.0	37	284.1	37	3.00
286.0	39	289.7	32	3.40
296.0	32	297.0	29	3.60
304.0	25	301.3	28	4.40
312.0	19	306.5	26	5.00
		310.5	24	5.10

Activation energy from A = 5.1 kcal/mole
B (half-lives) = 2.3 kcal/mole
B (intensity) = 3.5 kcal/mole

6-chloro,9-(N,N-dimethylaminopropyl)-isoalloxazine delayed fluorescence (λmax = 523 mμ)

Series A		Series B		
Temperature (°K)	Half-life (m.sec.)	Temperature (°K)	Half-life (m.sec.)	Relative intensity of delayed emission
282.1	24	275.9	21	1.45
286.2	24	282.8	20	1.60
291.1	22	287.8	21	1.80
296.0	16	292.8	21	2.20
299.3	16	297.3	20	2.90
308.3	12	301.2	19	2.30
314.0	11	305.0	16	2.36
		312.0	16	3.00

Activation energy from A = 4.5 kcal/mole
B (half-lives) = 1.8 kcal/mole
B (intensity) = 3.3 kcal/mole

6-methyl,9-(N,N-diethylaminoethyl)-isoalloxazine delayed fluorescence (λ max = 523 mμ)

Series A		Series B		
Temperature (°K)	Half-life (m.sec.)	Temperature (°K)	Half-life (m.sec.)	Relative intensity of delayed emission
284.5	38	280.7	41	1.10
291.1	32	286.3	38	1.25
294.4	32	291.3	34	1.45
301.7	28	297.6	32	1.68
305.8	22	302.3	32	1.80
312.4	22	305.7	28	2.00
		310.8	27	2.10

Activation energy from A = 3.3 kcal/mole
B (half-lives) = 2.5 kcal/mole
B (intensity) = 3.9 kcal/mole

Lumichrome phosphorescence (λ max = 550 mμ)

Temperature (°K)	Half-life (m.sec.)	Relative intensity
264.0	54	1.9
276.3	59	1.7
289.4	49	1.5
296.8	49	1.3

Appendix 4

Derivation of Steady State Kinetics for Flavin Photoreduction

Rate of production of $F^* = k_{+1}[F]I_o + k_{+4e}[F^t]2$

As no delayed emission is observed for fluid solutions step +4e can be neglected in comparison with step +1.

Rate of disappearance of F^*

$$=[F^*](k_{-1} + k_{+2a}[\text{solv.}] + k_{+3a} + k_{+3c}[\text{solv.}])$$

In the steady state, $[F^*] = \dfrac{k_{+1}[F]I_o}{(k_{-1} + (k_{+2a}+k_{+3c})[\text{solv.}] + k_{+3a})}$

Rate of production of F^t

$$= [F^*](k_{+3a} + k_{+3c}[\text{solv.}])$$

Rate of disappearance of F^t

$$=[F^t](k_{+4a}+(k_{+4b}+k_{+5})[RH_2] + k_{+4c}[\text{solv.}] + k_{+4e}[F^t] + k_{+4f}[O_2])$$

In the steady state $[F^t] = \{[F^*](k_{+3a}+k_{+3c}[\text{solv.}])\} /$
$(k_{+4a}+(k_{+4b}+k_{+5})[RH_2] + k_{+4c}[\text{solv.}] + k_{+4e}[F^t] + k_{+4f}[O_2])$

If the rate determining step is +5, reaction rate = $k_{+5}[RH_2][F^t]$. Applying steady state approximations to $[F^*]$ and $[F^t]$ this is given by :-

$\{k_{+1}k_{+5}(k_{+3a}+k_{+3c}[\text{solv.}]).I_o[RH_2][F]\} / \{(k_{-1}+(k_{+2a}+k_{+3c})[\text{solv.}]$
$+k_{+3a})(k_{+4a}+(k_{+4b}+k_{+5})[RH_2] + k_{+4c}[\text{solv}] + k_{+4e}[F^t] + k_{+4f}[O_2])\}$

This is the expression given in chapter 5. The expression in chapter 4 for the anaerobic situation is similar, only with $[O_2]$ = zero, the +4f step is omitted.

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The Chemistry of Flavines and Flavoproteins

PHOTOREDUCTION OF FLAVINES BY AMINO ACIDS

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1. Flavines are photoreduced through their triplet states by amines and amino acids (e.g. EDTA and DL-phenylglycine). The anaerobic photoreduction of FMN and several other flavines with DL-phenylglycine was analysed in terms of a detailed kinetic scheme. 2. The reaction produces equimolar amounts of benzaldehyde, carbon dioxide and reduced flavine. 3. The sensitivity of the rates to substituents in the DL-phenylglycine can be described by a Hammett ρ-value of $-1{\cdot}1$. 4. Phenylacetic acid behaves differently from DL-phenylglycine or benzylamine towards a series of flavines. 5. The photoreductions are quenched by several aromatic compounds. From the effects of light-intensity and temperature, and by comparison with potassium iodide quenching, it is concluded that inhibition by the aromatic compounds is not simply a collisional process. 6. FAD reacts more slowly than FMN both in the photoreduction and in dark reduction by NADH. Urea and dimethyl sulphoxide decrease the intramolecular interaction in FAD, but they have no effect on the rate of dark reduction of FAD compared with FMN. In contrast, the photoreduction of FAD is quicker in urea.

The potential function of flavines in several photobiological processes has prompted many studies on the photochemistry of riboflavine, FMN and their analogues (for a summary see Penzer & Radda, 1967). Though no direct evidence has been put forward for the action of excited flavines in photosynthesis or vision, flavines participate in photosynthetic electron transport (Whatley, Allen & Arnon, 1959; Shin, Tagawa & Arnon, 1963), and riboflavine occurs in the eye of some fish and mammals (Pirie, 1960). Flavines photosensitize *cis–trans* isomerizations (Posthuma & Berends, 1966; Gordon-Walker & Radda, 1967) and aerobic photo-oxidations of amino acids and proteins (Galston, 1950; Frisell, Chung & MacKenzie, 1959). The flavine nucleus is anaerobically photoreduced by electron donors, e.g. NADH, EDTA (Vernon, 1959; Radda & Calvin, 1964), methionine (Strauss & Nickerson, 1961; Yang, Ku & Pratt, 1967) and the ribityl side chain of riboflavine (Holmström & Oster, 1961). Flash photolysis (Holmström, 1964) and isotope effect studies (Moore, Spence, Raymond & Colson, 1963) implicate hydrogen atom abstraction in these reactions.

Recently the photoreaction of several flavines with the phenylacetate anion was shown to follow a different route (Hemmerich, Massey & Weber, 1967). This reaction leads to the substitution of a benzyl group in the flavine nucleus, suggesting that in addition to its redox function the chemical activity of the isoalloxazine ring may be important in flavoprotein catalysis. It is also possible that the redox function itself may involve group transfer rather than electron or hydrogen transfer (Hemmerich *et al.* 1967). One such suggestion has already been made (Coffey, Neims & Hellerman, 1965), and the lack of electron-spin-resonance signals during the reaction of several flavoproteins would be consistent with this interpretation.

The present paper is primarily concerned with the reaction of amino acids with excited flavines. We chose DL-phenylglycine and its derivatives for our investigations for two reasons. First, because their reaction with several amino acid oxidases has been studied (Neims, DeLuca & Hellerman, 1966; Radda, 1964), and, secondly, because phenylglycines are the amino acids closest in structure to phenylacetic acid. Thus we hoped that by comparing the several mechanisms of interaction of excited flavine with these substrates the biochemical significance of the model studies might be clarified.

MATERIALS

FMN was purchased from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany), FAD from Sigma Chemical Co. (St Louis, Mo., U.S.A.), and riboflavine from British Drug Houses Ltd. (Poole, Dorset). Lumiflavine was

synthesized in this Laboratory by the method of Hemmerich, Fallab & Erlenmeyer (1956). The isoalloxazine derivatives listed in Table 4 were gifts from the following: compounds (I) and (II), Dr P. Hemmerich; compounds (III) and (V), Professor T. Wagner-Jauregg; compound (IV), Dr R. M. Acheson. Substituted DL-phenylglycines were given by Dr J. H. C. Nayler, and 3-(*p*-chlorophenyl)-1,1-dimethylurea and 3-(2,4-dichlorophenyl)-1,1-dimethylurea were gifts from E. I. Du Pont de Nemours and Co. Inc. (Wilmington, Del., U.S.A.). All other reagents were commercial products in their purest form. Dimethyl sulphoxide was further purified by redistillation under vacuum.

METHODS

Reaction rates were measured by following flavine concentration spectrophotometrically at the adsorption maximum (for a Table of the maxima of flavine derivatives see Penzer & Radda, 1967) where reduced flavine does not absorb (Holmström & Oster, 1961). Reactants were mixed in a 1 cm. stoppered cuvette (concentrations are given in the Tables presented below). The solutions were deoxygenated by bubbling N_2 (passed through a solution of vanadous sulphate and H_2SO_4 containing amalgamated zinc to remove traces of O_2) for at least 20 min.

Solutions were illuminated by a 500 w tungsten lamp with a potentiometer to regulate light-intensity. A filter (OY 18; Chance Bros., Birmingham) was used to cut out wavelengths below 400 mμ, and a water bath absorbed the infrared. Light-intensity was quantitatively varied by neutral-density filters. The cuvette was supported in a thermostatically controlled water bath during illumination. Reaction rates were reproducible to $\pm 5\%$.

Absorption spectra were recorded on a Cary 14 recording spectrophotometer, and fluorescence measurements were made on a Zeiss PMQII spectrofluorimeter.

pH measurements were made with an Electronic Instruments Ltd. type 23A pH-meter. pK_b values were measured by titration, with a Radiometer Titrator type TTT 1b. The amino acid was dissolved in 0·1 M-NaCl and titrated with 2 N-NaOH solution made up in CO_2-free water.

Benzaldehyde was estimated by vapour-phase chromatography. The instrument was a Perkin–Elmer F11, with a 2 m. column packed with diethylene glycol adipate, pentaerythritol and phosphoric acid in the proportions 25:2:73 (by wt.). The method was as follows. A 40 ml. sample of the reaction mixture containing lumiflavine (0·10–0·15 mM in different runs) and DL-phenylglycine (10 mM) was adjusted to pH 8·3 with Na_2HPO_4 and placed in a vessel thermostatically controlled at 25°. This was thoroughly deoxygenated by bubbling N_2 for 2 hr. with stirring. The anaerobic mixture was illuminated until the extinction showed that lumiflavine concentration had fallen below 0·01 mM (typically 2 hr.). Then 1 μl. of hexan-1-ol was added to 25 ml. of this solution to act as an internal standard for the vapour-phase chromatography. The aqueous solution was extracted with 10 ml. of ether, which was subsequently dried with anhydrous Na_2SO_4. Samples (2 μl.) of the ethereal solution were injected into the chromatograph with a Hamilton micro-syringe. With N_2 carrier gas at 15 lb.in.$^{-2}$ pressure and a column temperature of 100°, the retention times were: hexan-1-ol, 4·1 min.; benzaldehyde, 13·0 min. The detector response was calibrated by standard mixtures of benzaldehyde and hexan-1-ol in ether. The extraction procedure was standardized with known aqueous solutions of benzaldehyde and hexan-1-ol at pH 8. Area ratios for a given solution and extraction were consistent to $\pm 5\%$, and for separately made and extracted solutions to $\pm 10\%$. Unilluminated blanks gave no benzaldehyde.

For estimation of benzoic acid and phenylglyoxylic acid the method was modified. The reaction mixture was acidified to pH 2 with conc. HCl before extraction. The same column was used at 190°. The retention times were: benzaldehyde, 1·6 min.; benzoic acid, 12·2 min.; phenylacetic acid, 19·5 min.; phenylglyoxylic acid, 36·0 min. The ethereal extracts were evaporated to a very small volume before injection to give maximum response. Blanks showed that the method should have detected 0·01 mM-acid in the aqueous solution.

Phenylglyoxylic acid was also estimated spectrophotometrically by the reaction with *o*-phenylenediamine, by the method of Bruice & Topping (1963).

CO_2 was determined by acidifying an anaerobic reaction mixture (identical with that described for the benzaldehyde experiments) with conc. HCl. The CO_2 released was then blown by a stream of N_2 into a solution of 0·5 mM-$Ba(OH)_2$ for 1 hr. Both these operations were performed without allowing the reaction mixture to contact the atmosphere. The $Ba(OH)_2$ solution was now titrated with standard HCl in the automatic titrator, and the titre was subtracted from that obtained by using an identical procedure with an unilluminated reaction mixture. The quantities of CO_2 involved were insufficient to precipitate $BaCO_3$, so the reaction occurring was:

$$CO_2 + OH^- \rightleftharpoons HCO_3^-$$

Thus the change in concentration of OH^- ions is equivalent to the amount of CO_2 absorbed.

This technique gave results accurate to $\pm 15\%$. The inaccuracies were a result of the extreme sensitivity of the method to absorption of atmospheric CO_2 and the irreproducibility of the bubbling procedure, which was 70% efficient ($\pm 10\%$) in runs with solutions of known CO_2 concentration.

RESULTS AND DISCUSSION

Kinetic studies. It is convenient to present our results in terms of the scheme outlined below, which is more detailed than that given by Radda & Calvin (1964):

$$\simeq + h\nu \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} F^\star \qquad (1)$$

The rate of production of $F^\star$ is proportional to the amount of light adsorbed according to the Beer–Lambert law. This may be simplified to:

$$I_0 - I = I_0(1 - e^{\epsilon[F]l})$$

$$\simeq I_0 \epsilon[F]l \text{ when } \epsilon[F]l < 0{\cdot}25$$

$$F^\star + \text{solv.} \xrightarrow{k_{+2a}} F + \text{solv.} \qquad (2a)$$

$$F^\star + RH_2 \xrightarrow{k_{+2b}} F + RH_2 \qquad (2b)$$

$$F^{\star} \xrightarrow{k_{+3a}} F^{t} \quad (3a)$$

$$F^{\star} + RH_2 \xrightarrow{k_{+3b}} F^{t} + RH_2 \quad (3b)$$

$$F^{\star} + \text{solv.} \xrightarrow{k_{+3c}} F^{t} + \text{solv.} \quad (3c)$$

$$F^{\star} + \dot{F}H \xrightarrow{k_{+3d}} F^{t} + \dot{F}H \quad (3d)$$

$$F^{t} \xrightarrow{k_{+4a}} F \quad (4a)$$

$$F^{t} + RH_2 \xrightarrow{k_{+4b}} F + RH_2 \quad (4b)$$

$$F^{t} + \text{solv.} \xrightarrow{k_{+4c}} F + \text{solv.} \quad (4c)$$

$$F^{t} + \dot{F}H \xrightarrow{k_{+4d}} F + \dot{F}H \quad (4d)$$

$$2F^{t} \xrightarrow{k_{+4e}} F + F^{\star} \quad (4e)$$

$$F^{t} + RH_2 \xrightarrow{k_{+5}} \dot{F}H + \dot{R}H \quad (5)$$

$$\text{or } F^{t} + RCO_2H \xrightarrow{k'_{+5}} FH + CO_2 \quad (5')$$
$$\qquad\qquad\qquad\qquad\quad |$$
$$\qquad\qquad\qquad\qquad\quad R$$

$$\dot{F}H + \dot{R}H \underset{k_{-6}}{\overset{k_{+6}}{\rightleftharpoons}} FH_2 + R \quad (6)$$

$$2\dot{F}H \underset{k_{-7}}{\overset{k_{+7}}{\rightleftharpoons}} F + FH_2 \quad (7)$$

$$\dot{F}H + RH_2 \underset{k_{-8}}{\overset{k_{+8}}{\rightleftharpoons}} FH_2 + \dot{R}H \quad (8)$$

F, $\dot{F}H$ and FH_2 are oxidized, half-reduced and fully reduced flavine respectively, and RH_2, $\dot{R}H$ and R are reduced, half oxidized and fully oxidized reducing agent. $F^{\star}$ is excited singlet flavine and F^{t} the flavine triplet. ϵ is the extinction coefficient of the flavine, l is the cell path length and I_0 is the incident light-intensity. This scheme supposes that the reactive species in photoreduction is the flavine triplet. This has already been demonstrated for photobleaching (Radda & Calvin, 1964), and was confirmed by observing that paramagnetic ions lower the rate of photoreduction (Table 1).

The rates of some of the processes listed are negligible. Neither EDTA nor DL-phenylglycine quenches flavine fluorescence, so k_{2b} and k_{3b} are unimportant. Second-order triplet deactivation (reaction 4e) is not always negligible, because the rate of photoreduction of FMN does not depend linearly on light-intensity (Fig. 1). In contrast, fluorescence intensity is directly proportional to excitation light-intensity. By restricting our considerations to initial reaction rates deactivations by the flavine radical (reactions 3d and 4d) can be neglected.

Reaction (8) has been independently studied by investigating the behaviour of the flavine radical produced in solution by catalytic hydrogenation of FMN. Following the radical concentration spectrophotometrically at 600mμ (Gibson, Massey & Atherton, 1962), we observed no reaction with EDTA in the dark, or on illumination by light with wavelengths greater than 550mμ. Light of wavelengths lower than 500mμ caused rapid reaction. This was caused by excitation of oxidized flavine followed by a change in radical concentration described by the equilibrium:

$$FMN + FMNH_2 \rightleftharpoons 2FMNH^{\cdot}$$

The radical concentration reached its maximum at half reaction. These observations suggest that k_{+8} is negligible.

Considering initial rates only, reactions (2b), (3b), (3d), (6), (7) and (8) are unimportant. Under these

Table 1. *Effect of metal ions on the photoreduction of FMN by EDTA*

All measurements were made at 40° on solutions containing 0·1 M-NaCl, 0·1 M-EDTA, 0·04 mM-FMN and 1 mM-metal chloride. Under these conditions fluorescence quenching by the metal chlorides was less than 1%.

Metal chloride added	Initial rate (fall in E_{447}/min.)	Percentage inhibition $\left[\left(1-\frac{k_{\text{inhib.}}}{k_{\text{uninhib.}}}\right)\times 100\right]$
None	0·078	0
$ZnCl_2$	0·079	0
$MgCl_2$	0·080	0
$NiCl_2$	0·067	14
$MnCl_2$	0·055	29

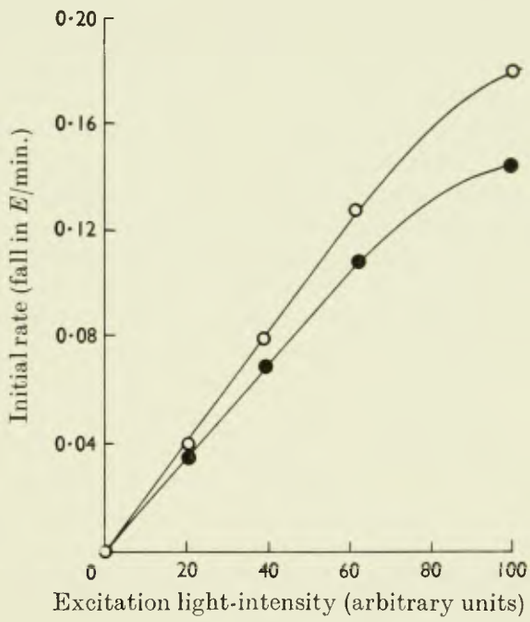


Fig. 1. Effects of temperature and light-intensity on the rate of photoreduction of FMN by EDTA. Reaction mixtures contained 0·1 M-phosphate buffer, pH 7·0, 1 mM-EDTA and 0·04 mM-FMN. ○, 40°; ●, 10°.

conditions, and assuming that flavine concentration is sufficiently low for the rate of excited singlet production to be given by $k_{+1}I_0[F]$, it can be shown that:

$$-\left(\frac{d[F]}{dt}\right)_{init.} = \frac{k_{+1}k_{+5}(k_{+3a}+k_{+3c}[solv.])}{(k_{+2a}[solv.]+k_{+3a}+k_{+3c}[solv.]+k_{-1})} \cdot \frac{I_0[RH_2][F]}{(k_{+4a}+(k_{+4b}+k_{+5})[RH_2]+k_{+4c}[solv.]+k_{+4c}[F^t])}$$

This expression is obtained by applying steady-state approximations to the concentrations of flavine excited states, and is independent of the relative magnitudes of the k_{+6} and k_{+7}, and of whether route (5) or (5′) is followed. It can account for the apparently contradictory reports that the rate of photoreduction is proportional to the concentration of reducing agent (Holmström, 1962) and that the two are independent (Radda & Calvin, 1964). At low reducing agent concentrations the $(k_{+4b}+k_{+5})[RH_2]$ term is unimportant relative to the other quenching terms in the denominator, so linear concentration-dependence should be found. At high concentrations of reducing agent solvent quenching becomes relatively unimportant as reaction with RH_2 reduces triplet lifetime. The anaerobic photoreductions of lumiflavine by EDTA and DL-phenylglycine both show this behaviour (Fig. 2).

Reaction products. The photoreaction of DL-phenylglycine with a variety of flavines is completely reversible in the dark by oxygen, indicating that reduced flavine is a reaction product. The reaction of phenylacetic acid is not reversible in this way. DL-Phenylglycine is oxidized to benzaldehyde and carbon dioxide, which are produced in a mole-for-mole ratio to lumiflavine reduced (Table 2). Neither benzoic acid nor phenylglyoxylic acid was detected. Our analytical methods were capable of detecting a yield of 10% or more phenylglyoxylic acid in spite of its slow photosensitized decomposition.

Effect of reducing agent structure on photoreduction. As benzaldehyde is the major product of reduction by DL-phenylglycine the primary reaction centre involves the α-carbon atom. Thus the effect of *meta* and *para* substituents in the benzene ring should indicate the nature of the rate process. The same substituents also alter the dissociation constants of the amino groups, and hence a knowledge of the pK_b values and of the influence of pH on reaction rate is essential for understanding the substituent effects. The pH-dependences of the reaction rates of a lumiflavine derivative with DL-phenylglycine and phenylacetic acid are recorded in Fig. 3. Whereas the phenylacetic acid curve does not correspond to the titration curves of the reactants, the DL-phenylglycine result is readily explained if only the anion, $Ph\cdot CH(NH_2)\cdot CO_2^-$, but not its zwitterion, reacts. The dissociation constants measured by titration, and the reaction rates at pH 7·0, are recorded in Table 3. The substituent effects may be analysed in terms of the Hammett (1940) equation. The photoreduction

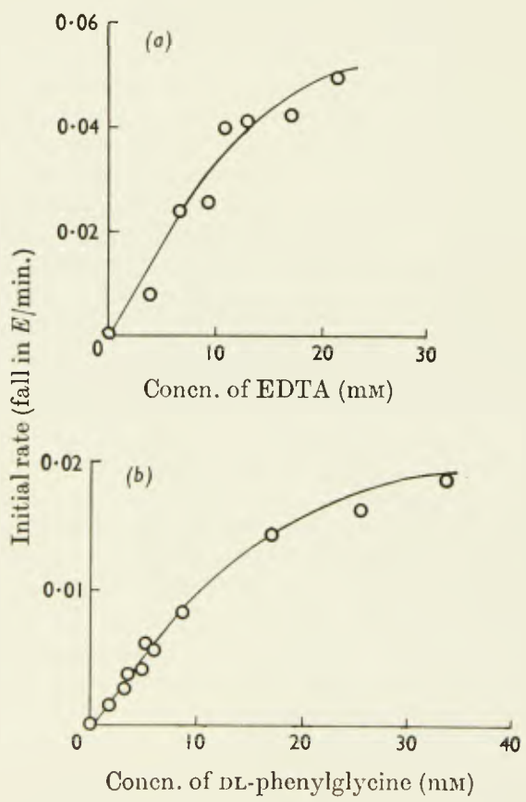


Fig. 2. Effects of concentration of (*a*) EDTA and (*b*) DL-phenylglycine on the rate of photoreduction of flavines. Reaction mixtures contained 0·1 M-phosphate buffer, pH 7·0 (*a*) or pH 8·3 (*b*), and 0·02 mM-FMN (*a*) or 0·035 mM-lumiflavine (*b*). Temperature was 25°.

Table 2. *Reaction products of the photoreduction of lumiflavine by phenylglycine*

All reactions were at 25° and pH 8·2. For details of the analyses see the Methods section.

Amount of lumiflavine reacting (μmoles)	Amount of benzaldehyde formed (μmoles)	Amount of CO_2 formed (μmoles)
4·8	5·2	
4·8	4·8	
4·8	5·6	
5·2		5·0
5·0		4·8
2·8		3·0

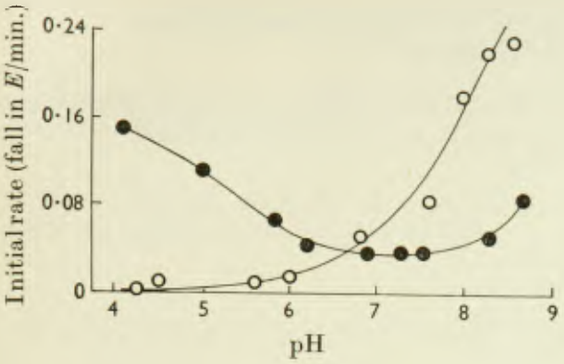


Fig. 3. pH-dependence of the rate of photoreaction of 9-acetoxyethyl-3-methyl-lumiflavine with DL-phenylglycine and with phenylacetic acid. Reaction mixtures contained 0·1 M-phosphate buffer of appropriate pH, 0·025 mM-flavine and 5 mM-acid at 25°. ○, DL-Phenylglycine; ●, phenylacetic acid.

Table 3. *Dissociation constants and corrected rates for the photoreduction of lumiflavine by substituted phenylglycines*

pK_b values were measured in 0·1 M-NaCl at 25° (±0·2°). Reaction rates were measured in solutions containing 0·1 M-phosphate buffer, pH 7·0, 0·015 mM-lumiflavine and 5 mM-phenylglycine at 30°.

Acid	pK_b	Corrected rate [Initial rate × anti-log(pK_b (phenylglycine) − pK_b (substituted acid))]
DL-Phenylglycine	5·09	0·78
DL-*m*-Fluorophenylglycine	5·45	0·36
DL-*m*-Chlorophenylglycine	5·52	0·28
DL-*p*-Methylphenylglycine	5·04	0·86
DL-*p*-Fluorophenylglycine	5·16	0·67
DL-*p*-Methoxyphenylglycine	4·94	2·10

rates give a ρ-value of $-1{\cdot}1$, with a correlation coefficient of 0·94. This relatively small ρ-value is consistent with a radical mechanism. Its negative sign suggests a transition state with an electron-deficient α-carbon atom on the amino acid. This is possible if the flavine triplet behaves like an electrophilic radical (the extraction of benzylic hydrogens by bromine atoms may be an analogous reaction; Kooyman, van Helden & Bickel, 1953). In contrast, the photoreduction of lumiflavine by substituted phenylacetic acids gives a very different pattern (J. A. Taylor & G. K. Radda, unpublished work).

Influence of flavine structure on reaction rates. We have observed that flavines are photoreduced by the unprotonated form of a variety of amines (e.g. diethanolamine, benzylamine, triethylamine). If decarboxylation is in the rate-determining step for the acids (as expected for phenylacetic acid) a comparison of relative photoreaction rates of different flavines with amines, amino acids and phenylacetic acid might distinguish between different mechanisms. Such a comparison is shown in Table 4. It is apparent that, whereas the ratio of reactivity of DL-phenylglycine to benzylamine remains constant within experimental error, the reactivity of phenylacetic acid ranges from the slowest to the fastest of the three substrates with different flavines. This behaviour suggests a dissimilarity of mechanism.

It was also observed that the DL-phenylglycine and benzylamine reactions were invariably rapidly reversed by air, but the phenylacetic acid one was not.

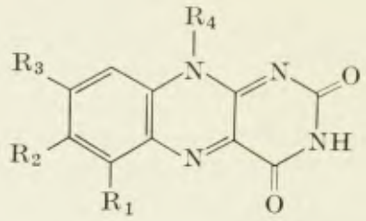
Inhibition of photoreductions. The interaction of some aromatic amines with excited flavines leads to inhibition of reactivity rather than photoreduction (Radda & Calvin, 1963; Homann & Gaffron, 1964; Radda, 1966). The elucidation of these interactions (cf. step 4b in our scheme) is important for an understanding of the mechanism of reduction. Some of the inhibitors are tryptophan, tyrosine, serotonin, 3-(*p*-chlorophenyl)-1,1-dimethylurea and 3-(2,4-dichlorophenyl)-1,1-dimethylurea. The first three inhibit the photoreduction of other dyes, but the substituted ureas do not. This has provoked the suggestion that there is a special type of interaction between the ureas and flavines (Homann & Gaffron, 1963). We have therefore compared the photoreductions of flavines and methylene blue in their sensitivity towards inhibitors.

The inhibition must involve quenching of the flavine triplet, because, though some of the inhibitors form ground-state complexes with flavines and are also fluorescence quenchers (Penzer & Radda, 1967), these effects are too small to explain the magnitude of the effect on reactivity [e.g. 3-(*p*-chlorophenyl)-1,1-dimethylurea quenches fluorescence by less than 1%; for the others see Radda (1966)]. The triplet may be quenched by either collision or complex-formation with the inhibitor (Radda, 1966). These are potentially distinguishable on the basis of the temperature-dependence of reaction rate. The effects of temperature on the inhibited and uninhibited photobleaching and photoreduction of FMN by EDTA are summarized in Table 5. The uninhibited reactions both have small positive temperature coefficients, in spite of the increase in collisional quenching by the solvent. This means that there is a small activation energy for the reaction. Photoreduction of FMN by NADH has a small negative temperature coefficient (Radda & Calvin, 1964), and the increase in coefficient in the order NADH, EDTA and ribityl side chain corresponds to decreasing reducing power.

Potassium iodide is generally assumed to be a

Table 4. *Relative rates of photoreduction of flavine derivatives by phenylglycine, phenylacetic acid and benzylamine*

Flavine concentrations were all approx. 0·02mM. Solutions contained 5mM-reducing agent in 0·1M-phosphate buffer, pH8·00 (for DL-phenylglycine), pH7·89 (for phenylacetic acid) or pH7·93 (for benzylamine).



Flavine				Rate of reduction by DL-phenylglycine / Rate of reduction by phenylacetic acid	Rate of reduction by DL-phenylglycine / Rate of reduction by benzylamine
R_1	R_1	R_3	R_4		
H	Me	Me	Ribityl phosphate (FMN)	8·25	5·5
H	Me	Me	Me (lumiflavine)	7·0	5·5
H	Me	Me	Me (N^3-Me) (3-methyl-lumiflavine, compound I)	8·0	6·0
Me	Me	H	Ribityl (isoriboflavine, compound II)	10·0	5·3
H	Cl	MeO	$[CH_2]_2$–N(CH_2–CH_2)$_2$ (compound III)	2·7	4·0
H	Cl	H	$[CH_2]_3$–NEt_2 (compound IV)	3·5	6·4
H	H	H	$[CH_2]_2$–NMe_2 (compound V)	0·4	5·0

collisional quencher, yet its inhibition of photobleaching falls with temperature. Triplet lifetime is decreased as a result of increased reactivity at the higher temperature, so that changes in potassium iodide quenching are not dominated by variations in collision frequency alone. For the other inhibitors the $k_{\text{inhib.}}/k_{\text{uninhib.}}$ ratio values at the two temperatures are greater than or equal to [for 3-(*p*-chlorophenyl)-1,1-dimethylurea] that found for potassium iodide (Table 5). This suggests that inhibition by serotonin and tryptophan has at least a component from complex-formation.

A given concentration of inhibitor affects photoreduction less than photobleaching. The temperature effects are smaller too. These can be explained as follows. The relative contribution of an inhibition term to the denominator of the rate equation is less when $k_{+5}[RH_2]$ is large. The rate constants for those processes that involve an activation energy increase more with temperature than for simple collision processes. Now, the activation energy for photobleaching is higher than for photoreduction, so the increase of the k_{+5} term relative to the quenching term (k_{+4b}) is more important for photobleaching.

The variation of inhibition with light-intensity (Fig. 1 shows this dependence for uninhibited reaction) depends on second-order triplet deactivation (k_{+4e}). This is greater for uninhibited reactions at a particular light-intensity because of the higher $[F^t]$. It is not possible to detect significant changes of inhibition over the light-intensity range covered, because $[F^t]$ is never large. At higher intensities the uninhibited reaction rate is too fast for accurate measurement, but approximate measurements indicate that inhibition falls under intense illumination, as expected (Table 5).

Neither DL-phenylglycine nor its derivatives inhibit the photoreduction of FMN by EDTA at pH7·0 (where the contribution due to reduction by the DL-phenylglycine is negligible), showing that for phenylglycines k_{+4b} is very small.

Photoreduction of methylene blue by EDTA has qualitatively similar temperature- and light-intensity-dependences to the FMN reaction (Table 6). The temperature coefficient, however, is higher ($k_{41°}/k_{15°}$ ratio 2·4) and inhibition by a given concentration of inhibitor is lower. Tryptophan and serotonin inhibit, but 3-(*p*-chlorophenyl)-1,1-dimethylurea and 3-(2,4-dichlorophenyl)-1,1-

Table 5. *Temperature and light-intensity effects on the rates of inhibited and uninhibited photobleaching and photoreduction of FMN*

Photobleaching: unless stated to the contrary all solutions contained 0·1 M-phosphate buffer, pH 7·0, 0·4 mM-inhibitor and 0·06 mM-FMN:

$$k_{\text{uninhib.}} \text{ at } 38°/k_{\text{uninhib.}} \text{ at } 17° = 1{\cdot}44$$

Photoreduction: unless stated to the contrary all solutions contained 0·1 M-phosphate buffer, pH 7·0, 1 mM-EDTA, 0·5 mM-inhibitor and 0·04 mM-FMN:

$$k_{\text{uninhib.}} \text{ at } 40°/k_{\text{uninhib.}} \text{ at } 10° = 1{\cdot}20$$

Abbreviation: CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea.

Photobleaching

Inhibitor	Temp.	$\frac{k_{\text{inhib.}}}{k_{\text{uninhib.}}}$	$\frac{k_{\text{inhib.}} \text{ at } 38°}{k_{\text{inhib.}} \text{ at } 17°}$
CMU	17°	0·18	1·33
CMU	38	0·24	
Tryptophan	17	0·04	2·00
Tryptophan	38	0·08	
Serotonin (0·1 mM)	17	0·04*	~14
Serotonin (0·1 mM)	43	0·57*	
KI (0·01 mM)	17	0·40	1·30
KI (0·01 mM)	38	0·52	

Photoreduction

Inhibitor	Temp.	Light-intensity (relative units)	$\frac{k_{\text{inhib.}}}{k_{\text{uninhib.}}}$	$\frac{k_{\text{inhib.}} \text{ at } 40°}{k_{\text{inhib.}} \text{ at } 10°}$
CMU	40°	100	0·35	1·00
CMU	10	100	0·35	
CMU	40	60	0·39	1·05
CMU	10	60	0·37	
CMU	40	40	0·40	1·08
CMU	10	40	0·37	
CMU	40	20	0·41	1·11
CMU	10	20	0·37	
				Average 1·06
Tryptophan	40	100	0·32	0·89
Tryptophan	10	100	0·36	
Tryptophan	40	20	0·32	0·86
Tryptophan	10	20	0·37	
				Average 0·87 (5)
Serotonin (0·1 mM)	40	100	0·29	>1·32
Serotonin (0·1 mM)	20	100	0·22	
CMU (FMN conc. 0·08 mM)	20	>200	0·55	
Tryptophan (FMN conc. 0·08 mM)	20	>200	0·40	

* Taken from Radda (1966).

dimethylurea do not. Inhibition by tryptophan increases slightly as the temperature is raised.

These differences between the indole and urea derivatives may be a result of the ability of the former to form stronger complexes with the dyes. The methylene blue complexes are generally weaker than flavine ones, so that 3-(*p*-chlorophenyl)-1,1-dimethylurea may just fall outside the range of significant interaction.

Effects of urea on inhibitions. Both substituted ureas (Hansch & Deutsch, 1966) and flavines (Weber, 1966) participate in hydrophobic interactions in their ground states. This is shown by the effect of urea and non-aqueous solvents on flavine–aromatic molecule complexes (Weber, 1966; Gascoigne & Radda, 1965). If similar interactions occur with flavine triplets they could explain the photoinhibitions. Table 7 shows that urea slightly inhibits photobleaching, but it accelerates the reaction in the presence of other inhibitors. This

Table 6. *Temperature and light-intensity effects on the rates of inhibited and uninhibited photoreduction of methylene blue by EDTA*

All solutions contained 0·1 M-phosphate buffer, pH 7·0, 0·5 mM-inhibitor and 0·02 mM-methylene blue:

$k_{\text{uninhib.}}$ at 41°/$k_{\text{uninhib.}}$ at 15° = 2·4

Abbreviations: CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea; DCMU, 3-(2,4-dichlorophenyl)-1,1-dimethylurea.

Inhibitor	Temp.	Light-intensity (relative units)	$\frac{k_{\text{inhib.}}}{k_{\text{uninhib.}}}$	$\frac{\frac{k_{\text{inhib.}}}{k_{\text{uninhib.}}} \text{ at } 41°}{\frac{k_{\text{inhib.}}}{k_{\text{uninhib.}}} \text{ at } 15°}$
Tryptophan	41°	100	0·68	0·79
Tryptophan	15	100	0·86	
Tryptophan	41	60	0·73	0·88
Tryptophan	15	60	0·83	
Tryptophan	41	40	0·72	0·84
Tryptophan	15	40	0·86	
Tryptophan	41	20	0·73	0·97
Tryptophan	15	20	0·75	
CMU	41	100	1·0	
CMU	15	100	1·0	
DCMU	20	100	1·0	
Serotonin	20	100	0·05	
Tryptophan	20	>200	0·95–1·0	

Table 7. *Effects of* 6·5 M-*urea on the rates of inhibited and uninhibited photobleaching, photoreduction and fluorescence of FMN*

Solutions contained 0·1 M-phosphate buffer, pH 7·0, and 0·015 mM-FMN. Abbreviation: CMU, 3-(*p*-chlorophenyl)-1,1-dimethylurea.

Reactants	$\frac{\text{Reaction rate in absence of urea}}{\text{Reaction rate of FMN alone}}$	$\frac{\text{Reaction rate in 6·5 M-urea}}{\text{Reaction rate in absence of urea}}$	$\frac{\text{Fluorescence intensity}}{\text{Fluorescence intensity of FMN alone}}$	$\frac{\text{Fluorescence intensity in 6·5 M-urea}}{\text{Fluorescence intensity in absence of urea}}$
FMN	1·00	0·84	1·00	0·98
FMN+KI (0·01 mM)	0·34	1·16	—	—
FMN+KI (20 mM)	—	—	0·67	1·07
FMN+tryptophan (0·5 mM)	0·06	2·00	—	—
FMN+tryptophan (2 mM)	—	—	0·89	1·07
FMN+CMU (0·5 mM)	0·18	1·52	—	—
FMN+serotonin (2 mM)	—	—	0·59	1·41
FMN+EDTA (1 mM)	—	1·01	—	—

Table 8. *Effect of urea on the rates of photobleaching and photoreduction of FAD by EDTA*

All solutions contained 0·1 M-phosphate buffer, pH 7·0, 0·025 mM-FAD and 1 mM-EDTA.

Reactants	Conc. of urea (M)	$\frac{\text{Reaction rate in urea}}{\text{Reaction rate in absence of urea}}$
FAD	6·5	1·00
FAD+EDTA	1·6	1·04
FAD+EDTA	3·2	1·15
FAD+EDTA	4·9	1·24
FAD+EDTA	6·5	1·30

suggests that complex-formation, partly stabilized by hydrophobic interaction, is a likely contributor to the inhibition (cf. Radda, 1966). The small acceleration that urea induces in the potassium iodide-inhibited reaction is probably caused by a change in viscosity. This is also reflected in a slight fall in fluorescence quenching of FMN by potassium iodide in urea. Urea removes the ability of tryptophan to quench flavine fluorescence, and decreases quenching by serotonin by 60% (Table 7). The negligible change in the rate of photoreduction of FMN by EDTA in urea solutions corresponds to

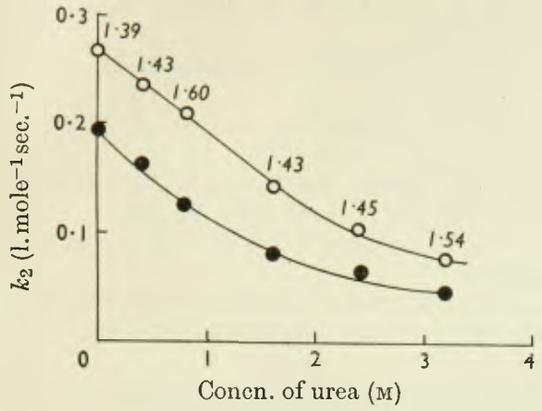


Fig. 4. Effect of urea on the second-order rate constant (k_2) for dark reduction of FAD and FMN by NADH. Reaction mixtures contained 0·02M-phosphate buffer, 0·06mM-FAD or -FMN and 2mM-NADH at 25°. ○, FMN; ●, FAD; *italicized* numbers give k_2 (FMN)/k_2 (FAD).

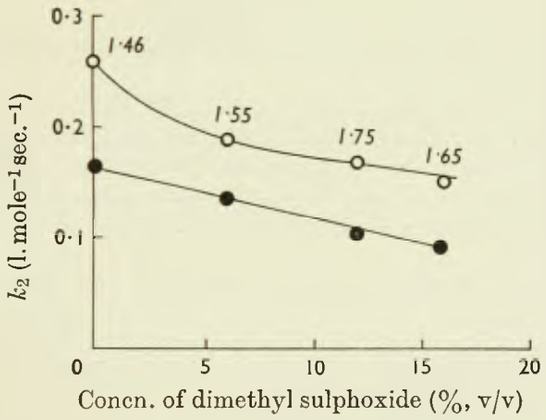


Fig. 5. Effect of dimethyl sulphoxide on the second-order rate constant (k_2) for dark reduction of FAD and FMN by NADH. Reaction mixtures contained 0·02M-phosphate buffer, 0·06mM-FAD or -FMN and 2mM-NADH at 25°. ○, FMN; ●, FAD; *italicized* numbers give k_2 (FMN)/k_2 (FAD).

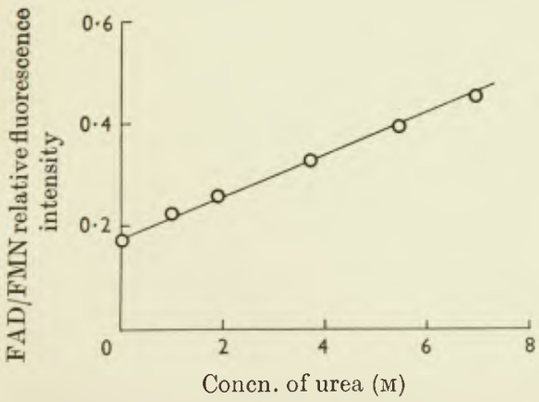


Fig. 6. Dependence of the FAD/FMN relative fluorescence intensity on urea concentration. Flavine concentrations were 0·015mM.

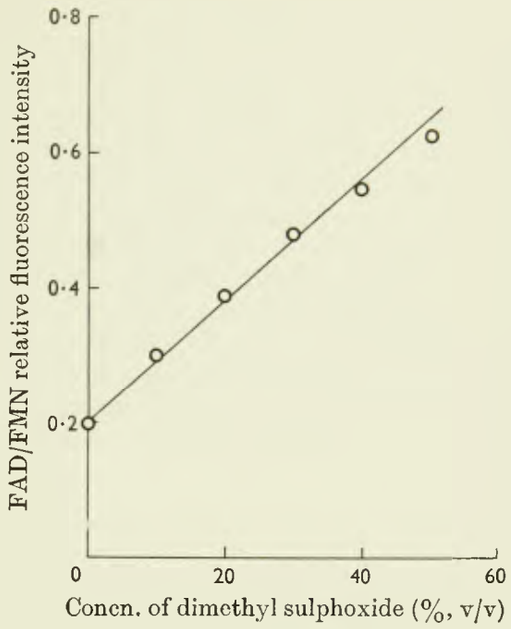


Fig. 7. Dependence of the FAD/FMN relative fluorescence intensity on dimethyl sulphoxide concentration. Flavine concentrations were 0·015mM.

the lower inhibitions generally observed for photoreduction in accordance with our kinetic scheme.

The low rates of photoreduction and photobleaching of FAD compared with those of FMN (Radda, 1966; McCormick, Koster & Veeger, 1967) have been attributed to the formation of an intramolecular complex in the former (Weber, 1950). When urea is added there is no change in the rate of photobleaching (Table 8), though there is a large increase in fluorescence, indicating unfolding (Gascoigne & Radda, 1965). With the inhibitory effect of urea on the photobleaching of FMN being taken into account, unfolding must result in a faster rate. The effect is more clearly shown for photoreduction by EDTA, where inhibition by urea is less important. Even so, in 6·5M-urea the increase in reactivity (1·30-fold) is less than the increase in fluorescence (2·13-fold).

The dark reactions of FMN and FAD with NADH (cf. Radda & Calvin, 1964) are not significantly altered on unfolding. This has been shown by comparing the rates of reaction of FMN and FAD in solvents containing urea or dimethyl sulphoxide (Figs. 4 and 5). The relative fluorescence intensities change markedly under these conditions (Figs. 6 and 7). This shows that in the dark reaction steric hindrance is about the same in the folded and unfolded forms. We would expect this to apply equally to photoreactions, so we attribute the increase in rate here to decreased triplet quenching (cf. the effect of urea on intermolecular inhibitions).

In conclusion, we consider that all experimental results on the photoreduction of flavines by amino acids are most readily understood in terms of the steps presented in our scheme. It is likely that the first step is a one-electron process between the amino acid and the excited flavine. This is probably not the case for the reaction between excited flavines and phenylacetic acid, which shows several marked differences from the other photoreductions: (1) it is not completely reversed by air in the dark when the others are; (2) the pH-dependence of reaction rate is not simply explicable in terms of the pK_a values of the reactants; (3) the order of photoreactivity with various flavine derivatives is widely different.

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