KINETIC STUDIES OF REDOX AND RELATED REACTIONS OF SOME NEW ISOALLOXAZINES

A thesis
submitted in partial fulfilment
of the requirements for the Degree
of
Doctor of Philosophy in Chemistry
at the
University of Waikato
by

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University of Waikato
1976

Abstract

This thesis describes the synthesis of three new isoalloxazines with special structural characteristics which we hoped would, and in fact did, modify their chemical reactivity so as to improve our understanding of mechanisms of flavin reactions generally: kinetic studies of their reactions with a dithiol, reduced nicotinamide and sulphite were made.

A new general synthesis is described for the preparation of o-nitro-(N-alkylamino)aromatic compounds via nucleophilic displacement by alkylamine of a methoxy group ortho to a nitro group. Two new compounds, the isomers 1-(2'-hydroxyethylamino)-2-nitronaphthalene and 2-(2'-hydroxyethylamino)-1-nitronaphthalene, were prepared by this method.

These compounds were used to synthesize two isoalloxazines which each have an additional benzene ring fused either at the 6,7-bond of the isoalloxazine nucleus (7-(2'-hydroxyethyl)-10-methylnaphtho[1,2-g]pteridine-9,11(7H,10H)-dione; isoalloxazine A) or at the 8,9-bond (12-(2'-hydroxyethyl)-9-methylnaphtho[2,1-g]pteridine-8,10(9H,12H)-dione; isoalloxazine B). The third new isoalloxazine (10-(2'-hydroxyethyl)-3-methylbenzo[g]-pteridine-2,4(3H,10H)-dione; isoalloxazine C) was prepared by a conventional route.

Kinetic studies, the first recorded for isoalloxazines of the type of A and B, were undertaken for reactions with 1,3-dithio-2-propanol, with NADH and with sulphite.

In the case of the dithiopropanol reaction, a linear free-energy relationship was observed between the logarithm of the second order rate constant and the polarographic

half-wave potential, $E_{\frac{1}{2}}$ (slope 26 ± 1 V^{-1}). The conclusion drawn from this is that, if the redox reaction occurs through an addition-elimination sequence, the 5-, 6- and 8-positions are not essential centres for thiol oxidation.

In the case of the reaction with NADH, direct evidence was obtained for the formation of a kinetically important complex between the isoalloxazine and NADH from the observation of saturation kinetics. This is the first reported instance of the observation of such direct evidence for complex formation with NADH. The rate constants for the three isoalloxazines follow the reverse order expected from the $E_{\frac{1}{2}}$ values and from the rates of reaction with DTP. The equilibrium constants (K_e) for the complexing of tryptophan with the isoalloxazines were measured by fluorescence quenching. A linear free-energy relationship was not observed between the logarithm of K_e and the logarithm of the rate constant for NADH oxidation. It was concluded that the unexpectedly high rate of reaction of A relative to that of B was due to the formation by A of a complex with NADH closer to the hydride acceptor site, the 5-position, than is the case for isoalloxazine B; and that the productivity of the complex therefore is markedly determined by the orientation of the NADH molecule with respect to the isoalloxazine within the complex.

The anaerobic nucleophilic addition reaction of sulphite with isoalloxazines A and C is a two step process of the form $X \rightleftharpoons Y \rightarrow Z$, whereas the reaction with B is a single equilibrium step. The reduced isoalloxazine products from A and C are oxidized by molecular oxygen to new isoalloxazines but that from B is not. The new isoalloxazine

from C reacts further with sulphite to yield a reduced species different from that of the anaerobic reaction, while the product from A does not react further. These observations provide support for the mechanism previously suggested in the literature for the reaction of sulphite with isoalloxazines. A small rate depression was observed for A relative to B and C in a plot of logarithm of rate constant against $E_{\frac{1}{2}}$. This is interpreted as being due to steric crowding at the 5-position caused by the α-hydrogen on the naphthalene moiety, an effect not observed in the dithiol reaction which more likely involves nucleophilic addition at the 4a- rather than the 5-position.

Acknowledgements

The author gratefully thanks the following people for their assistance and discussion during the period of the D.Phil. study.

In particular, Dr L. Main for suggesting the topic and his helpful discussion and advice throughout the course of the work.

Messrs R. Page and B. Cadman for their prompt servicing of the instruments used in the work.

Mr P. Holland and Miss O. Johnson, of Ruakura Animal Research Centre, for running the mass spectra.

Professor A.D. Campbell and his associates, of the University of Otago, New Zealand, for microanalysis of the compounds.

My fellow M.Sc. and D.Phil. colleagues for their comments, often facetious.

My parents for their unremitting patience.

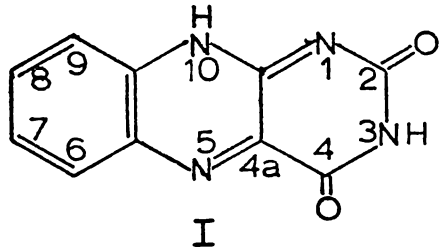
The University of Waikato for financial assistance.

Contents

1. INTRODUCTION

The term 'biological oxidation' covers a large area of reactions which occur in the metabolism of living organisms. This work is concerned with the smaller, but still large field of flavin-mediated redox reactions. The flavin coenzymes involved in these processes are riboflavin (II), flavin mononucleotide (FMN, III) and flavin adenine dinucleotide (FAD, IV).

The basic skeleton of flavins is an isoalloxazine nucleus (I), which is systematically named a benzo[g]pteridine-2,4(3H,10H)-dione. However, there must be a substituent other than hydrogen at the N(10) position or otherwise the molecule exists as the corresponding isomeric alloxazine. The numbering system adopted for this thesis is that of Chemical Abstracts. Two names are in common use for compounds with skeleton I. These are flavin and isoalloxazine.

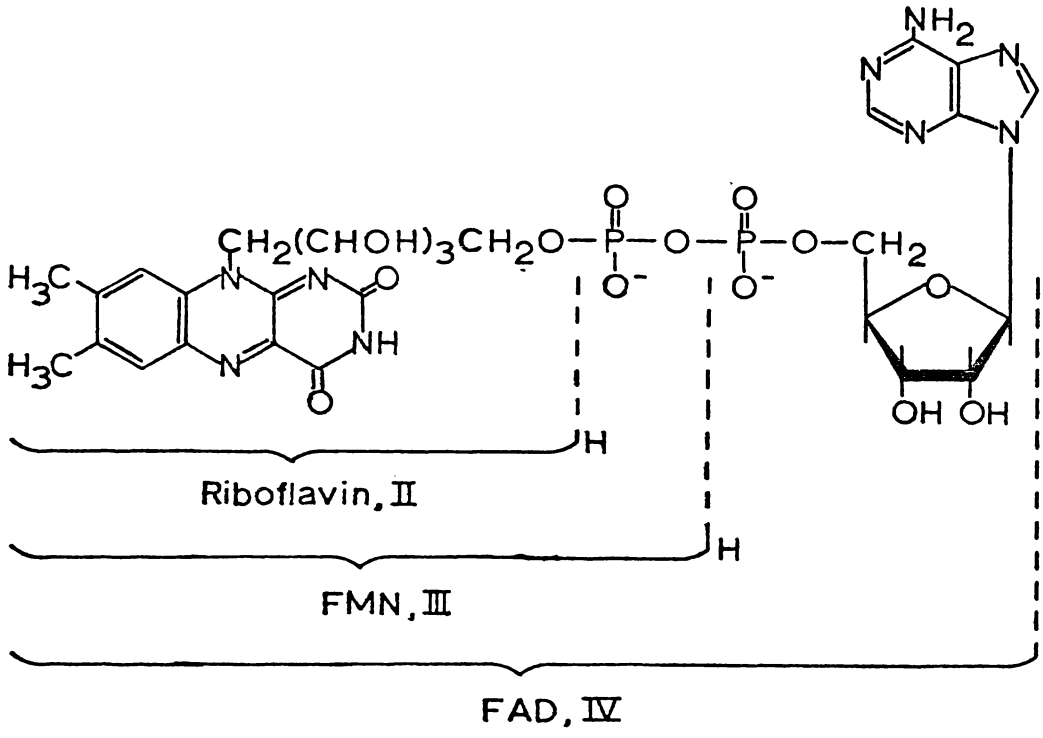


I

The former is usually retained to describe naturally occurring 7,8-dimethylsubstituted isoalloxazines while the latter term is used when the substitution pattern in the benzene ring is other than 7,8-dimethyl. These terms are used throughout this thesis as defined above.

1-1 HISTORICAL ASPECTS[1]

The initial detection of riboflavin or a derivative of riboflavin as an impure resinous pigment was made by Blyth in 1879. Blyth called this pigment lactochrome.



Further research into naturally occurring flavins did not begin until half a century later, when several impure yellowish pigments were isolated from several sources: lactochrome from whey by Bleyer and Kallmann; lyochrome from animal tissues by Ellinger and Koschara; cytoflav from heart muscle by Banga and Szent-Gyorgyi; ovoflavin from egg white by Wagner-Jauregg and coworkers. The latter pigment was the first pure crystalline product and this achievement made it possible to determine the biological effect of riboflavin. It was shown, in fact, to be a nutritional factor for mammals and was later called vitamin B_2. Subsequently, lactoflavin was isolated in sufficient quantities for structural determinations to be carried out. These showed the molecule to consist of a ribitol moiety and an isoalloxazine nucleus. The structure was confirmed by synthesis in 1935 by Karrer and Kuhn and their associates. The compound was now called riboflavin.

Riboflavin (II) is found universally in plant and animal tissues, either free or bound in two coenzymes,

riboflavin mononucleotide (FMN, III) and riboflavin adenine dinucleotide (FAD, IV). 'Old yellow enzyme' which catalyses the reoxidation of the reduced coenzyme nicotinamide adenine dinucleotide phosphate (NADPH) was found to give two fractions when treated with methanol: a colourless protein and a protein-free pigment. Kuhn established that the latter pigment was related structurally to riboflavin and Theorell demonstrated that in fact it was the riboflavin-5'-phosphate ester, FMN. The second prosthetic group containing riboflavin, FAD, was discovered in 1938 by Warburg and Christian in D-amino acid oxidase. The structure of this second cofactor was confirmed by synthesis in 1954 by Todd and his coworkers.

Recently many derivatives of riboflavin have been found in which the 8-methyl group is modified. Examples of 8-substituents on the isoalloxazine nucleus include $N\text{-}CH_2\text{-}$[2], $\text{-}S\text{-}CH_2\text{-}$[3], $\text{-}OH$[4], and $\text{-}N(CH_3)_2$[5].

1-2 FLAVOENZYMES

The two flavin prosthetic groups FMN and FAD vary in their binding to the apoprotein from one enzyme to another. They generally act as prosthetic groups rather than freely dissociating coenzymes. The binding position on the flavin is not known with any certainty in many cases, but several possible sites are:

(a) ionic interactions between cationic groups on the protein and the phosphate residues of the coenzyme;

(b) hydrogen bonding to the nitrogen and oxygen functions of the isoalloxazine nucleus;

(c) hydrogen and apolar bonding to the ribityl side chain; and

(d) through covalent bonds to the 8-α position on the isoalloxazine ring system.

Flavoproteins occupy an intermediate position between two other types of dehydrogenases, pyridinoproteins and cuproproteins. They catalyze the oxidation of substrates of intermediate redox potential, such as amines to aldehydes, carbonyl compounds to α,β-unsaturated carbonyl compounds, nonheme iron and NAD(P)H. Their reduced forms are oxidized by interaction either with oxygen directly or with a number of alternative acceptors in the respiratory chain.

Flavoproteins are mainly concerned with three important areas of oxidative metabolism; these are as follows:

(a) the oxygen-linked dehydrogenation of substrates (e.g. amino acids);

(b) the cytochrome-linked dehydrogenation of the initial members of the particle-bound respiratory chain utilizing substrates such as NADH, succinate, α-glycerophosphate, choline, sarcosine and D- and L-lactate;

(c) the NAD(P)-linked dehydrogenation of certain low potential substrates, e.g. dihydrolipoate, dihydroorotate and reduced ferredoxin.

The latter two groups sometimes have additional cofactors associated with the catalysis which are usually metal ions.

The flavoenzymes are involved in many facets of the metabolism of living organisms. They have considerable importance in the respiratory system; the flavin moiety seems to be the collecting point for electrons from differ-

ent substrates[6a]. Reactions of flavins may, in fact, be rate limiting in mitachondrial respiration, since all other chain components are present in excess (e.g. nicotinamides:flavins:ubiquinone:cytochrome a ≒ 100:1:100:10)[6b]. In the photosynthetic processes, flavins also have a major role in the cyclic and non-cyclic electron flow and energy conservation. In both microsomal and mitochondrial oxygenation flavin seems to control not only cytochrome reduction but also, independently, oxygen-activation.

Classification of the flavoproteins is best made from the viewpoint of the substrate utilized; a classification by this method is given below:

(a) C-H dehydrogenases: this is the major grouping in this classification. The enzymes are involved with deaminations of amino acids and amines, decarboxylations, oxidations of alcohols and aldehydes and the reoxidation of reduced nicotinamide coenzymes (equation 1.1).

$$\text{NADH} \xrightarrow{\text{Fl}} \text{NAD}^+ \qquad (1.1a)$$

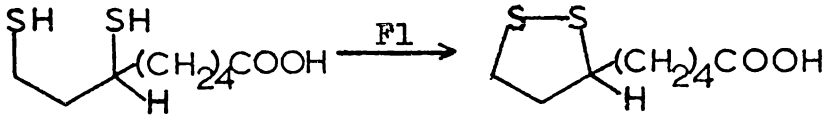
$$\text{RCH(NH}_2\text{)COOH} \longrightarrow \text{RCOCOOH} + \text{NH}_3 \qquad (1.1b)$$

$$\text{HOOCCH}_2\text{CH}_2\text{COOH} \longrightarrow \text{HOOCH=CHCOOH} \qquad (1.1c)$$

$$\text{CH}_3\text{COCOOH} \longrightarrow \text{CH}_3\text{COOH} + \text{CO}_2 \qquad (1.1d)$$

$$\text{CH}_3\text{CH(OH)COOH} \longrightarrow \text{CH}_3\text{COCOOH} \qquad (1.1e)$$

(b) S-H dehydrogenases: such enzymes catalyse the oxidation of dithiols, e.g. dihydrolipoamide, to the corresponding disulphide compounds (equation 1.2).



(1.2)

(c) Electron transferases: as the name implies these enzymes are involved in electron transfer systems, examples of these enzymes being ferrodoxin-NADP reductase and flavodoxin.

(d) Other redox-active flavoproteins: these enzymes are concerned with the reduction of such ions as sulphite (to sulphide) and nitrate (to nitrite).

1-3 FLAVIN CHEMISTRY[8]

Active flavin can operate between different chemical oxidation states. There are three such states: the terms used to describe them are flavoquinone for the fully oxidized flavin molecule, flavosemiquinone or flavin radical for the half-reduced state and flavohydroquinone for the totally reduced form. As indicated in Figure I, these flavin states may exist in dissociated or protonated forms, depending on pH. Thus the spectra of the various flavin states exhibit a dependence on the pH.

1-3.1 Flavoquinone

In the absence of reducing agents and in the presence of oxygen the flavoquinone ($Fl_{ox}H$, Vb) is the only stable flavin state. $Fl_{ox}H$ exhibits a very characteristic absorption spectrum with maxima for riboflavin at 445 and 373 nm. These two prominent visible absorption bands of oxidized flavins appear to be π-π^* transitions due to electronic transitions in different parts of the isoalloxazine ring structure[6c].

Deprotonation at N(3), to give the flavoquinone anion Fl^-_{ox} (Vc), does not have any pronounced effect on the spec-

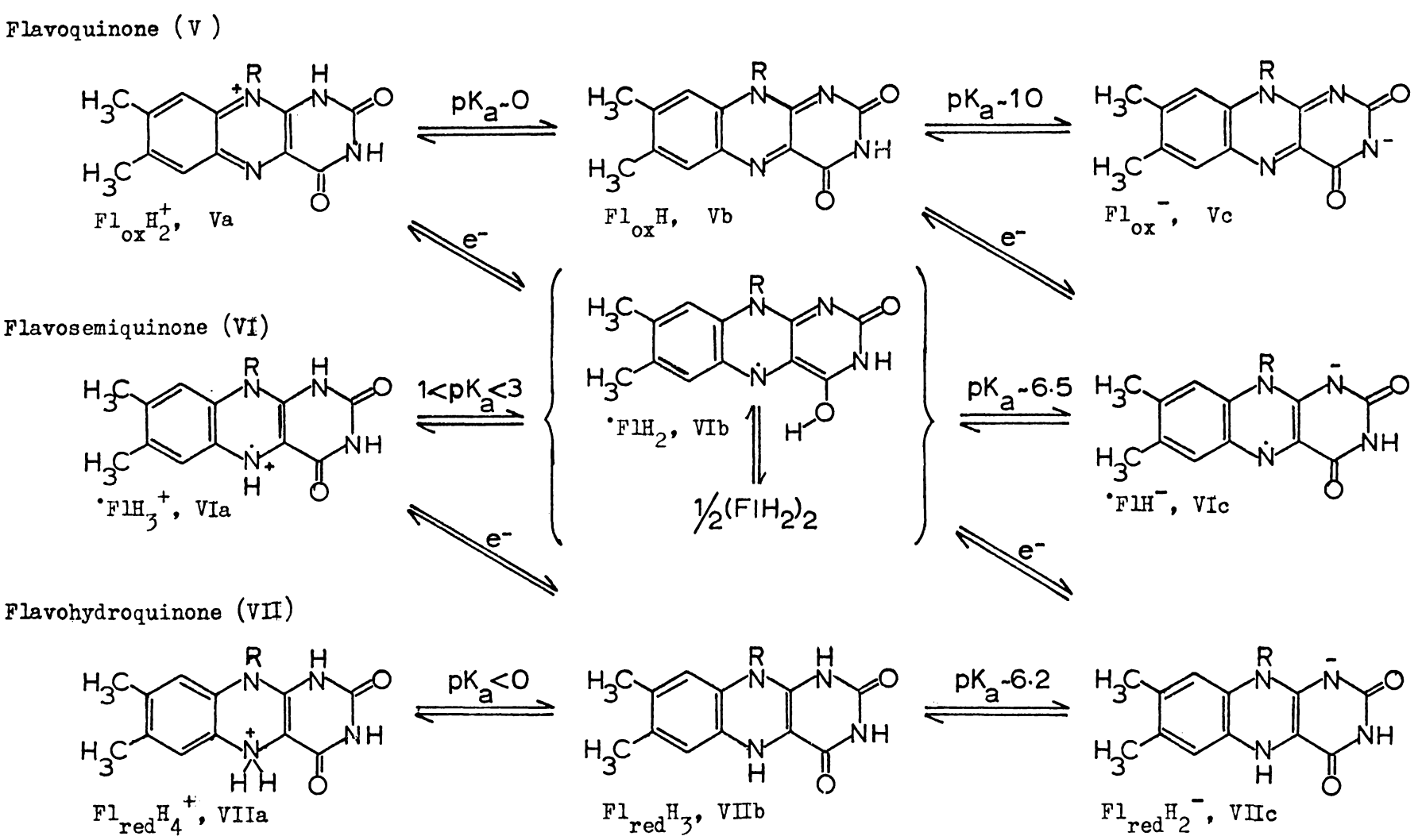


Figure I. Flavin species, as they occur at different pH and redox states. Approximate buffer regions of the singlet species are given[8b].

trum. This indicates that there is negligible π-conjugation of electrons of this part of the pyriminoid ring with the rest of the flavin nucleus. Protonation of the flavoquinone at pH<1 on N(1) gives $Fl_{ox}H_2^+$ (Va). This causes drastic changes in the flavin spectrum and elimination of the characteristic 'flavin' colour. This cation probably has no physiological significance however since the pK_a is so low.

Flavins are subject to strong solvatochromism. Solvent changes from polar to non-polar increase the resolution observed in the 450 nm band and shifts the 370nm band to shorter wavelengths.

The flavins exhibit a strong green fluorescence and this has a maximum emission at 530nm. The fluorescence is extremely sensitive to environmental influences. In some cases the emission is quenched by complex formation between the isoalloxazine nucleus and some aromatic compound. The fluorescence also disappears at extremes of pH.

1-3.2 Flavohydroquinone

In a two electron reaction, oxidized flavin reacts with the reduced form of a substrate molecule, AH_2, to yield the flavohydroquinone ($Fl_{red}H_3$, VIIb) and oxidized substrate, A (equation 1.3).

$$Fl_{ox}H + AH_2 \longrightarrow Fl_{red}H_3 + A \qquad (1.3)$$

The reduced flavin becomes itself a substrate for other electron acceptors, thereby regenerating the oxidized flavin. Artificial electron acceptors can be utilized in the absence of natural acceptors. Examples of these are methylene blue, indophenol, phenazine, tetrazolium dyes and the

most common, molecular oxygen.

On full reduction of the flavoquinone to the flavohydroquinone, which can be achieved chemically with sodium dithionite[9], the characteristic visible absorption spectrum of the flavin is bleached, leaving bands at approximately 260 and 350nm. This spectrum consists of a series of weak overlapping bands which are probably due to several n-π^* transitions[6c].

The more acidic protons in the flavohydroquinone are situated at the N(1) and N(3) positions. Alkylation at N(3) causes no change in the acidity of the reduced flavin which has a pK_a of approximately 6. Therefore the most acidic proton in the flavohydroquinone is at N(1). Tautomerism of the C(2) and C(4) carbonyls to the 'iminols' can occur quite readily contrary to the situation for oxidized flavin. This is reflected by the ease with which the carbonyl oxygens are alkylated. Although N(3) can also be alkylated, there is no corresponding reaction with N(1). This is probably due to steric hindrance from the N(10) substituent. Thus the chemistry of the N(3) position appears to remain unaffected by the change in the oxidation state of the flavin.

The greatest change which occurs on reduction of $Fl_{ox}H$ is that the product flavohydroquinone is folded about the central axis (N(5),N(10)) in a 'butterfly wing' conformation in contrast to the planar flavoquinone[10] as is consistent with the loss of conjugation between the benzenoid and pyriminoid rings. The flavohydroquinone cation ($Fl_{red}H_4^+$, VIIa) is present at pH 1 and N(5) becomes a quaternary ammonium tetrahedral centre. Deprotonation at N(1) of the

neutral reduced flavin molecule at pH 6-7 gives the anion, $Fl_{red}H_2^-$ (VIIc). The full negative charge on the pyrimidine ring prevents delocalization of electrons from the other rings and thereby enhances the folding of the molecule.

1-3.3 Flavosemiquinone

The flavin radicals ($^{\bullet}FlH_2$, VIb) formed by the one-electron oxido-reduction of free flavohydroquinone and flavoquinone, respectively, are difficult to demonstrate as the radicals react more rapidly to form the oxidized or reduced flavins. The flavosemiquinone is more readily shown with flavoproteins as the presence of the apoprotein stabilizes the flavin radical.

In partially reduced flavin solutions flavosemiquinone molecules exist in equilibrium with the oxidized and reduced forms by comproportionation (equation 1.4).

$$Fl_{ox}H + Fl_{red}H_3 \rightleftharpoons 2^{\bullet}FlH_2 \qquad (1.4)$$

Similarly to the two other oxidation states the flavosemiquinone can exist in neutral, cationic and anionic forms. In acid solution the flavosemiquinone exhibits a characteristic reddish colour due to the light absorption spectrum of the cationic radical, $^{\bullet}FlH_3^+$ (VIa). This has maxima at about 360 and 490nm. Deprotonation at pH>2 gives the neutral species, $^{\bullet}FlH_2$ (VIb), which also has a characteristic blue colour with a long wavelength absorption about 560-620nm. Spectral assignments for the anion have not as yet been made with any certainty.

Experimental difficulties experienced with the absorption spectrum of the flavosemiquinone is counteracted by their paramagnetism and hence the exhibition of ESR spectra.

As with light absorption the ESR spectra show pH dependence. The radical anion has the simplest spectrum of 12 evenly spaced lines. The cationic and neutral spectra are more complex with hyperfine lines occurring. Alkylation of N(3) has only very slight effects on the ESR spectrum and also there is only a very small spin density localized on the pyriminoid nitrogens. Thus the unpaired spin is virtually isolated from the pyriminoid nitrogens.

1-3.4 Molecular Complexes

Bimolecular complexes of the charge-transfer type have been demonstrated to be formed between the flavin molecules of different redox states. These produce broad light absorptions with maxima in the 800-1000nm region. These complexes form for a number of aromatic compounds and flavin molecules. The effect on the visible spectrum of the flavin is to broaden the peaks and to shift them to longer wavelengths.

X-ray data has shown the existence of face-to-face complexes of isoalloxazines with various aromatic molecules in the crystalline state. For example, lumiflavin associates with 2,3-naphthalenediol in a 1:2 complex[11] and with benzoquinone in a 1:1 complex[12]. Purines and pyrimidines are known to associate in aqueous solutions by forming stacks of parallel planar molecules[13]. The crystal structures of the isoalloxazine-complex crystal would suggest similar stacking.

Proton magnetic resonance and circular dichroism spectra[14] of aqueous FAD solutions corroborate the association of the aromatic rings to form parallel stacks. In photo-

chemical reduction in the presence of EDTA, FAD exhibits a slower rate of reduction when compared with FMN[15]. This fact was attributed to the formation of an intramolecular complex between the flavin nucleus and the adenine moiety of FAD. Fleischman and Tollin[16] found that isoalloxazine derivatives, such as lumichrome, FMN and riboflavin, produced highly coloured solutions in strong acid with electron-rich phenols due to the formation of 1:1 charge-transfer complexes between the phenol and monoprotonated flavin. With very good donor molecules, such as 1,4-naphthalenediol, the complexes formed in neutral solution. The formation of the complexes was corroborated by spectral evidence; decreases and broadening of the flavin absorptions and the appearance of long wavelength absorptions, in the case of 1,4-naphthalenediol, were observed.

The characteristic fluorescence of isoalloxazines is quenched by a wide variety of aromatic compounds[17]. In fact the fluorescence of FAD is 90% quenched relative to an equivalent concentration of FMN[18] due to complexing between the adenine and isoalloxazine ring systems of FAD. Fluorescence quenching of isoalloxazines has also been shown to occur in solutions containing tryptophan[19]. The observation of such quenching by these types of molecules is interpreted as implying complex formation of some form although not necessarily of the charge-transfer type. Purines and pyrimidines, although forming complexes with isoalloxazines, do not give colour changes with isoalloxazines in acid unlike the phenols[16a].

1-4 FLAVIN AND FLAVOPROTEIN MECHANISMS

The mechanisms by which flavins and flavoproteins catalyze reactions will be discussed in two parts. Firstly, the oxidation states between which the flavin nucleus operates and secondly, on a molecular level, the types of flavin-substrate complexes which have been suggested from enzyme and model studies as likely intermediates in the redox reactions.

1-4.1 Flavin Oxidation States in the Flavoproteins

One of the unsolved problems in the field of biological oxidations is the mechanism by which the two-electron transfers occurring in the early stages of the respiratory chain are linked to the one-electron transfer steps occurring in the cytochrome region of the respiratory system. When redox equivalents are transferred through flavin from, say, NADH to cytochrome or ubiquinone, the flavin accepts two electrons at a time together with a proton. However, the acceptor sites will not accept more than one electron at a time. Thus the flavin effectively 'splits' the electron pair. However, if this occurs then there must be some way in which the two electrons can be yielded with the same potential since the change from flavoquinone to flavosemiquinone and the latter to flavohydroquinone represents a great difference in the potential of the two levels. This problem can be solved if there is a second redox-active group which can comproportionate or disproportionate the system so that the second electron has equivalent energy to the first. The simplest method would be to have an addit-

ional flavin moiety present thereby producing two flavin radicals which would necessarily be of similar potential (equation 1.5). This process would necessitate that there

$$2Fl_{ox}H \xrightarrow{2e^-} Fl_{red}H_3 + Fl_{ox}H \rightleftharpoons 2^{\cdot}FlH_2 \quad (1.5)$$

be some form of inter-flavin contact. Most of the flavoenzymes have more than one coenzyme associated with them, suggesting that this is a real possibility.

With the existence of three oxidation levels, the flavoenzymes can utilize pairs of these states to carry out their catalysis. Some enzymes appear to function between the oxidized and radical levels, while others operate between the reduced and radical levels and then again others use only the oxidized and reduced states of the flavin.

Glucose oxidase provides the only authenticated example of an enzyme functioning solely between flavoquinone and flavohydroquinone[20]. Spectral intermediates have not been detected and the half-reduced enzyme has been found to be unreactive to substrate. This would also indicate that the di-radical is not an intermediate in the mechanism. Snake venom L-amino acid oxidase provides an example of the mechanism involving flavosemiquinoid intermediates from the oxidized flavin[21]. Transient long-wavelength absorbing intermediates form on addition of L-leucine to a solution of the enzyme[22]. This enzyme is very susceptible to inhibition by excess substrate. It was suggested that the enzyme was being completely reduced to the flavohydroquinone state in the presence of excess substrate. However, the half-reduced enzyme was found to be inactive towards the substrate.

Catalysis involving transfer between the flavohydro-

quinone and the flavin radical levels appears to be demonstrated by NADPH cytochrome c reductase[23]. The cytochrome is reduced at the expense of the flavohydroquinone to yield an intermediate exhibiting a long-wavelength absorption consistent with a radical structure.

1-4.2 Mechanisms at the Molecular Level

The interesting aspect of flavin catalysis is the mechanism of electron transfer and the accompanying question of the type of contact between the isoalloxazine nucleus and the reductant molecule which allows such electron transfer to occur.

The reduction of the flavoquinone state to the flavohydroquinone state is a two electron process as shown in equation 1.6.

$$Fl_{ox}H + 2e^- + 2H^+ \longrightarrow Fl_{red}H_3 \qquad (1.6)$$

Therefore, in flavin-mediated redox reactions there is a requirement for the transfer of these two electrons to the flavin from the reductant molecule. The methods by which these electrons may be transferred from reductant to isoalloxazine are as follows:

(a) the electrons are transferred as a pair with a proton, i.e. as in a hydride ion equivalent;

(b) the electrons are transferred singly, i.e. a radical mechanism involving transfer of the hydrogen radical, $H^\bullet$, and one electron;

(c) the electrons are transferred via the formation and subsequent breaking of a covalent bond from the reductant molecule to the flavin.

Depending on which of these mechanisms operates the nature of the contact between isoalloxazine and reductant would be expected to differ. Contact in cases (a) and (b) might be expected to occur through the formation of a complex, possibly of the charge-transfer type, from within which direct transfer of hydride ion, hydrogen atom or electron occurs. Such prior complexing would not be excluded in the case of (c), but the formation of a σ-bond between reductant and isoalloxazine requires specifically that there must be a receptor site for adduct formation on the isoalloxazine nucleus.

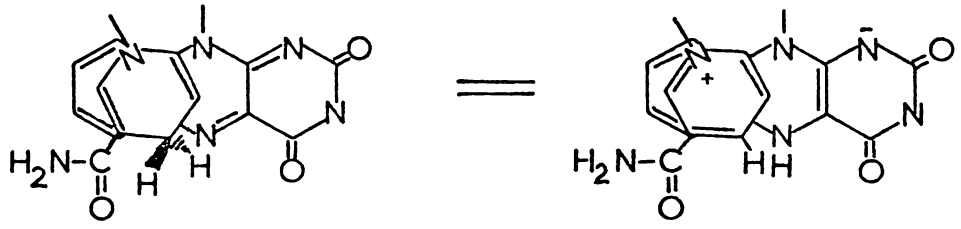
These factors are discussed in more detail in the following sections.

1-4.2.1 The Hydride Ion Mechanism

Support for this mechanism would come from evidence for complex formation of flavin with reductant and for direct hydrogen transfer to isoalloxazine. Many oxidations of reduced nicotinamide species involve the direct transfer of hydrogen to the oxidant[24]. This has been shown by isotopic labelling with deuterium and these observations have generally been interpreted as implying the transfer of a hydride ion from within a molecular complex of reductant and oxidant.

Isoalloxazines certainly do form complexes with many molecules (section 1-3.4) but whether such complexes with the reductant lie on the reaction pathway for redox reactions is another question: a representation of such a complex between reduced nicotinamide and the isoalloxazine nucleus and ensuing hydride transfer to the isoalloxazine

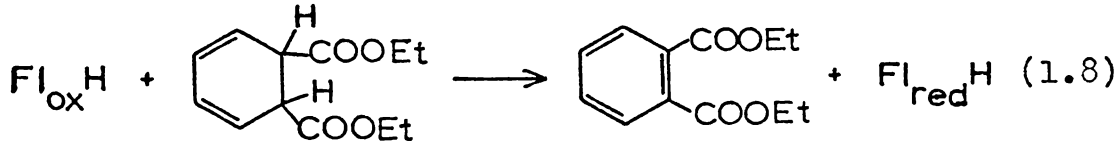
may be envisioned as in equation 1.7.



NADH·····FI NAD⁺·····FIH⁻ (1.7)

Isenberg and coworkers[25] suggested that oxidized isoalloxazine and reduced nicotinamide form a charge-transfer complex of this type which is important in the redox process, but no conclusive evidence to support this hypothesis had been obtained until recently when direct evidence was presented for a preequilibrium complex between oxidized lumiflavin and N-methyl-1,4-dihydronicotinamide[26]. This evidence was obtained from a Lineweaver-Burke plot (k_{obs}^{-1} against [nicotinamide]$^{-1}$) which had a negative intercept on the x-axis. Such plots are generally interpreted as implying the formation of some form of molecular complex between the reactants. This was corroborated by the observation of a long wavelength absorption during the reduction reaction. The rate of disappearance of this peak was identical to the rate of formation of reduced lumiflavin.

The oxidation of dimethyl trans-1,2-dihydrophthalates[27] (equation 1.8) and NADH[19] by certain isoalloxazines showed



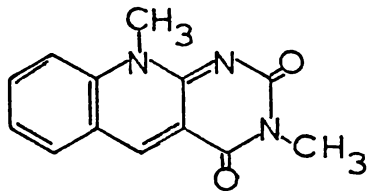
(1.8)

a linear relationship between the logarithm of the rate of reduction of the isoalloxazine and the logarithm of the equilibrium constant for the binding of tryptophan to the isoalloxazine nucleus. The interpretation of these observations was that 'kinetically important preequilibrium

complex formation takes place'.

As mentioned above, reduced nicotinamides are known to participate in reduction reactions with direct transfer of a hydrogen to the oxidant[24]. The transfer of hydrogen from NADH to a substrate molecule via an enzyme-bound flavin has been demonstrated[28]. This implies that the hydrogen is initially directly transferred from NADH to the oxidized flavin in the enzyme with subsequent transfer from the reduced flavin to the substrate which is reduced.

Deazaflavin (VIII) has the nitrogen at the N(5) posi-



VIII

tion replaced with a carbon atom. This molecule behaves in a similar fashion to the isoalloxazines in the usual flavin reductions[29] forming the 1,5-dihydro molecule. Reaction of deazaflavin with NADH in deuterium oxide occurs with direct hydrogen transfer to the C(5) position[30]. The interpretation of this evidence is that both the hydrogen atom and the two electrons are transferred internally to deazaflavin from within a complex between NADH and deazaflavin. Such a test cannot be made for direct transfer with isoalloxazines themselves as proton exchange occurs at N(5) of the reduced isoalloxazine, but the result for deazaflavin suggests that direct hydrogen transfer would probably occur for isoalloxazines themselves.

1-4.2.2 Electron Transfer via Covalent Bond Formation

Hamilton[31] was the first to clearly consider the possibility of electron transfer occurring through a coval-

ent link between the isoalloxazine moiety and the substrate.

The basic requirement for such a mechanism is an electrophilic centre on the isoalloxazine nucleus to which a nucleophile can add and that the covalent complex thus formed can break down as completion of the electron transfer occurs.

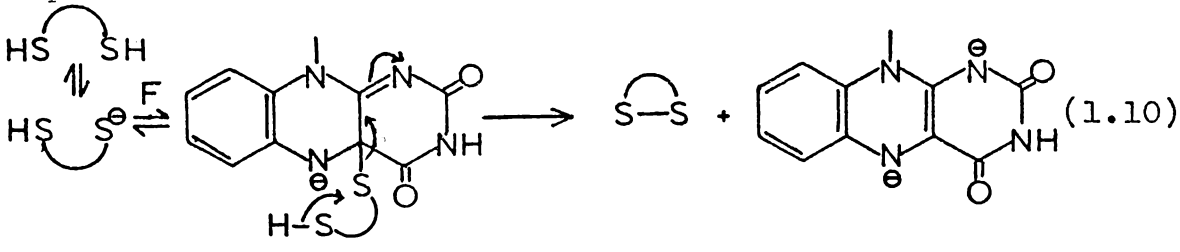
For the oxidation of a generalized species HA-BH, the mechanism can be outlined as in equation 1.9.

$$HA{+}BH \overset{-H^+}{\rightleftharpoons} HA{-}B^- \overset{F}{\rightleftharpoons} H{-}A{-}B{-}F^- \longrightarrow$$
$$H^+ + A{=}B + F^= \xrightarrow{2H^+} FH_2 \qquad (1.9)$$

The hydrogen atoms are therefore transferred externally as protons during the reduction of the isoalloxazine in contrast to the case involving direct hydride transfer in 1-4.2.1 above, while the electrons are transferred via the formation of the covalent bond between the reductant molecule and the isoalloxazine.

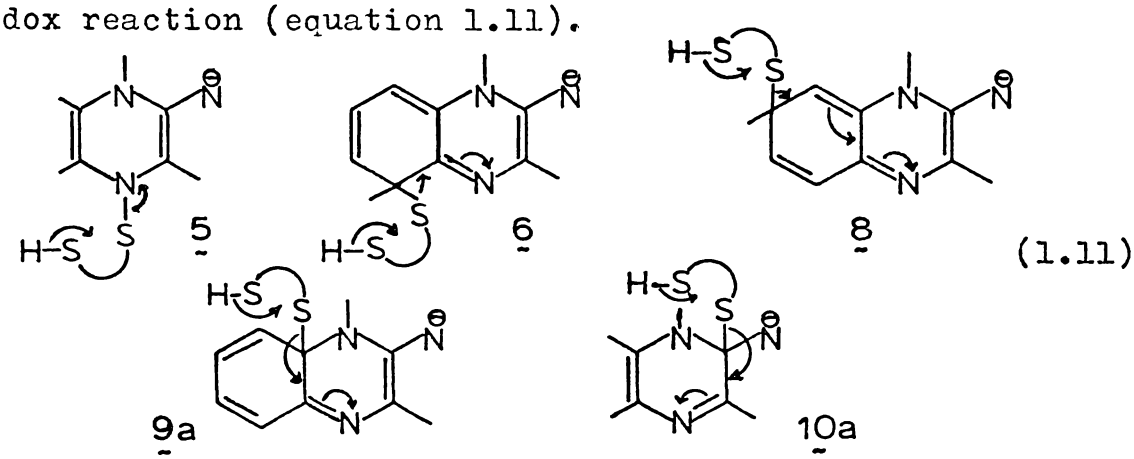
Theoretical calculations[32] show that the N(5) position on the isoalloxazine nucleus is the most electrophilic closely followed by the 4a, 6, 8, 9a and 10a positions, and all are possible sites for addition of nucleophiles.

Hamilton considered the possible generality of such a mechanism for a number of reductants with the 4a-position as the most likely electrophilic centre for covalent bond formation. Thus, for example, if the timing of proton transfer to the isoalloxazine is ignored for the oxidation of a dithiol the mechanism may be visualized as in equation 1.10.



(1.10)

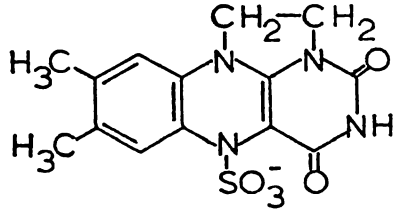
The other possible sites for covalent bond formation would also allow the completion of electron transfer in a redox reaction (equation 1.11).



(1.11)

The necessary step for vindication of such a mechanism is the observation of a covalent adduct of the isoalloxazine as an intermediate on the pathway of a redox reaction, but so far these have not been observed.

Addition compounds of isoalloxazines have been observed and in some cases isolated from non-redox reactions. Nucleophilic addition of sulphite has been observed with flavoenzymes[33] and with free isoalloxazines[34]. In the case of the enzymes spectral evidence only is available since the instability of the sulphite-enzyme complexes does not allow their isolation. Isoalloxazine-sulphite addition complexes have been isolated where the isoalloxazine has been alkylated at the N(1) position[34a]. The position of attack was suggested to be the N(5) nitrogen (IX). This

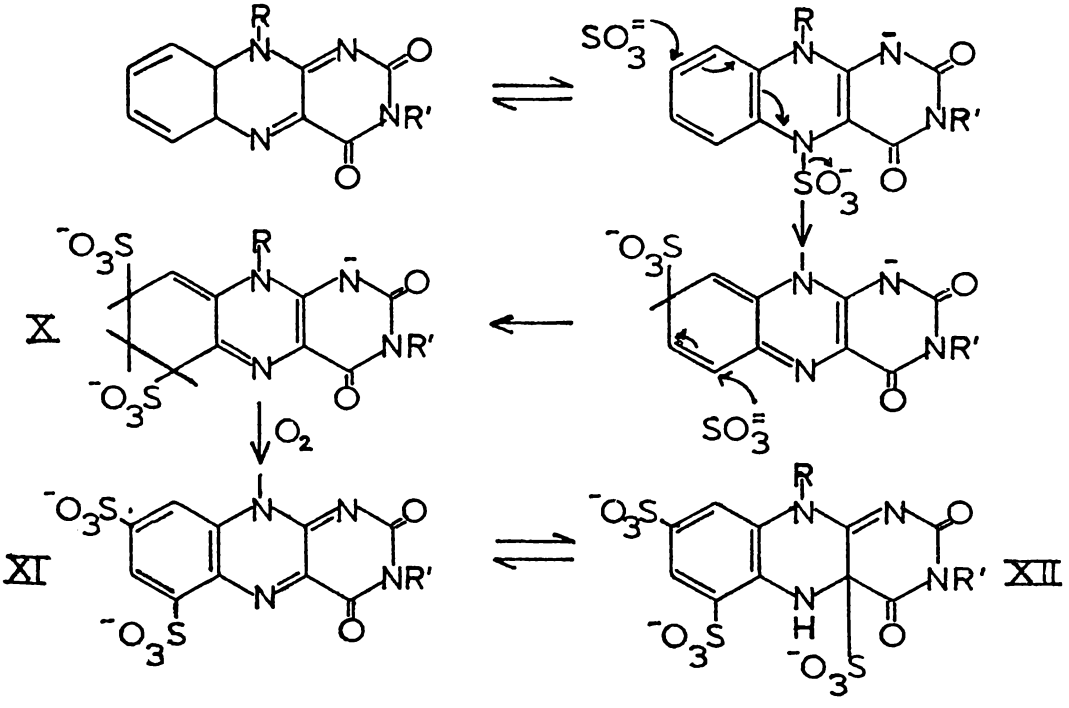


IX

was reasonable since this position is in fact the most electrophilic in the isoalloxazine nucleus[34b]. Hevesi and Bruice[34b] have shown that although the sulphite initially adds to N(5) in isoalloxazines carrying no N(1)-substituent

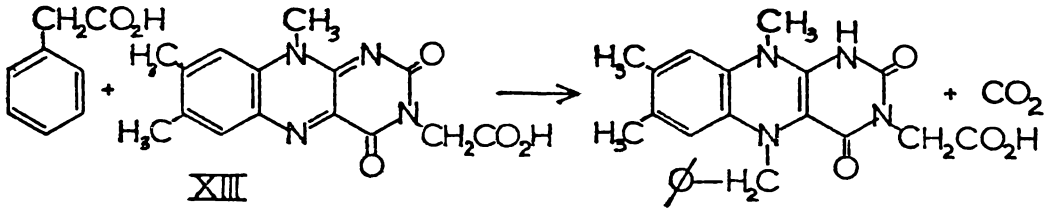
further attack occurs at the C(8) and C(6) positions with the expulsion of the N(5) sulphonate group to yield the 1,6,7,8-tetrahydroisoalloxazine-6,8-disulphonate (equation 1.12, X). When this new reduced isoalloxazine is reoxidized by air the isoalloxazine, XI, is formed and this when

R = 2,6-dimethylphenyl ; R' = CH_3



treated with sulphite was found to form the 4a-adduct (XII). This was thought to be because the N(5) position was electrostatically hindered by the sulphite group at C(6) in XI. The structure of XI was confirmed by elemental analysis. This study establishes that 4a-, 5-, 8- and possibly 6-positions are potential acceptor sites for nucleophiles.

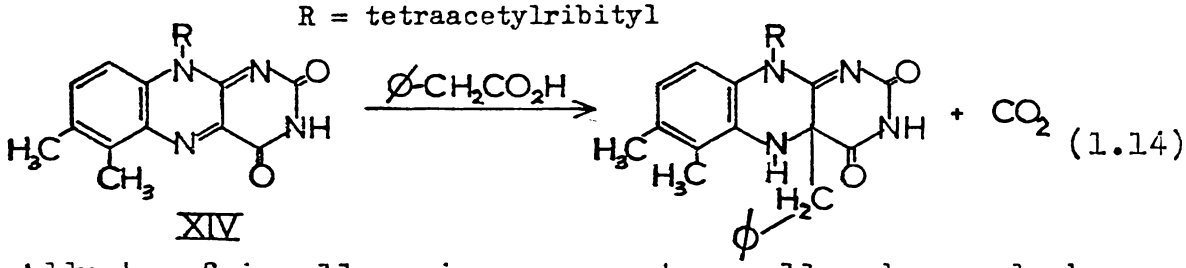
Isoalloxazines are readily reduced in photochemical reactions in the presence of a variety of compounds such as EDTA[35] and phenyl acetic acid[36]. Both 4a- and 5-adducts have been observed in the case of phenylacetic acid[36]. The adduct which is formed depends on the steric conditions at the N(5) position in the isoalloxazine. With XIII, which is not crowded at the 5-position, the 5-adduct was produced



XIII (1.13)

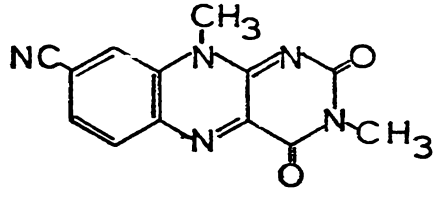
(equation 1.13) but if there was a methyl group at C(6)(XIV) phenylacetic acid added to the 4a-position (equation 1.14).

R = tetraacetylribityl



XIV (1.14)

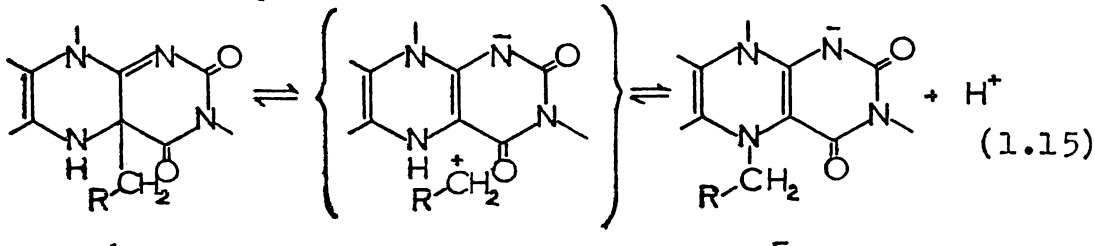
Adducts of isoalloxazines are not usually observed when they are photochemically reduced in the presence of EDTA, but when electron deficient isoalloxazines (XI, XV) were used 4a-adducts were observed to form[35]. In fact the adduct



XV

produced from XV was so stable that it resisted further reduction to the 1,5-dihydroisoalloxazine. Thus the formation and stability of the adducts are controlled by steric and electronic factors.

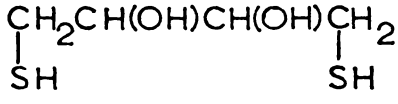
Isomerization of 4a- to 5-adducts and vice versa (equation 1.15) has been observed in addition complexes from both photochemical[36,37] and dark non-redox reactions[38]. The direction of the isomerization depends on the thermodynamic stability of the individual adducts and the ease of isomerization depends on the carbonium ion stability of



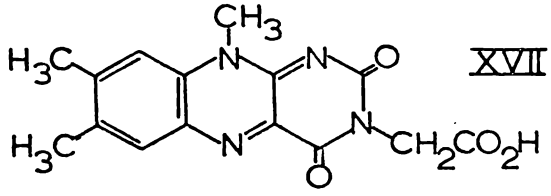
4a 5 (1.15)

the migrating group[37]. The conclusion which may be drawn from this is that there is the possibility of a general covalent reaction mechanism, say through a 4a-adduct. Thus this could form directly or if the steric and electronic factors operating in the flavin cause a 5-adduct to form this could then rearrange to the 4a-isomer.

Although there has been the observation of the adducts mentioned above, there is as yet no spectral evidence for an adduct being an essential intermediate on a non-photochemical redox reaction pathway. Despite lack of spectral corroboration, kinetic evidence has been presented, however, which implicates an adduct intermediate in the reaction of dithiothreitol (XVI) and 3-carboxymethyllumiflavin (XVII)[39]. Through kinetic analysis of the pH-rate profile



XVI



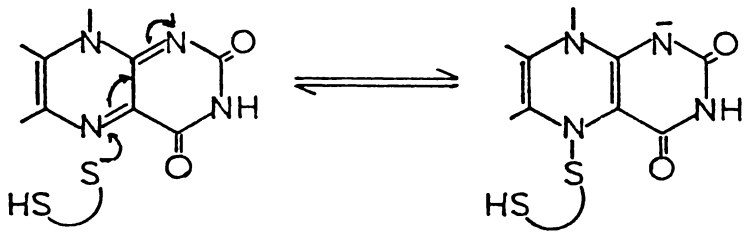
XVII

of this reaction Loechler and Hollocher have deduced that

(a) there is change in the rate-determining step from adduct formation (pH 7-11) to adduct breakdown above and below that pH range, and that

(b) general acid catalysis (by buffer) of the adduct formation step occurs.

This is consistent with adduct formation at the 4a- rather than the 5-position for the following reasons. From equation 1.10 it can be seen that when the adduct forms at 4a-position a formal negative charge develops at N(5), whereas if attack occurs at N(5), the formal negative charge is generated at N(1) as in equation 1.16. The pK_a of N(5)H in a 4a-adduct has been estimated to be very high



(1.16)

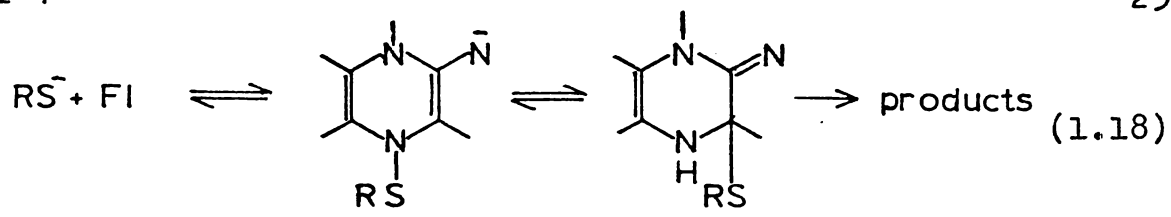
(pK 24[39]) and thus 4a-addition would be expected to be subject to general acid catalysis; the pK_a of N(1)H in a 5-adduct, on the other hand, is much lower (pK_a= 6.6[39]) and consequently protonation of the anion would not occur at the pH values used and general acid catalysis would not be observed.

Further kinetic evidence has been obtained by Yokoe and Bruice[40] from the reaction of 8-cyano-3,10-dimethyl-isoalloxazine (XV) and thiophenol. The reaction of XV with excess thiophenol followed first order kinetics in isoalloxazine (pH 4.8-9.9) and was found to be second order in total thiol. General acid catalysis was not observed. Despite the latter observation these facts are consistent with 4a-addition and with rate limiting breakdown of the adduct to the product (equation 1.17).

$$RS^- + XV \rightleftharpoons 4a^- \underset{k_{gb}B}{\overset{k_{ga}BH}{\rightleftharpoons}} 4aH \xrightarrow{k_3RS^-} \text{products} \qquad (1.17)$$

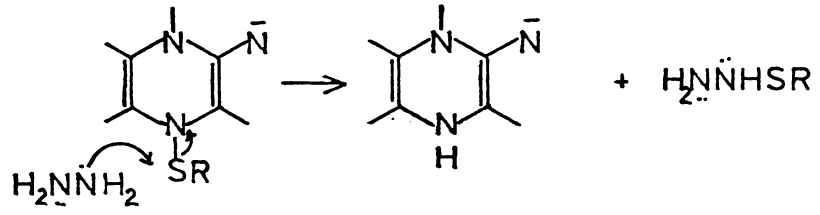
General acid catalysis is not observed because the breakdown of the adduct rather than the attack of the thiol anion is rate limiting. This difference between mono- and di-thiols is caused by the close proximity of the second thiol group in the latter which allows rapid completion of electron transfer; formation rather than breakdown of the intermediate is rate determining and general acid catalysis is observed.

A mechanism for monothiols could also be postulated[40] involving addition of thiol to N(5) (equation 1.18) to



(1.18)

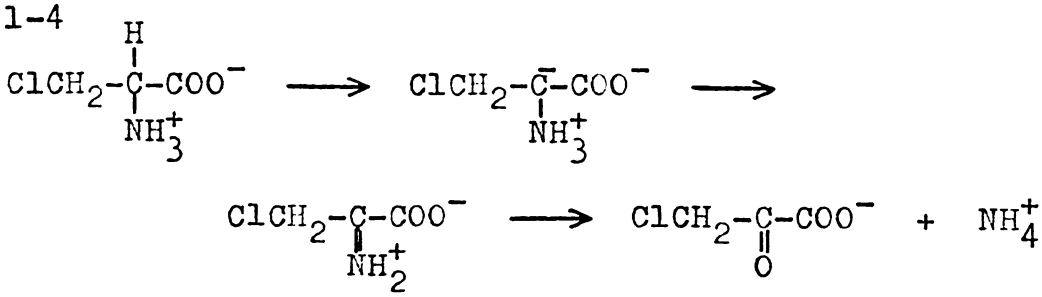
account for the lack of general acid catalysis. Migration of RS^+ to the 4a-position is suggested, with subsequent breakdown to the products. However, this mechanism was discounted as the 5-adduct would be a sulphenamide which would be susceptible to transamination by strong α-effectors such as hydrazine (equation 1.19), but this was found to have no effect on the rate of reaction[40].



(1.19)

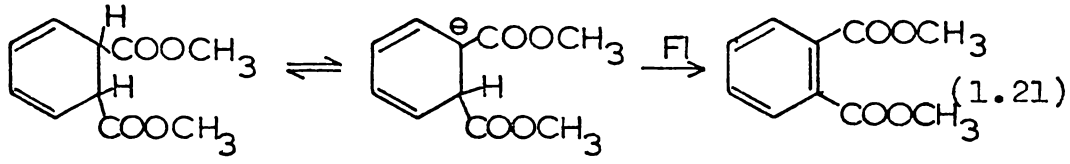
The kinetic evidence of the dithiol reaction also eliminates the other electrophilic sites for attack of thiols at the isoalloxazine nucleus since the anion at N(1) is formed when addition occurs to these centres (equation 1.11). The 9a and 10a sites have been eliminated as obligatory positions for covalent adduct formation as the rate of reduction of the isoalloxazine was found not to be depressed when these positions were sterically crowded[19].

The mechanism proposed by Hamilton requires the formation of an anion leading to the attack on the isoalloxazine. Flavoenzyme and model isoalloxazine studies demonstrate that a carbanion or some other nucleophile derived from the substrate is an important intermediate in certain dehydrogenation reactions. The oxidation of β-chloroalanine by D-amino acid acid oxidase is dependent on the dissociation of an α-C-H bond in the substrate, i.e. the formation of a carbanion is rate limiting[41] (equation 1.20).

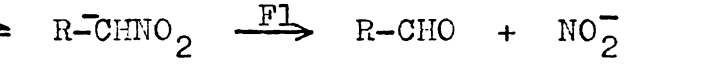


(1.20)

Model dehydrogenations of dimethyldihydrophthalates[27] (equation 1.21) and nitroalkanes[40] (equation 1.22) by isoalloxazines have also been found to be dependent on the



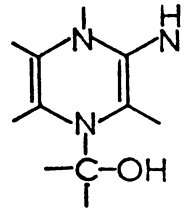
(1.21)



(1.22)

formation of a carbanion intermediate, but this in itself is insufficient evidence to exclude direct hydrogen transfer.

A mechanism involving a 5-adduct is a possibilty for the reaction of nitroalkanes with isoalloxazines. The reaction of nitromethane and 2-nitropropane was not buffer catalyzed[40] suggesting involvement of a 5-adduct rather than a 4a-adduct and there is evidence for the formation of a 5-adduct from the reaction of D-amino acid oxidase with nitroalkanes[42]. A 5-carbinolamine intermediate (XVIII)



XVIII

has been suggested for the interaction of reduced isoalloxazine with carbonyl compounds[43], but this species has been shown not to be on the redox reaction pathway[44].

1-4.2.3 The One-Electron Transfer Process

With the transfer of a single electron to the oxidized

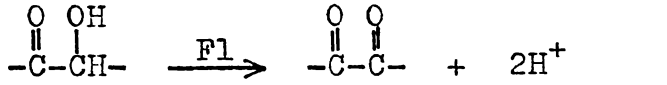
isoalloxazine nucleus the flavosemiquinone must be an intermediate species. The observation of the semiquinone state would be more likely to occur during enzyme studies where the surrounding protein molecule could stabilize the reactive radical species. It must be emphasized that there is a complicating factor which may occur with the observation of the semiquinone state; this is that oxidized and reduced isoalloxazine molecules can interact to yield two radical molecules as in equation 1.23.

$$Fl_{ox}H + Fl_{red}H_3 \rightleftharpoons 2^{\cdot}FlH \qquad (1.23)$$

These need not, of course, be on the redox pathway. There are several cases of flavosemiquinones appearing in enzyme systems; these have been mentioned in section 1-4.1.

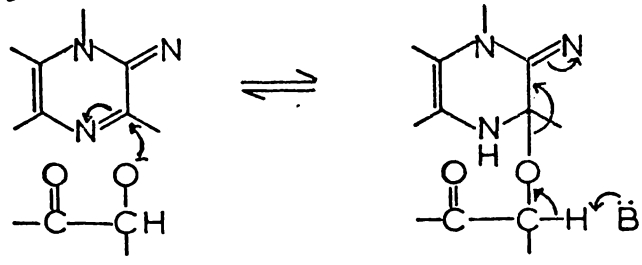
A stable semiquinone intermediate of deazaFAD bound to D-amino acid oxidase has been reported[45]. The radical was formed in a photochemical reaction in the presence of EDTA; the absorption spectrum was similar to the known spectrum of the 'red' semiquinone of normal D-amino acid oxidase. The formation of the radical was confirmed by the observation of an ESR signal under the same conditions.

Isoalloxazines oxidize α-ketols to the corresponding 1,2-diketone compounds (equation 1.24).



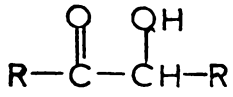
(1.24)

Brown and Hamilton[46] have suggested that the reaction might proceed via a 4a-adduct (see section 1-4.2.2) as in equation 1.25.

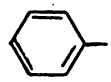


(1.25)

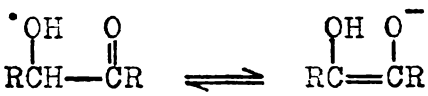
However, α-ketols are also oxidized to their corresponding diketones by molecular oxygen and Fehling's solution. The rates of these reactions are proportional to the hydroxyl ion concentration and they are the same at identical pH[47]. Bruice and coworkers[47] found that the rates of oxidation of furoin (XIXa) and benzoin (XIXb) to furil and benzil



XIX (a) R = 

(b) R = 

respectively by molecular oxygen, 2,6-dichlorophenolindophenol and lumiflavin were also identical at the same pH. It appears therefore that the oxidation of α-ketols by all these oxidants depends on the rate-limiting formation of the same intermediate, that is, the enediolate anion (XX) (equation 1.26).

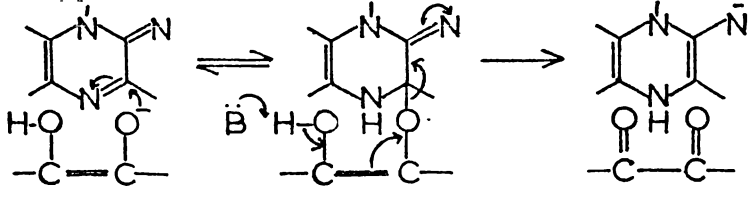


(1.26)

This is in contrast to the anion suggested by Hamilton (equation 1.25).

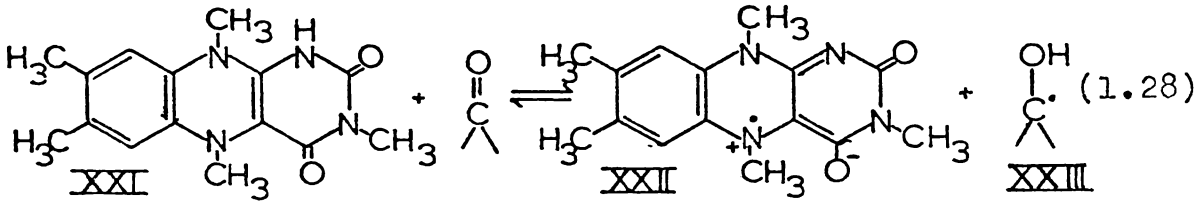
When an alcoholic mixture of benzoin and benzil is treated with base a purple colouration appears. This coloured compound has been shown to have an oxidation level between those of benzoin and benzil[48] and has been interpreted as formation of a radical species. The mechanism of the oxidation of benzoin and ascorbic acid by molecular oxygen has been shown clearly to involve a radical intermediate[49,50].

Thus although the covalent mechanism, as in equation 1.27, is applicable to the oxidation of α-ketols by iso-



(1.27)

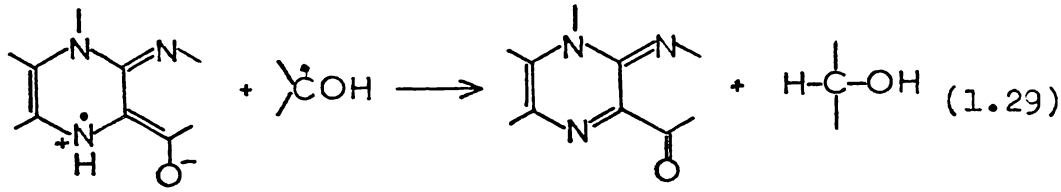
alloxazines there is the possibility that the reduction of isoalloxazines by enediols may involve radical species in the redox reaction pathway. This appears to be even more likely as a result of observations made recently in the reaction of reduced isoalloxazines with carbonyl compounds. Bruice and Yano[51] have demonstrated one-electron transfer in these reactions. For example, the reactions of 1,5-dihydro-3,5-dimethyllumiflavin (XXI) with such carbonyl compounds as ethyl pyruvate, phenyl glyoxal, chloral, barbituric acid, benzoquinone, naphthoquinone and ninhydrin yield the blue zwitterionic 3,5-dimethyllumiflavin radical (XXII) (equation 1.28).



(1.28)

XXI XXII XXIII

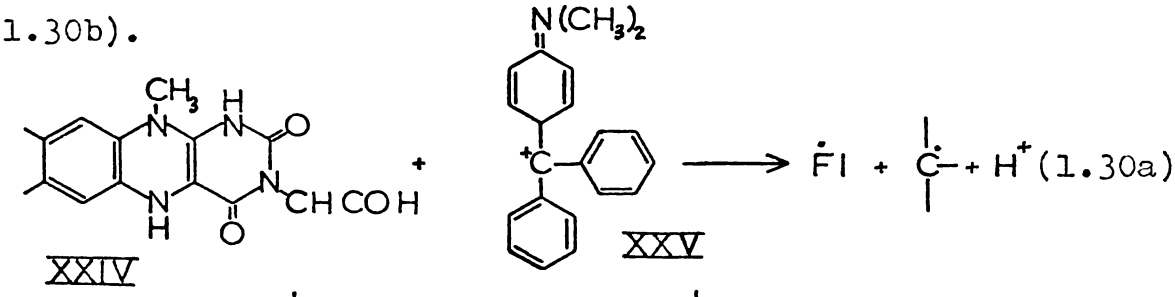
The observation of the radical is possible because the substitution of an alkyl group at N(5) enhances the stability of the radical formed and also it prevents further reaction with another carbonyl group, or with the radical formed from the carbonyl (XXIII), to the fully oxidized isoalloxazine as Bruice and Yano have suggested might occur for the reaction of 'normal' reduced isoalloxazines (equation 1.29).



(1.29)

That single electron transfer is a distinct possibility for isoalloxazines is shown by the reaction of 1,5-dihydrolumiflavin-3-acetic acid (XXIV) with Malachite Green cation (XXV) which was first order in isoalloxazine and second

order in Malachite Green[51]. This is consistent with a radical process where the isoalloxazine radical, formed from the initial reaction (equation 1.30a) between reduced isoalloxazine and Malachite Green cation, reacts with a second cation to give the oxidized isoalloxazine (equation 1.30b).



XXIV XXV

$$\cdot FlH + {}^{+}\!\!\begin{array}{l}|\\ C-\end{array} \rightleftharpoons Fl_{ox} + {}^{\cdot}\!\!\begin{array}{l}|\\ C-\end{array} + H^{+} \qquad (1.30b)$$

$$2\ {}^{\cdot}\!\!\begin{array}{l}|\\ C-\end{array} \longrightarrow \text{product} \qquad (1.30c)$$

Whether radical mechanisms of this type operate in the reverse direction, e.g. in the oxidation of alcohols to ketones (i.e. the reverse of equations 1.29 and 1.28) remains to be seen.

Much of the work done with isoalloxazines which is aimed at studying specific effects related to either the hydride transfer mechanism (section 1-4.2.1) or the covalent mechanism (section 1-4.2.2) is equally relevant to the single electron transfer processes. For example, the evidence relating to formation of bimolecular complexes is relevant since there might be contact of this form prior to electron or hydrogen atom transfer. Likewise single electron transfer followed by covalent bond formation between the resulting radicals (equation 1.31) is chemically

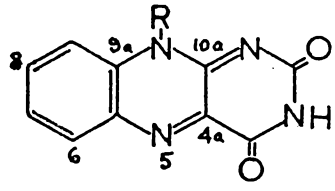
$$Fl + \begin{array}{c} HR^{-} \\ \Downarrow\Uparrow \\ H_2R \end{array} \longrightarrow {}^{\cdot}Fl^{-} + {}^{\cdot}RH \longrightarrow F\text{-}RH \qquad (1.31)$$

equivalent to direct attack on isoalloxazine by the reduct-

ant anion, and the mere observation of a covalent intermediate does not exclude initial one electron redox processes of this type.

1-5 AIM OF THIS WORK

As mentioned earlier (section 1-4.2.2) there are several possible electrophilic centres (XXVI) on the isoalloxazine ring system at which nucleophiles might attack



XXVI

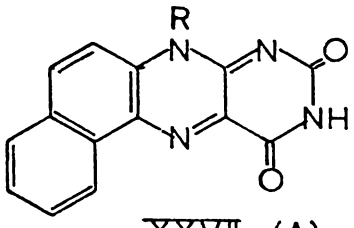
to form covalent adducts. Before the present study commenced the 9a and 10a sites had already been eliminated as essential for adduct formation along the redox reaction pathway for dithiol oxidation but the roles of the 4a-, 5-, 6- and 8-positions were unknown. We therefore set out with the intention of synthesizing isoalloxazines in which some of these other electrophilic centres are blocked or sterically crowded.

It is difficult to imagine how the 4a-position could be blocked or sterically crowded as there is no possibility of introducing substituents at this or any of the adjacent ring positions and we were left therefore to concentrate on the 5-, 6- and 8-positions.

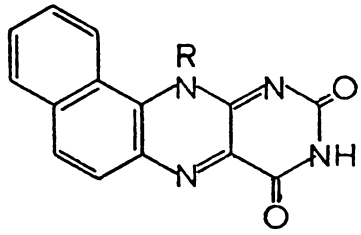
The 6- and 8-positions could clearly be blocked by introducing bulky substituents at these positions. Such a substitiuent in the 6-position would also have a steric crowding effect on the N(5)-position but it would be weak unless the substituent were particularly bulky, and in this case synthetic difficulties were envisaged. What was

required was clearly a substituent held rigidly in the region of N(5) yet not sufficiently bulky that it would affect any attack of nucleophiles at the 4a-position (from above the plane of the isoalloxazine ring).

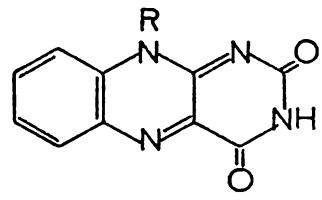
To meet all these requirements, we decided to synthesize the following series of isoalloxazines, which for convenience will be subsequently be referred to as A, B and C:



XXVII (A)



XXVIII (B)

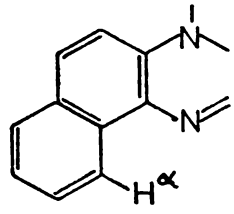


XXIX (C)

Incorporation of the additional benzenoid substituents in A and B has the following effects:

(a) Attack of a nucleophile at the 6- and 8-positions in XXVII and XXVIII respectively would result in complete loss of aromaticity in both benzenoid rings of each isoalloxazine and consequently would be thermodynamically unfavourable.

(b) The α-hydrogen in isoalloxazine A (XXX) is held rigidly in a position which should lead to steric crowding



XXX

at the N(5)-position. Hemmerich[10] has stated that "5-addition involves strictly in-plane attack" and we predict that the hydrogen atom in question would provide more effective hindrance of such a reaction than 6-substituents generally while at the same time having no out-of-plane steric crowding and consequently no effect on any addition

at the 4a-position.

(c) The additional benzene rings of A and B might also influence reactions which are dependent on molecular complexing since the area available for complexing has been increased and the differences in the orientations of the additional benzene rings with respect to the isoalloxazine moiety might have effects which throw light onto the question of orientation effects in the molecular complex.

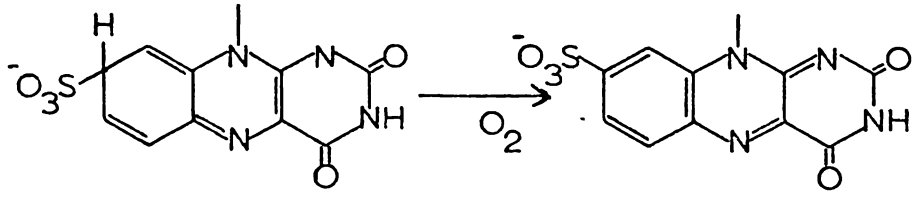
Since our studies commenced, kinetic evidence concerning general acid catalysis in the reaction of dithiothreitol and 3-carboxyluniflavin has been adduced as evidence that adduct formation at the 4a- rather than 5-, 6- and 8-positions is essential for dithiol oxidation (see section 1-4.2.2). The conclusions drawn in this kinetic study are based on a multi-parameter kinetic analysis of a pH-rate profile and while they are established within reasonable doubt, we felt that more direct evidence concerning essential sites for adduct formation which our isoalloxazines were able to provide was still desirable either to confirm or repudiate the kinetic interpretation.

With regard to the reaction of sulphite with isoalloxazines, the interpretation which has been made (see section 1-4.2.2) is that initial 5-addition is followed by a second sulphite attack at the 8-position and then a third at the 6-position. Three questions we hoped to be able to answer from our studies were:

(a) Does the steric crowding at N(5) in isoalloxazine A redirect initial sulphite addition to the 4a-position or possibly to the 8-position?

(b) Does attack of the second sulphite at the 8-posi-

tion lead to an air-oxidizable sulphonated dihydroisoalloxazine (equation 1.32) even if the 6-position is not avail-



(1.32)

able to allow the formation of a disulphonated tetrahydroisoalloxazine (isoalloxazine A)?

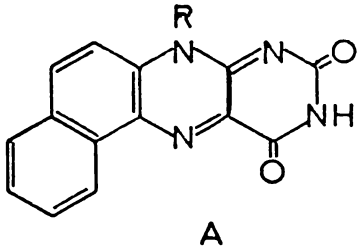
(c) Does the second sulphite attack occur at the 6-position if the 8-position is not free (isoalloxazine B)?

Finally, the reaction of reduced nicotinamides such as NADH is known to involve kinetically important preequilibrium complex formation prior to electron transfer (see section 1-4.2.1). The additional area available for such complexing in isoalloxazines A and B and the variation in the orientation of the extra benzene rings with respect to the N(5) position, which is probably the hydride acceptor site in the reaction with reduced nicotinamides, makes our series an interesting one to study. This is particularly so from the point of view of how the orientation of, for example, NADH with respect to the isoalloxazine in the complex determines the rate of subsequent hydride transfer.

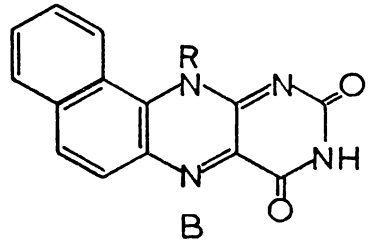
2. SYNTHESIS OF ISOALLOXAZINES

2-1 PLANNING THE SYNTHESIS

As outlined in the previous chapter, we wished to synthesize isoalloxazines with the following basic structures.



A



B

The requirements for the synthesis were therefore as follows

(a) to incorporate the required naphthalene nucleus into the molecule in the correct orientation;

(b) to introduce a substituent at N(10) which would help to offset the anticipated reduced solubility in water of the isoalloxazines resulting from the additional fused benzene ring;

(c) to incorporate a substituent at N(3) so as to eliminate problems of interpretation of kinetic resulting from N(3)-H ionization, which occurs around pH 10[52].

The first of these is the most important and is discussed first as it predetermines the options available to meet the requirements (b) and (c).

2-1.1 Incorporation of the Naphthalene Nucleus

There are several general methods for the preparation of isoalloxazines as summarized by Lambooy[53]:

(a) The condensation of N-substituted ortho-phenylene-diamines with various pyrimidines, such as

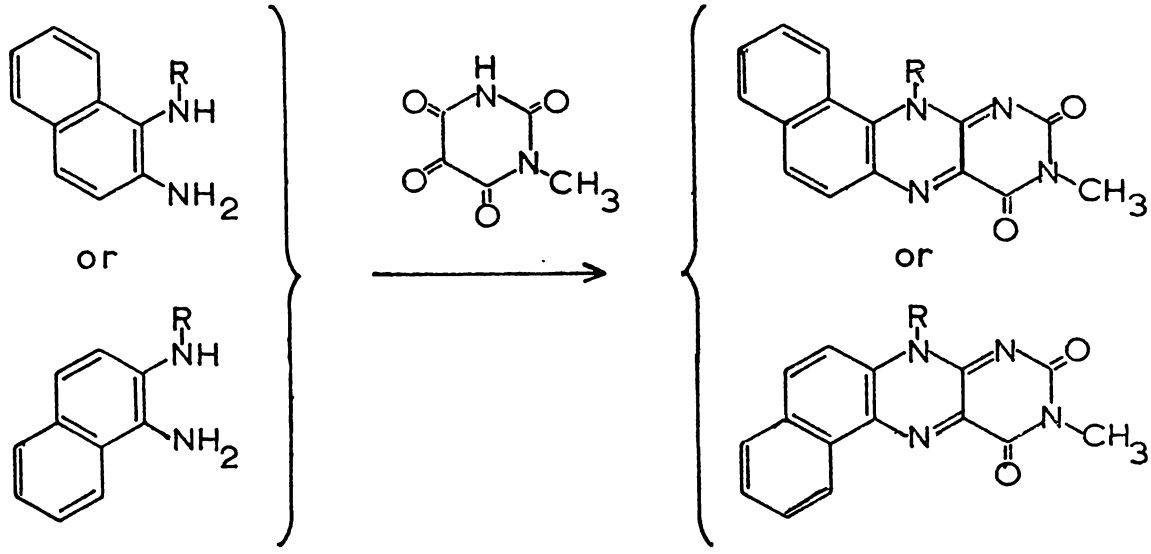
alloxan and the barbituric acids.

(b) The reaction of violuric acid with substituted anilines.

(c) The condensation of diaminouracils with benzoquinones and with biacetyl.

(d) The cyclization of suitable 2,3-quinoxalines.

From the point of view of accessibility of suitable naphthalene derivatives as starting materials, only the first of these methods was open to us. A convenient reaction leading to isoalloxazines of the required structure seemed to involve the condensation of 1-methylalloxan with mono-(N)-substituted-1,2-diaminonaphthalenes (Scheme I).

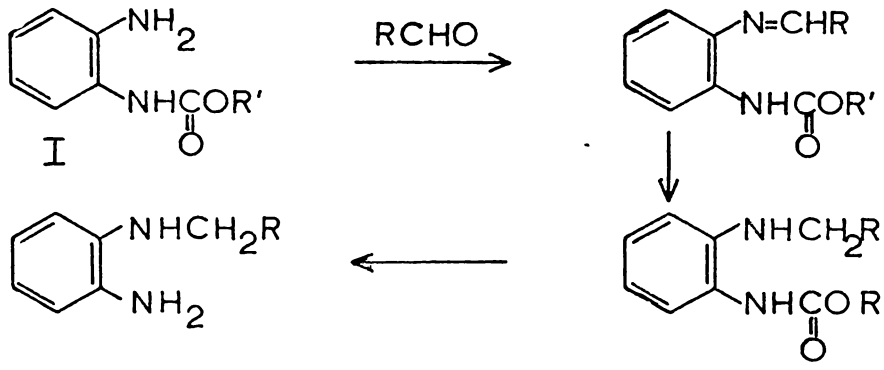


Scheme I

The synthetic problem was reduced therefore to the preparation of a suitable pair of isomeric N-alkyl-1,2-diaminonaphthalenes. The following standard synthetic methods[53] for the preparation of mono-(N)-substituted-1,2-diaminobenzenes were considered from the point of view of applicability to the naphthalene derivatives.

(a) The reaction of 1-amino-2-acylaminobenzenes (I) with aldehydes gives the corresponding imino compound which

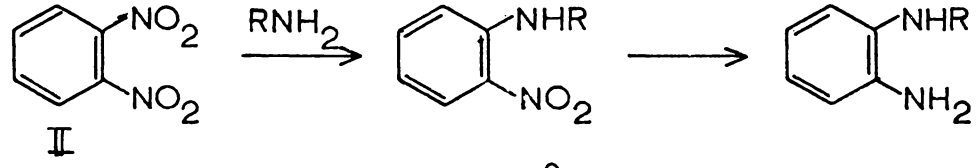
is then reduced to give the secondary amine (equation 2.1).



(2.1)

The free diamine is obtained by mild alkaline hydrolysis of the acylamino group.

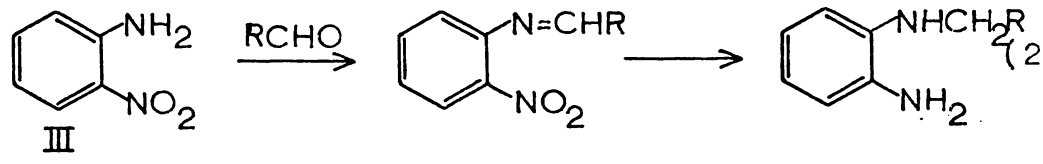
(b) One of the nitro groups in ortho-dinitrobenzenes (II) can be substituted by primary amines to give a product which is easily reduced to the diamine (equation 2.2).



(2.2)

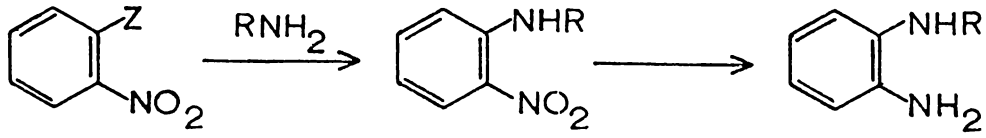
Elevated temperatures (130-140°) have been found to be necessary to promote this reaction, in some cases for several hours [54-63].

(c) This procedure is similar to (a) in that it involves the condensation of an aldehyde with an arylamine, specifically a 2-nitroaniline (III). The condensation product is then reduced to the diamine (equation 2.3).



(2.3)

(d) In 1-halo-2-nitrobenzenes (IV) the halogen is activated by the ortho-nitro group such that it undergoes nucleophilic substitution by amines (equation 2.4). This reaction is the most commonly used preparation of the diamine in the synthesis of isoalloxazines. The halogens

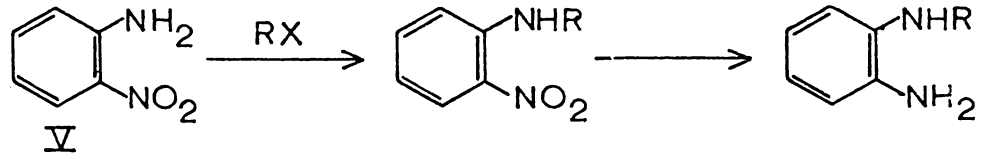


(2.4)

IV Z = F, Cl, Br, I

appear to follow the usual order of decreasing reactivity for aryl halides: F > Cl > Br > I. The nitroamine is reduced by any of the standard methods.

(e) The fifth sequence involves the reaction of a 2-nitroaniline (V) with an alkyl or aryl halide at refluxing temperature in the presence of potassium carbonate and cuprous iodide or potassium acetate and cupric hydroxide[64]. The N-substituted nitroamine can then be reduced by the common reagents.(equation 2.5).

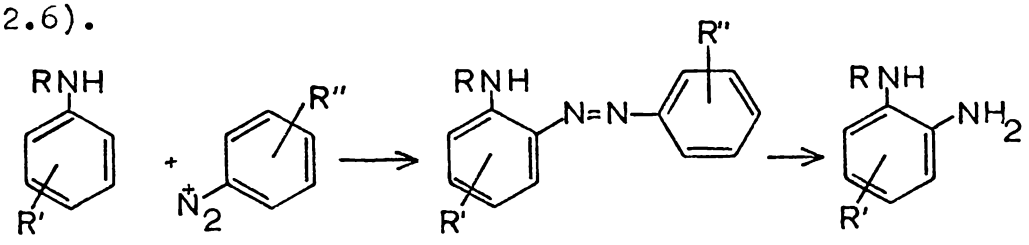


(2.5)

V

The N-tolylsulphonamides and N-acylnitroanilines may also be used in this procedure but extra steps involving the hydrolysis of the N-substituent are necessary.

(f) Aryl diazonium salts (VII) react with N-substituted anilines (VI) to form 2-azoamino compounds (VIII) if the 4-position is blocked and this can be followed by reduction to give the required phenylenediamine (equation 2.6).



(2.6)

VI VII VIII

None of the above methods (a) to (f) were considered to be ideal for our syntheses for the following reasons:

(a) A specifically acylated 1,2-diaminonaphthalenes

would be required and these are not readily accessible.

(b) 1,2-dinitronaphthalene is not readily available and the non-equivalence of the nitro groups would probably lead to a mixture of isomeric products which would be difficult to separate.

(c) and (e) 1-Amino-2-nitronaphthalene could be obtained via nitration of N-acylated-1-aminonaphthalene but only as a minor byproduct since 4-substitution would predominate. 2-Amino-1-nitronaphthalene could probably be obtained via nitration of N-acylated-2-aminonaphthalene but this would have required the handling of highly toxic 2-aminonaphthalene.

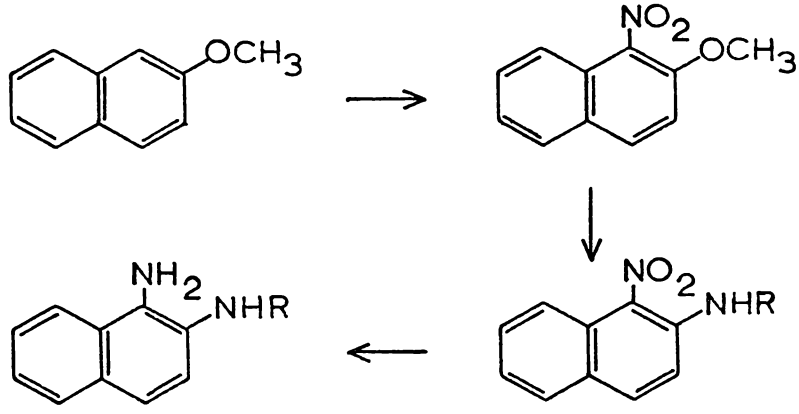
(f) Objections exactly equivalent to those of (c) and (e) apply.

(d) For this method 1-halo-2-nitro- and 2-halo-1-nitro-naphthalenes are required. Preparation of these via Sandmeyer type reactions of nitro-substituted diazonium salts requires the same aminonitronaphthalenes as for (c) and (e) and the same objections therefore apply. The alternative, direct nitration of halonaphthalenes is of no value as the deactivating effect of the halogen would probably determine that most substutution would occur in the unsubstituted fused benzene ring.

We were left, therefore, to consider alternatives to the above methods. The one method which seemed most subject to suitable modification is (d); the substituent Z (in IV) displaced in the nucleophilic aromatic substitution reaction with amine need not necessarily be a halogen. More importantly, if an alternative substituent Z could be found which was not only o,p-directing in electrophilic

aromatic substitution but also activating, then the major objection applying to the halo-substituent outlined above would not apply.

The alkoxy group is known to be displaced by hydroxide and thiophenoxide in activated nucleophilic aromatic substitution, but we have been unable to find any reference to substitution of the alkoxy group by amines, not even in kinetic studies, but we pursued this possibility since the group is a powerfully activating and o,p-directing group in electrophilic aromatic substitution. Also, monosubstitution in 2-substituted naphthalenes generally occurs almost exclusively at the 1-position with o,p-directing substituents[65a]. Thus a suitable route to a 2-alkylamino-1-nitronaphthalene appeared to be nitration of 2-methoxynaphthalene followed by nucleophilic aromatic substitution of methoxide with amine, and finally reduction (equation 2.7).

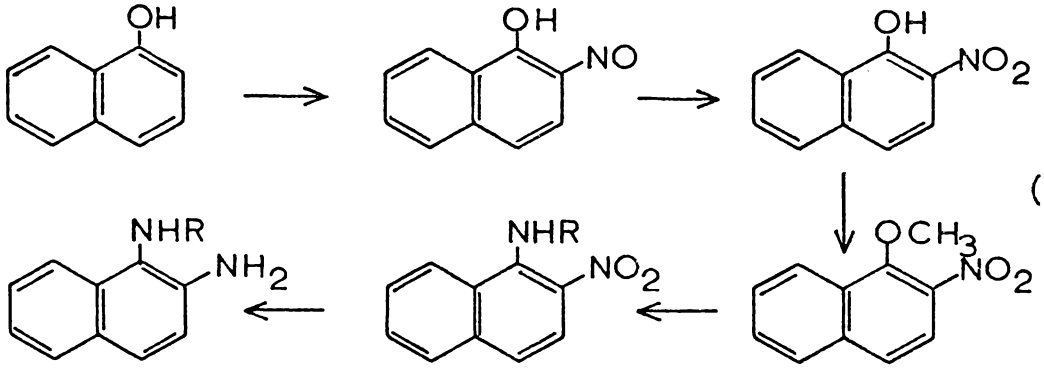


(2.7)

This turned out to be a successful route.

The equivalent sequence starting with 1-methoxynaphthalene would give not only the required 1-methoxy-2-nitronaphthalene but also the 4-nitro derivative, the latter probably in considerable excess[66]. In view of the probable difficulty of separation and purification of the required

2-nitro derivative we looked into the alternative of phenol nitration prior to methylation and found that 2-nitro-1-naphthol can be prepared in good yield via nitrosation of 1-naphthol followed by oxidation of 2-nitroso-1-naphthol[67]. Steam distillation can be used to separate it from the 4-nitro-1-naphthol. The following synthetic route was thus attempted and found to be successful (equation 2.8).



Although we have used the hydroxy and methoxy groups to achieve a particular substitution pattern in the naphthalene derivatives, the method is quite general and its application to benzene derivatives should allow preparation of isoalloxazines with substituent patterns at the 6, 7, 8 and 9 positions in the isoalloxazine nucleus not previously readily accessible.

2-1.2 The N(3)- and N(10)-Substituents of the Isoalloxazine Nucleus

As already indicated in the above section, a methyl group can be readily incorporated at the N(3)-position through the use of 1-methylalloxan in the final condensation reaction.

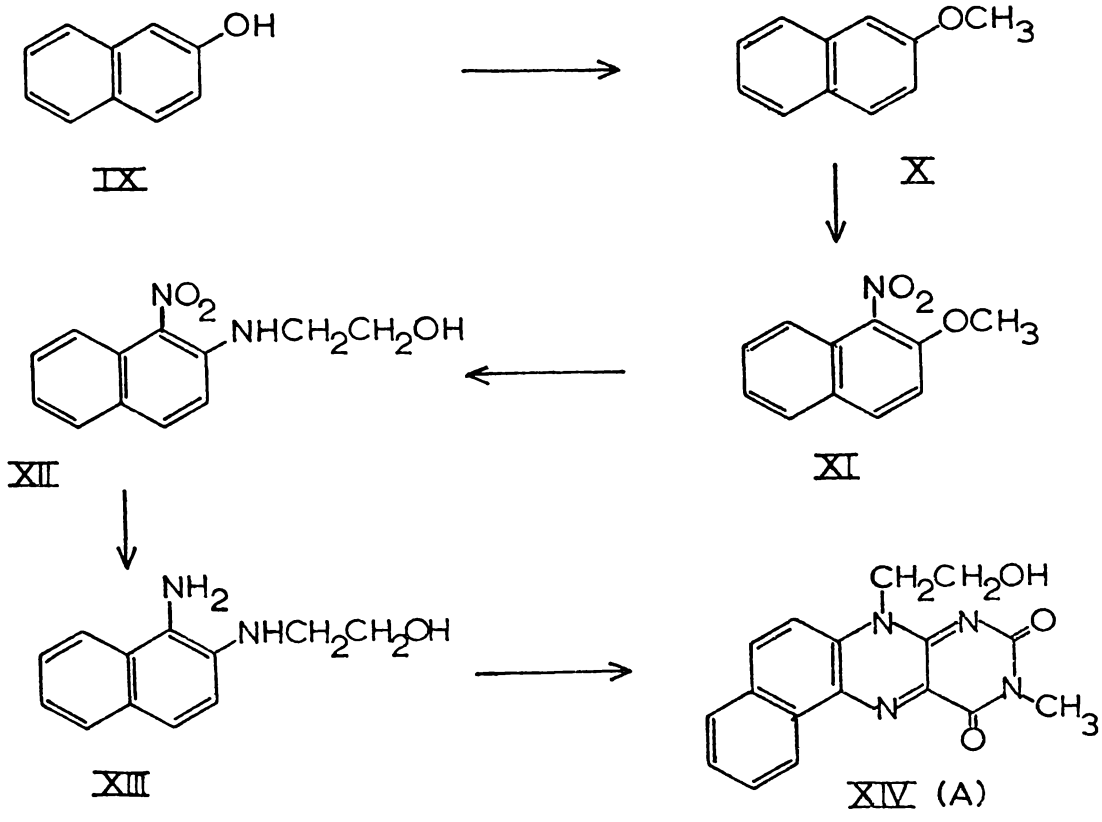
The N(10)-substituent of the isoalloxazine is derived from the amine used in the nucleophilic aromatic substitution stage of the synthesis described above in section

2-1.1. We chose therefore to use 2-aminoethanol as the amine in the hope that the 2'-hydroxyethyl substituent at the N(10)-position of the isoalloxazine would improve the solubility. 2-Aminoethanol is less basic and less nucleophilic than the alkylamines[68] but it reacted sufficiently fast for our purposes with the methoxynaphthalenes.

2-2 THE SYNTHETIC STAGES

The following reaction sequences (Schemes II, III, IV and V) were carried out during the synthesis of the three isoalloxazines, labelled for convenience A, B and C.

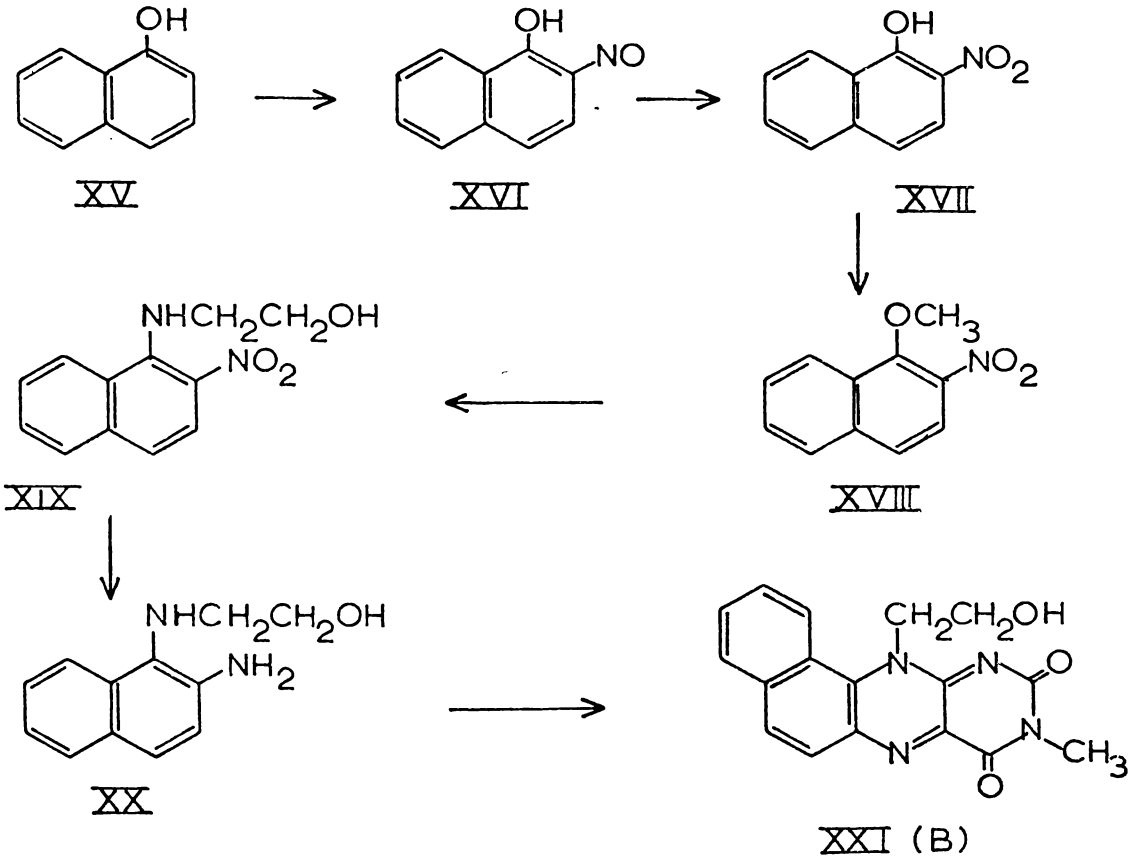
(a) Isoalloxazine A: (7-(2'-hydroxyethyl)-10-methylnaphtho[1,2-g]pteridine-9,11(7H,10H)-dione)



IX X XII XI XIII XIV (A)

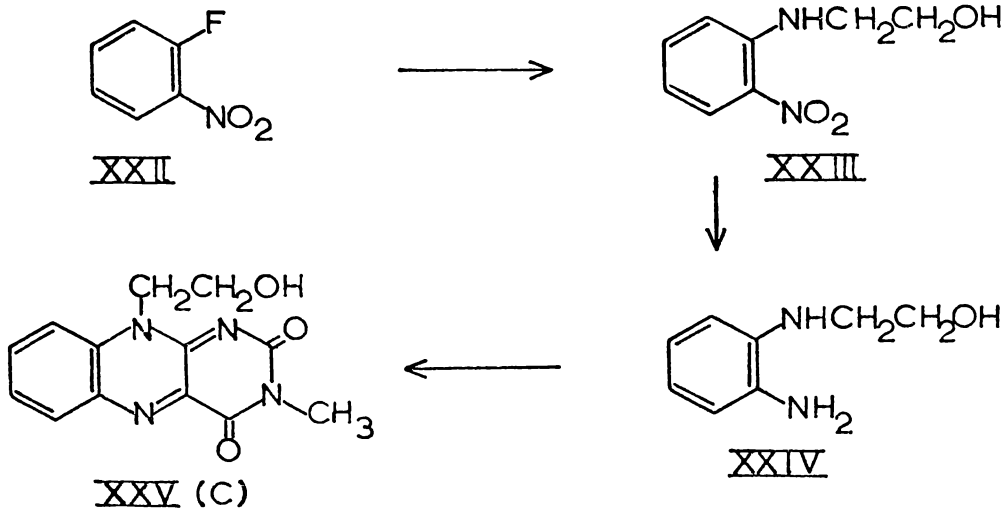
Scheme II

(b) Isoalloxazine B: (12-(2'-hydroxyethyl)-9-methyl-naphtho[2,1-g]pteridine-8,10(9H,12H)-dione)



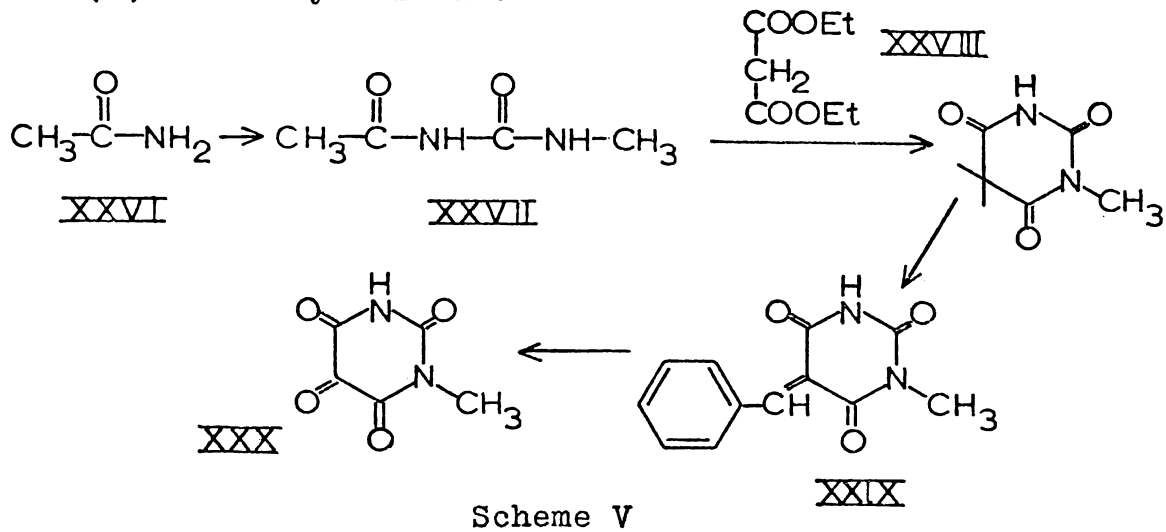
Scheme III

(c) Isoalloxazine C: (10-(2'-hydroxyethyl)-3-methyl-benzo[g]pteridine-2,4(3H,10H)-dione)



Scheme IV

(d) 1-Methylalloxan:



Scheme V

2-2.1 Preparation of 1-Methylalloxan

Alloxans can be synthesized by the oxidation of the corresponding barbituric acid derivatives. The latter compounds are obtained as products from the condensation of diethylmalonate and N-substituted ureas. Attempts were made to carry out the condensation of diethylmalonate with methylurea in the presence of sodium ethoxide[69] and the subsequent reaction of the product with benzaldehyde to give 5-benzal-3-methylbarbituric acid[70], but only low yields (7-10%) of the barbituric acid were obtained. Better yields were achieved when diethylmalonate was condensed with 1-acetyl-3-methylurea in the presence of magnesium methylate[71]. Benzalbarbituric acids are readily oxidized to the corresponding alloxans by chromium trioxide[70] in good yield.

2-2.1.1 Preparation of 1-Acetyl-3-methylurea (XXVII)[72]

Acetamide (XXVI, 59g, 1 mol) was dissolved in liquid bromine (88g, 0.55mol) by warming gently. Sodium hydroxide (40g, 1 mol) in 160ml of water was then added slowly with

stirring. The yellow solution was heated on a water bath until an effervescence began. The mixture was cooled in an ice bath and the crystallized product was filtered at the pump and washed with cold water. Recrystallization from hot water gave pure 1-acetyl-3-methylurea, m.p. 181° (lit.[73] 180-1°). Yield 32g (55%).

2-2.1.2 Preparation of 5-Benzal-3-methylbarbituric acid (XXIX)[70,71]

Magnesium turnings (9.5g, 0.4mol) were dissolved in methanol (200ml). Diethylmalonate (XXVIII, 25g, 0.15mol) was added, followed by 1-acetyl-3-methylurea (32g, 0.3mol). The mixture was then refluxed for 72 hours. The solvent was removed under reduced pressure and the residue was taken up in hot water and acidified with dilute hydrochloric acid. Benzaldehyde (17g, 0.16mol) was then mixed with the hot solution of 3-methylbarbituric acid and left to cool. The solid material was collected and then suspended in absolute ethanol. 5-Benzal-3-methylbarbituric acid was filtered at the pump and dried over silica gel, m.p. 227° (lit.[74] 220.5-222.5°). Yield 23g (64%).

2-2.1.3 Preparation of 1-Methylalloxan (XXX)[70]

Chromium trioxide (20g, 0.2mol) was dissolved in water (10ml) and then glacial acetic acid (50ml) was added. With the temperature of this solution being maintained between 50° and 60°, 5-benzal-3-methylbarbituric acid (23g, 0.1 mol) was added in small portions with stirring. After the final addition of barbituric acid the temperature was maintained at 50-60° for a further 30 minutes. The mixture was then

cooled at 9° for 24 hours. 1-Methylalloxan was filtered at the pump and was washed with cold glacial acetic acid until colourless. The product was recrystallized from glacial acetic acid to give pure 1-methylalloxan, m.p. 135-40° (lit.[73] 150°. This is presumably the hydrate; after drying at 100° for 12 hours, m.p. 198°.). Yield 12g (76%).

2-2.2 Preparation of the Methoxy-nitronaphthalenes

The sequences for these syntheses have been outlined above in section 2-1.1. The experimental details are as follows.

2-2.2.1 Preparation of 2-Methoxy-1-nitronaphthalene (Scheme II)

(a) Preparation of 2-Methoxynaphthalene (X)[75a]

2-Hydroxynaphthalene (IX, 100g, 0.7mol) was mixed with sodium hydroxide (29g, 0.7mol) dissolved in water (420ml). The solution was cooled to 0° and dimethyl sulphate (88g, 0.7ml) was added with stirring. The mixture was then placed on a steam bath for one hour. The product was collected at the pump and washed with 10% sodium hydroxide solution. The solid was recrystallized twice from methanol to give pure 2-methoxynaphthalene, m.p. 72-4° (lit.[75a] 72°). Yield 97g (88%).

(b) Preparation of 2-Methoxy-1-nitronaphthalene (XI)[65b]

2-Methoxynaphthalene (50g, 0.32mol) was dissolved in acetic acid (160ml) and cooled to 5°. Nitric acid (22.5ml, sp.gr. 1.42) was added slowly with stirring, the

temperature being maintained below 20°. The solution was stirred for a further 30 minutes after the final addition of nitric acid and then cooled to 5° again. The crystallized product was filtered at the pump and washed with cold water. Recrystallization from glacial acetic acid gave pure 2-methoxy-1-nitronaphthalene, m.p. 127-8° (lit.[73] 128°). Yield 38g (59%).

2-2.2.2 Preparation of 1-Methoxy-2-nitronaphthalene (Scheme III)

(a) Preparation of 1-Methoxy-2-nitrosonaphthalene (XVI)[67]

A solution of 1-hydroxynaphthalene (XV, 20g, 0.14 mol) in 5% sodium hydroxide solution (200ml) was mixed with a solution of sodium nitrite (10g, 0.14mol) in 2.5l of ca. 0.1M sulphuric acid containing 500g of ice. The yellow precipitate was collected after 12 hours and it was than redissolved in a theoretical amount of very dilute sodium hydroxide solution (0.1%). The product was then reprecipitated with dilute hydrochloric acid. The mixture of 2- and 4-nitroso-1-naphthols was collected and dried over silica gel under reduced pressure.

(b) Preparation of 1-Hydroxy-2-nitronaphthalene (XVII)[67]

The nitroso mixture (17g) from above was made into a paste with hydrogen peroxide (59ml, 100vol) and 10% ferrous sulphate solution (2ml). A 20% solution of sodium hydroxide (13ml) was mixed with the paste and after the initial reaction had subsided a further 20ml of the

sodium hydroxide solution was added. After 12 hours the reaction mixture was diluted to 500ml with water. This solution was then boiled and filtered at the pump while still hot. When the solution was cool it was acidified with dilute hydrochloric acid and the 2-nitro isomer was isolated by steam distillation. The distillate was extracted with chlorofrom. The solution of the product in chloroform was dried over anhydrous magnesium sulphate and the solvent was then removed under reduced pressure. 1-Hydroxy-2-nitro-naphthalene was recrystallized from absolute ethanol, m.p. 127° (lit.[73] 127-8°). Yield 8,2g (31% from 1-naphthol).

(c) Preparation of 1-Methoxy-2-nitronaphthalene (XVIII)[75b]

A solution of diazomethane* in diethyl ether was mixed with 1-hydroxy-2-nitronaphthalene (XVII, 5.3g, 0.03 mol) in methylene chloride (100ml) until an excess of the reagent was present. The solution of the product was then dried over anhydrous magnesium sulphate and the solvent was removed under reduced pressure. The product was placed on a short column of alumina (BDH, Brockman, activity II), eluted with carbon tetrachloride and recrystallized from glacial acetic acid to give crystals of 1-methoxy -2-nitro-naphthalene, m.p. 79-80° (lit.[66] 80°). Yield 2.3g (40%).

2-2.3 Preparation of the (2'-Hydroxy-ethylamino)nitro Compounds

Test reactions of 2-aminoethanol with the nitro ethers

* Prepared from N-nitrosomethylurea and potassium hydroxide[75c].

showed an immediate deepening of the colour of the solution indicating that the ortho-nitroamine was forming. Comparison of the UV-visible and nmr spectra of the starting material and the coloured product confirmed this. When the replacement reaction was carried out in 100% 2-aminoethanol it was found that decomposition of the nitroamine occurred to give a brown product if the reaction was allowed to continue for too long or the temperature became too high. Increased yields were obtained when the substitution reaction was conducted in chloroform as solvent.

2-2.3.1 Preparation of 2-(2'-Hydroxyethylamino)-1-nitronaphthalene (XII, Scheme II)

2-Methoxy -1-nitronaphthalene (XI, 31g, 0.15mol) was mixed with 2-aminoethanol (100ml) and heated on a steam bath for 30 minutes. The cooled solution was then mixed with chloroform and repeatedly extracted with water. The chloroform fraction was dried over anhydrous magnesium sulphate and the chloroform was removed under reduced pressure. The solid was recrystallized twice from chloroform to give bright orange crystals of 2-(2'-hydroxyethylamino)-1-nitronaphthalene, m.p. 127-8°. Yield 8.7g (25%). (Found: C, 62.12; H, 5.25; N, 11.90%. Calculated for $C_{12}H_{12}N_2O_3$: C, 62.06; H, 5.21; N, 12.06%. The nmr spectrum (DMSO) showed signals at τ 6.32 (4H, multiplet, $-CH_2CH_2-$); 1.98-2.80 (5H, two doublets at 2.10 and 2.76, J=10.0±0.5Hz for both, other major peaks occur as a multiplet); 1.42 (1H, doublet, J=8.5 ± 0.5Hz); 1.07 and 4.88 (1H each, both broad, -NH and -OH). The ir spectrum (nujol) showed absorptions at (cm^{-1}) 3450 (w,b), 1640 (s), 1565 (s), 1522 (s)

1310(s), 1189(m), 1113(m), 1070(m), 899(m), 805(m), 779(m) and 749(m). The ir spectrum was run on a Shimadzu Infrared Spectrophotometer Model IR-27G; the nmr spectrum was run on a JEOL C-60HL instrument.)

2-2.3.2 Preparation of 1-(2'-Hydroxyethylamino)-2-nitronaphthalene (XIX, Scheme III)

1-Methoxy-2-nitronaphthalene (XVIII, 1.5g 0.007mol) and 2-aminoethanol (1.2g, 0.02mol) were dissolved in chloroform (5ml) and refluxed for 1 hour. The product was then purified by the procedure as described above for the 2-amino-1-nitro isomer. The compound was recrystallized from chloroform to give bright orange crystals of 1-(2'-hydroxyethylamino)-2-nitronaphthalene, m.p. 130-1°. Yield 1.4g (86%). (Found: C, 61.87; H, 5.41; N, 12.06%. Calculated for $C_{12}H_{12}N_2O_3$: C, 62.06; H, 5.21; N, 12.06%. nmr (DMSO) τ 6.35 (4H, multiplet, $-CH_2CH_2-$); 1.98-2.90 (5H, contains two doublets 2.07 and 2.82, J=10.0 ± 0.5Hz for each, other peaks occur as a multiplet); 1.05 and 4.97 (each peak is 1H, the former is broad and the latter is a triplet, J=8.0 ± 0.5Hz, -NH and -OH). ir (nujol, cm^{-1}) 3460(w,b), 1629(s), 1585(s), 1534(s), 1326(s), 1257(s), 1185(s), 1170(s), 1138(s), 1062(m), 953(m), 807(m) and 762(m).)

2-2.3.3 Preparation of 1-(2'-Hydroxyethylamino-2-nitrobenzene (XXIII, Scheme IV)

This synthesis involves the reaction of 1-fluoro-2-nitrobenzene with 2-aminoethanol as outlined in the general reaction sequence (d) in section 2-1.1.

1-Fluoro-2-nitrobenzene (XXII, 10g, 0.07mol) and 2-aminoethanol (12g, 0.2mol) were dissolved in chloroform (70ml) and refluxed for 30 minutes. The product was purified in a similar manner to that for the previous two nitroamines, except that it was recrystallized from carbon tetrachloride to give bright red crystals of 1-(2'-hydroxyethylamino)-2-nitrobenzene, m.p. 69-71° (lit.[76] 69-71°). Yield 8.6g (66%).

2-2.4 Reduction of the (2'-Hydroxyethylamino)nitro Compounds

The transition metals can be used as hydrogenation catalysts for the preparation of the phenylenediamine from its immediate precursor, the nitroamine. Nickel[56,77], palladium[54,78] and platinum[55,57-60,77e,77k,79] oxides have been used with varying degrees of success. The reduction has also been accomplished with palladium and platinum on carbon[77c,79g,80], calcium carbonate[55,79b,80a,81,82] and barium sulphate[55,81].

Chemical reducing reagents may also be used to give the diamine. Examples of reagents which have been utilized are stannous chloride[63,64], tin[82,83] and iron[84] in hydrochloric acid, refluxing water with iron filings[85], hydrogen sulphide or ferrous sulphate in ammonia[86], sodium dithionite[87] in 50% ethanol and zinc in acetic acid[87,88].

Isolation of the phenylenediamines is rarely carried out[82] because they are not especially stable. Generally a large loss is associated with any purification of these readily oxidizable compounds. Consequently they are used immediately without further treatment.

Three reagents were tried for the reduction of the nitroamines. These were Raney nickel and hydrazine hydrate in ethanol, tin in hydrochloric acid , and stannous chloride in hydrochloric acid. The last of these was found to be the best reductant for these compounds. The yields of isoalloxazine obtained were 15-30%, 50% and 60-65% respectively for the three methods in the case of isoalloxazine A. Stannous chloride was also a much more convenient reagent to use than either tin or Raney nickel. The experimental details are as follows.

Stannous chloride (3.6g, 0.016mol) in hydrochloric acid (20ml, sp.gr. 1.18) was added to the (2'-hydroxyethylamino)nitro compound (0.004mol) dissolved in hydrochloric acid (10ml, sp.gr. 1.18). The mixture was heated until it had decolourized. It was cooled to 0^{o} and an equivalent volume of ice-chilled sodium hydroxide solution (30%) was added. The alkaline solution of the diamine was extracted with diethyl ether. The ether was removed under reduced pressure and the residue taken up in a small volume of glacial acetic acid.

2-2.5 The Condensation Reaction Leading to the Isoalloxazine

The synthetic route for this condensation has been outlined above in section 2-1.1 (Scheme I). The condensation of the diamine and the alloxan is commonly carried out in glacial acetic acid with boric acid present[38]. The latter acts as a catalyst in the reaction[77].

The same procedure was employed for all three isoalloxazines prepared and it is as follows.

As mentioned above the diamine was dissolved in glacial acetic acid. This solution was then mixed with a solution of 1-methylalloxan (0.68g, 0.004mol) and boric acid (2g) also in glacial acetic acid and then refluxed for 5 minutes. The solvent was removed under reduced pressure and the residue was suspended in warm water for 30 minutes. The product was collected at the pump and dried over silica gel under reduced pressure. The isoalloxazines were then purified as described in the following section (2-3) and elemental analyses can be found in section 2-4.

2-3 PURIFICATION OF THE ISOALLOXAZINES

It has been found in some cases that if the reduction of the immediate precursor to the diamine becomes too vigorous, loss of the side chain[26d] or loss of other substituents[82] on the ring may occur. In such instances, impurities would occur which would be very similar to the desired isoalloxazine. These would therefore be extremely difficult to remove from the isoalloxazine sample.

Of the three isoalloxazines synthesized only A presented any great problem associated with its purification. Isoalloxazines B and C were found to be sufficiently soluble in glacial acetic acid such that recrystallization of the isoalloxazines from this solvent gave acceptable elemental analyses (section 2-4).

Initial trial tests showed that isoalloxazine A had low solubility in the solvents commonly used for recrystallization of isoalloxazines. Several attempts at the purification of A were made:

(a) recrystallization from dimethyl formamide (DMF);

(b) chromatography on an alumina column (BDH, Brockman, activity II) with methanol as eluant followed by recrystallization from DMF;

(c) recrystallization for a third time from DMF.

Although good crystals of the isoalloxazine were obtained from the recrystallizations, the analyses after each purification step were unsatisfactory. This fact was emphasized by running thin layer silica gel chromatographs with 1-butanol-acetic acid-water (8:2:2) and 1-butanol-ethanol-2M ammonia (3:1:1). Both chromatograms showed two spots very close together.

A second preparative sample of A was chromatographed on silica gel (BDH, 60-120 mesh) with formic acid-chloroform (1:4). Mass spectral analysis of the product obtained indicated that it was, in fact, the formate ester of the 2'-hydroxy function (m/e: 350(p), 322, 306, 292, 278, 221, 193, 179, 166, 152, and 140).

To hydrolyze the ester, the isoalloxazine (330mg) was dissolved in hot hydrochloric acid (25ml, sp.gr. 1.18). After 5 minutes the solution was cooled in an ice/salt bath for 1 hour. The isoalloxazine was filtered at the pump to give brown-orange crystals. The solid was washed with water and dried (under reduced pressure over silica gel) to give bright red crystals of isoalloxazine A, m.p. 287-288.5°.

Recrystallization of isoalloxazine B from glacial acetic acid also gave a bright red product, m.p. 278-9°. Isoalloxazine C was recrystallized twice from glacial acetic acid to give bright yellow crystals, m.p. 294-6°.

2-4 ANALYSIS OF THE ISOALLOXAZINES

Difficulty in obtaining satisfactory elemental microanalysis of isoalloxazines is a well known problem[57,64,90]. The isoalloxazines tenaciously retain solvents after recrystallization; these are only removed after drying at high temperatures and in some extreme cases sublimation in a high vacuum was required[64].

Removal of traces of 'bound water' is the more usual problem[57,90]. Elevated temperatures and reduced pressures are commonly used to eliminate the water. However with high temperatures there is the accompanying possibility of thermal decomposition. Consequently there must be a balance between the temperature used and the amount of water removed from the sample.

The three isoalloxazines were dried at 50° and 1 mm Hg over phosphorus pentoxide in a Towers drying pistol before analysis. The microanalysis data and mass spectral results obtained are as follows.

(a) Isoalloxazine A (XIV): 7-(2'-hydroxyethyl)-10-methylnaphtho[1,2-g]pteridine-9,11(7H,10H)-dione
Found: C, 62.42; H, 4.46; N, 16.62%. Calculated for $C_{17}H_{14}N_4O_3.\frac{1}{3}H_2O$: C, 62.19; H, 4.50; N, 17.06%.
M/e: 322(p), 292, 278, 249, 221, 193, 179, 166, 152 and 140.

(b) Isoalloxazine B (XXI): 12-(2'-hydroxyethyl)-9-methylnaphtho[2,1-g]pteridine-8,10(9H,12H)-dione
Found: C, 63.29; H, 4.53; N, 17.26%. Calculated for $C_{17}H_{14}N_4O_3$: C, 63.35; H, 4.38; N, 17.38%.
m/e: 322(p), 292, 278, 234, 221, 193, 179, 166, 152 and 139.

(c) Isoalloxazine C (XXV): 10-(2'-hydroxyethyl)-3-methylbenzo[g]pteridine-2,4(3H,10H)-dione

Found: C, 56.71; H, 4.51; N, 20.06%. Calculated for $C_{13}H_{12}N_4O_3$: C, 57.35; H, 4.44; N, 20.58%.

m/e: 272(p), 262, 256, 255, 242, 228, 215, 199, 185, 171, 156, 143 and 129.

(The mass spectra were run a Varian MAT CH5 mass spectrometer.)

3. EXPERIMENTAL

Reactions that were carried out with the three isoalloxazines A, B and C involved sulphite, 1,3-dithio-2-propanol (DTP) and reduced nicotinamide adenine dinucleotide (NADH).

3-1 REAGENTS AND PREPARATION OF SOLUTIONS

As the reduced isoalloxazines are readily reoxidized by molecular oxygen the solutions used for the kinetic studies must be made up such that air is excluded. For the aqueous solutions, deaerated (N_2) double distilled water was used in all cases. This water was prepared by reboiling a quantity of double distilled water for 15 minutes and then while the water was cooling oxygen-free nitrogen gas was flushed through it.

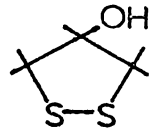
pH determinations made on the buffer solutions and on the reaction solutions were measured on a Radiometer model 26 pH meter with a calomel electrode (Radiometer type K401) and a glass electrode (Radiometer type G202C).

3-1.1 1,3-Dithio-2-propanol

1,3-Dithio-2-propanol (Aldrich) was distilled before use. The reactions of DTP with the isoalloxazines were carried out in 0.1M borax buffer at pH 9.2. A stock solution of the buffer was prepared with di-sodium tetraborate (BDH) and this was flushed with oxygen-free nitrogen prior to the preparation of each dithiol solution. These solutions were then standardized, immediately before each run, against iodine. An aliquot of the solution was

diluted with distilled water and acidified with sulphuric acid (sp.gr. 1.84), excess solid potassium iodide (M&B) was added and the solution was then titrated with standard solution of potassium iodate (BDH)[91,92a]. The equations for this reaction are given in equation 3.1.

$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 + 3H_2O$$

$$I_2 + HOCH(CH_2SH)_2 \longrightarrow 2I^- + 2H^+ + \text{  } \quad (3.1)$$

The endpoint of the titration was determined with starch indicator. This method of standardization of the dithiol solutions produces the iodine in vitro and is preferred tc titration of dithiol with a standard solution of iodine as it minimizes the risk of 'over oxidation' of the the dithiol to higher oxidation states than the disulphide, which might occur in pockets of high iodine concentration during a direct titration procedure[92b].

It was found to be necessary to carry out this standardization of the dithiol solutions each time prior to a run because it was found that the dithiol concentration decreased with time. This was found to cause no great problems for the reactions of isoalloxazines B and C with DTP as the reactions were completed within several minutes of commencing each run for both of these isoalloxazines. However, this apparent instability of DTP did cause problems with isoalloxazine A where the time intervals of the reactions were usually 30 to 60 minutes long and in some cases longer. It is over this length of time that the dithiol concentration changes. This consequently would be expected to give rise to a general irreproducibility inherent in the

reaction of A with DTP. This was in fact observed, the scatter of the points for isoalloxazine A in a plot of k_{obs} against the concentration of DTP (Figure 16, section 4-6) was greater than that observed for the two other isoalloxazines. Although thiols are subject to autooxidization, this requires the presence of metal ions and some oxidant[92c]; oxygen is the most readily available for the latter. As the solutions of the dithiol are anaerobic this possibility may be discounted, the lack of oxygen is also shown by the fact that no reoxidation of the reduced isoalloxazine species occurred when the reaction solution was shaken during or after the run had been completed and prior to the opening of the reaction cell. The instability of DTP may be due to closeness of the two thiol functions. It has been shown that the rate of autooxidation of dithiols is dependent on the distance of separation of the two groups[92d].

3-1.2 Reduced Nicotinamide Adenine Dinucleotide

Reduced nicotinamide solutions were prepared by mixing weighed amounts of NADH (Sigma) with a 0.05M phosphate buffer; the concentrations were then calculated using a molecular weight of 771 (supplied by Sigma). The buffer stock solution was prepared from sodium hydrogen phosphate (BDH) and sodium dihydrogen phosphate (M&B) to give a pH of 6.3. Each solution of reduced nicotinamide was flushed with oxygen-free nitrogen for at least 15 minutes before being used.

3-1.3 Sulphite

The reactions of sulphite with the three isoalloxazines were also conducted at a pH of 6.3. No secondary buffer system was required for these reactions since the concentrations of sulphite employed were such that they acted as their own buffer. The solutions were prepared from sodium sulphite (M&B) and sodium meta-bisulphite (M&B). The sulphite concentrations were calculated using a pK_a for sulphurous acid of 6.63 (30^{o})[34b].

3-1.4 Isoalloxazines

The three isoalloxazines were dissolved in redistilled dimethyl formamide (BDH). The concentrations of the stock solutions were such that a 0.05ml aliquot would give an isoalloxazine concentration in the reaction cell of approximately 5×10^{-5}M. These stock solutions were also rigorously flushed with oxygen-free nitrogen gas.

3-2 KINETIC MEASUREMENTS AND ANALYSIS OF DATA

The reaction cell used in the kinetic runs was a modified Thunberg tube (Figure 2). The isoalloxazine

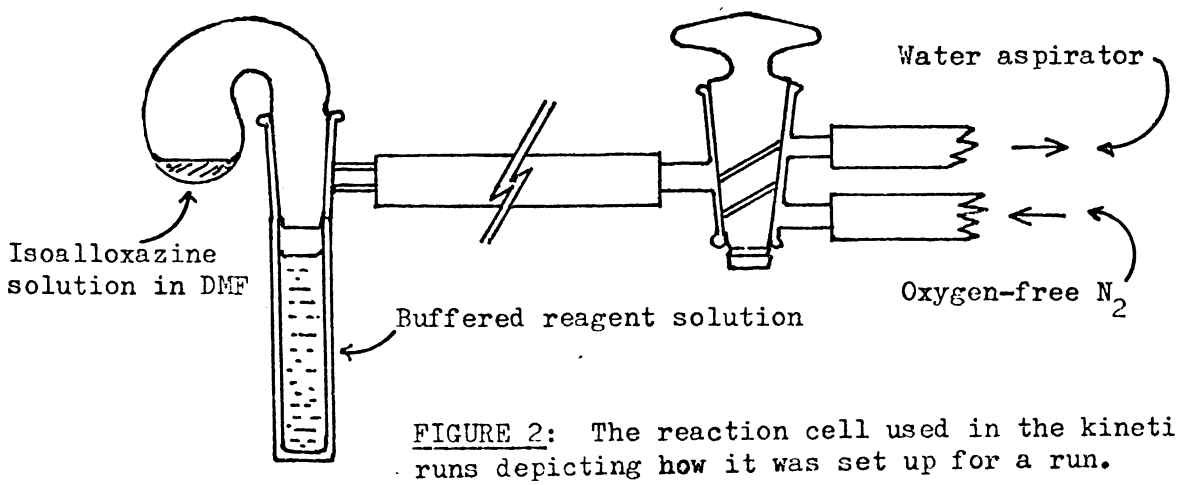


FIGURE 2: The reaction cell used in the kinetic runs depicting how it was set up for a run.

solution, normally 0.05ml, was placed in the cell cap as shown in Figure 2. The volume of the isoalloxazine solution was kept this small to minimize temperature drops when the two solutions were mixed to initiate the reaction. The amount of dimethyl formamide in the reaction solution after mixing was no greater than 2%.

The solution of the second reagent was placed in the cuvette and in the case of the sulphite and NADH reactions it was flushed for 15 minutes with oxygen-free nitrogen. This practice was not continued with the DTP solutions; due to the instability of the 1,3-dithio-2-propanol solutions this procedure was eliminated in favour of commencing the runs as soon as possible after the solutions had been prepared.

The cell system was assembled and sealed with Apiezon M grease after the cell cap and the remaining space above the solution in the cell had been carefully flushed with nitrogen. The Thunberg tube was then alternately evacuated with a water aspirator and filled with oxygen-free nitrogen. This cycle was repeated from 20 to 25 times. Care is necessary to eliminate oxygen from the system as the reduced isoalloxazine is readily reoxidized by molecular oxygen and even very small amounts of oxygen could clearly cause deviations in the observed kinetics of the reaction under study. The cell was finally closed at atmospheric pressure under nitrogen and placed in the thermostated cell holder of the spectrophotometer. The temperature was allowed to equilibrate for 15 minutes at 30° before the reaction was initiated by mixing the two reactants.

The reaction between the isoalloxazines and the reduc-

tants was followed at constant wavelength by recording the disappearance of the isoalloxazine continuously with time on a Cary 17 spectrophotometer. The absorption was measured at wavelengths of 460, 485 and 434 nm for isoalloxazines A, B and C respectively.

Repetitive scan experiments were also conducted on some of the runs in order to observe the formation of possible intermediates during the course of the reactions. These runs were also carried out on the Cary 17 and a Unicam SP 1800B Ultraviolet spectrophotometer with a Unicam SP 1805 program controller was also used; the spectra for the latter instrument were recorded on a Unicam AR linear recorder.

After each run had been in progress for approximately five half-lives, the reaction solution was allowed to stand for a further equivalent period of time to obtain an infinity reading as a check against the computed value for this parameter.

Before air was readmitted to the cell at the end of each run the Thunberg tube was shaken and the absorption rechecked for any increase over the infinity reading. Such a change would indicate the possible presence of oxygen in the cell. Air was then readmitted to the cell and mixed with the solution. The absorption was again measured. This is a test to determine the percentage regeneration of the oxidized isoalloxazine. Full regeneration of the isoalloxazine would indicate that the disappearance of the isoalloxazine was due to the redox reaction and not some irreversible process such as hydrolysis of the isoalloxazine nucleus. The pH of the reaction solution was measured

after the completion of each run.

The absorption-time data obtained from the kinetic runs were treated by a non-linear least squares computer program run on a Digital PDP 11/05 computer. An explanation of and the theory behind this program is in Appendix I. The observed rate constants were thereby obtained for several concentrations of the reductants for each isoalloxazine.

3-3 MISCELLANEOUS EXPERIMENTAL DETERMINATIONS

Several other experiments were conducted to obtain pertinent data on the reagents as described below.

3-3.1 Polarography

The polarographic half-wave potential is the potential at the point on the polarographic wave where the current is equal to one-half the diffusion current and it is designated by $E_{\frac{1}{2}}$.

The half-wave potentials of the three isoalloxazines were determined using a Heath polarograph model EUA-19-2 with the Heath dropping mercury electrode model EUA-19-6. The $E_{\frac{1}{2}}$ values obtained were measured against the standard calomel electrode (Coleman) at room temperature. It is not necessary to determine the $E_{\frac{1}{2}}$ values at the temperature of the kinetic runs (i.e. 30^{o}) as the half-wave potentials of reversible processes are nearly independent of the temperature[93].

The polarographic half-wave potentials are pH dependent[93] as shown in equation 3.4. This effect arises from

the dependence of $E_{\frac{1}{2}}$ on the difference in the number of protons in the oxidized and reduced species; thus if there is an ionizable proton in one of these two species the difference in the number of protons will clearly change with pH.

For the reaction in equation 3.2

$$Ox + mH^+ + ne^- \longrightarrow Re \qquad (3.2)$$

equation 3.3 can be written

$$E_{\frac{1}{2}} = E^o - \frac{RT}{nF} \ln \frac{D_{ox}^{\frac{1}{2}}}{D_{re}^{\frac{1}{2}}} + m\frac{RT}{nF} \ln a_{H^+} \qquad (3.3)$$

Equation 3.3 can be condensed to equation 3.4

$$E_{\frac{1}{2}} = E_{\frac{1}{2}}^o - 0.059\frac{m}{n}\,pH \qquad (3.4)$$

E^o is the formal potential; D_{ox} and D_{re} are the diffusion coefficients of the oxidized and reduced species respectively; $E_{\frac{1}{2}}^o$ is the value of $E_{\frac{1}{2}}$ at pH 0; m and n are the numbers of protons and electrons, respectively, involved in the process.

The half-wave potentials were therefore determined at the two pHs used in the kinetic experiments, e.g. 6.3 and 8.9. A 0.05M phosphate buffer with 1.0M potassium nitrate was used for the former pH and a 0.01M borax buffer containing 1.0M potassium chloride was used for the higher pH.

The concentration of the isoalloxazine solutions were approximately 1 x 10^{-4}M with the concentration of dimethyl formamide being no greater than 5%. The isoalloxazine solutions were flushed with oxygen-free nitrogen for 15 minutes immediately prior to the $E_{\frac{1}{2}}$ determinations being made under nitrogen.

3-3.2 Ionization Constants

To our knowledge the ionization constants of 1,3-dithio-2-propanol have not been reported in the literature. The dithiol dissociates as shown in equation 3.5.

$$HSCH_2CH(OH)CH_2SH \overset{K_1}{\rightleftharpoons} H^+ + HSCH_2CH(OH)CH_2S^- \qquad (3.5)$$
$$HSCH_2CH(OH)CH_2S^- \overset{K_2}{\rightleftharpoons} H^+ + {}^-SCH_2CH(OH)CH_2S^-$$

The ionization constants, K_1 and K_2, for this process are defined as in equation 3.6.

$$K_1 = \frac{[H^+][CH_2(SH)CH(OH)CH_2S^-]}{[CH_2(SH)CH(OH)CH_2SH]} \qquad (3.6)$$
$$K_2 = \frac{[H^+][CH_2(S^-)CH(OH)CH_2S^-]}{[CH_2(SH)CH(OH)CH_2S^-]}$$

These two parameters were measured by titration with base in aqueous solution at 30° and μ 0.2. Approximately 0.01M dithiol solutions were titrated with 1.006M potassium hydroxide using a Radiometer Autoburette ABU 11. The pH was measured with a Radiometer model 26 pH meter employing a Radiometer type K401 calomel electrode and a Radiometer type G202C glass electrode.

The ionization coefficients were obtained from the pH-volume data using a computer program[94] on a Digital PDP 11/05 computer. A printout of this program and an explanation of it may be found in Appendix II.

3-3.3 Extinction Coefficients

The molecular extinction coefficient is defined as the optical density of the absorbing solution for a concen-

tration of 1 g.mol. per litre of the absorbing species and a path length of 1 cm. The coefficient can be calculated from the Beer-Lambert law which is as shown in equation 3.7.

$$\log I_o/I_t = \varepsilon ct \qquad (3.7)$$

where $\log I_o/I_t$ is the optical density of the absorbing solution; ε is the molecular extinction coeficient; c is the concentration of the absorbing species and t is the path length.

The molecular extinction coefficients were measured for the three isoalloxazines in the visible and near UV regions. The determinations were made in aqueous solution containing 5% dimethyl formamide with isoalloxazine concentrations of 1.492, 2.566 and 1.822×10^{-5}M for isoalloxazines A, B and C respectively. The spectra of the isoalloxazines were recorded on a Cary 17 spectrophotometer with a path length of 1cm.

3-3.4 Equilibrium Constants for Complex Formation

The decrease in the fluorescence of isoalloxazines with increasing concentration of tryptophan has been interpreted as resulting from the formation (as in equation 3.8) of a non-fluorescent complex[27].

$$Fl + Q \rightleftharpoons FlQ \qquad (3.8)$$

The equilibrium constant, K_e, for the formation of the complex with the quenching agent is given by equation 3.9.

$$K_e = \frac{[FlQ]}{[Fl][Q]} \qquad (3.9)$$

The fluorescence of the quenched isoalloxazine solutions were measured relative to the fluorescence of an uncomplexed isoalloxazine solution of identical concentration on a Beckman model 772 Ratio Fluorometer. The measurements were made at 23^{o} and in a 0.05 phosphate buffer (pH 6.34). The tryptophan concentrations ranged from 0.5 to 2.0 x 10^{-3}M and the isoalloxazine concentrations were approximately 1 x 10^{-5}M.

4. RESULTS AND DISCUSSION

The results of the experiments described in Section 3-3 are given first followed by the results of the kinetic studies.

4-1 POLAROGRAPHY

The polarographic half-wave potentials were obtained from the polarograms by drawing straight lines through the residual and limiting currents (see Figure 3) and then drawing a third line which bisects the distance separating the first two lines at each end of the polarogram. The intersection of this bisector with the polarogram gave the $E_{\frac{1}{2}}$ value of the isoalloxazine under study. Seven to nine determinations were made for each of the three isoalloxazines and for riboflavin at pHs of 6.3 and 8.9. The mean values of $E_{\frac{1}{2}}$ and their standard deviations are given in Table 4-1. They decrease with pH in a similar manner and in agreement with the decrease expected from equation 3.4 (section 3-3.1). A typical polarogram of those obtained for the isoalloxazines is shown in Figure 3.

TABLE 4-1

$E_{\frac{1}{2}}$ (V) for isoalloxazines A, B, C and riboflavin against S.C.E. at 22°.

pH	6.3		8.9	
	$E_{1/2}$	s.d.	$E_{1/2}$	s.d.
A	-0.443	0.001	-0.549	0.003
B	-0.261	0.001	-0.410	0.002
C	-0.356	0.005	-0.466	0.002
Riboflavin	-0.392	0.005	-0.496	0.002
Riboflavin[95]	pH 8.0	-0.460		

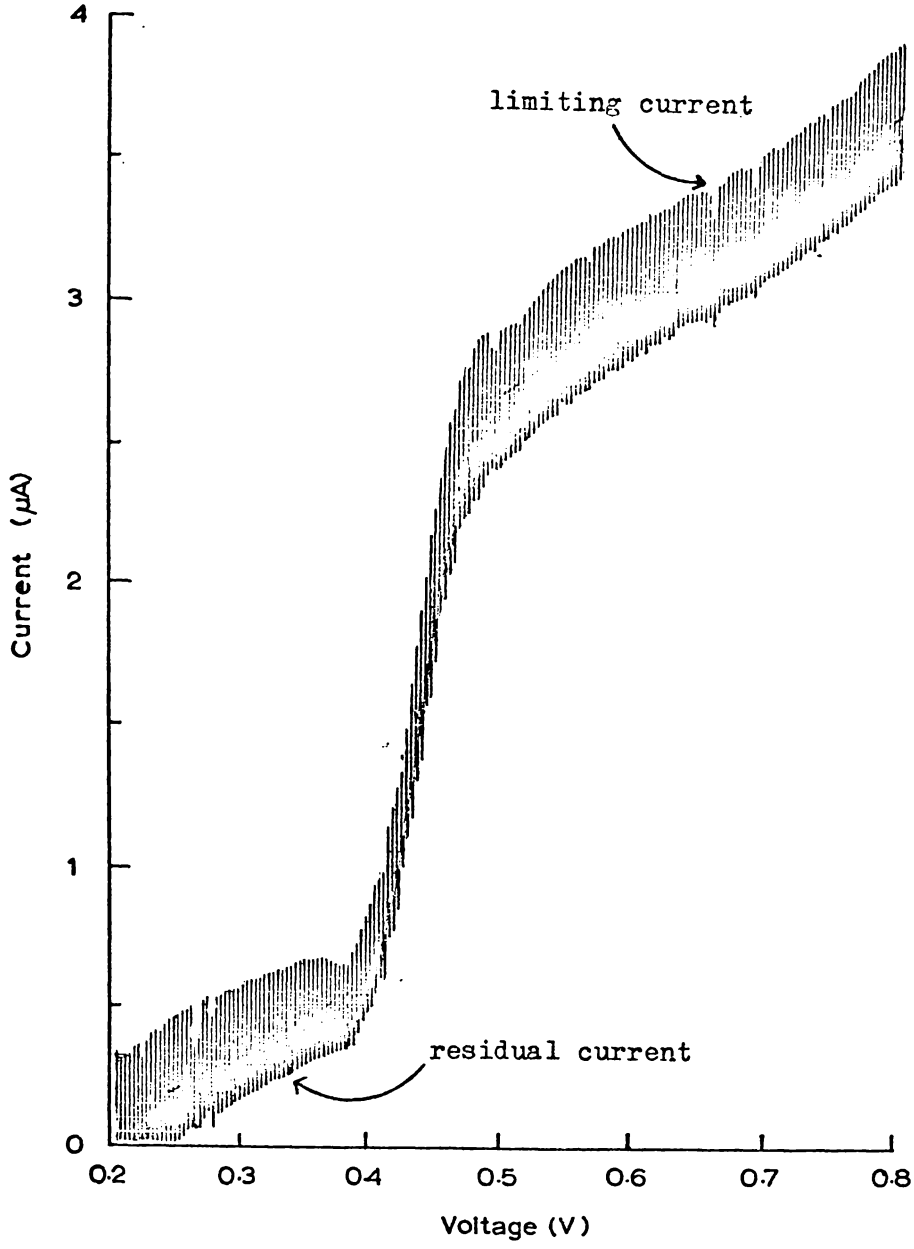


FIGURE 3: A typical example of the polarograms obtained for the isoalloxazines; this particular polarogram is of isoalloxazine A at pH 6.3.

4-2 IONIZATION CONSTANTS OF 1,3-DITHIO-2-PROPANOL

Five titrations of 1,3-dithio-2-propanol with potassium hydroxide were conducted. The pK_1 and pK_2 values for the processes shown in equation 4.1 obtained from the

$$\begin{aligned} HSCH_2CH(OH)CH_2SH &\overset{K_1}{\rightleftharpoons} H^+ + HSCH_2CH(OH)CH_2S^- \\ HSCH_2CH(OH)CH_2S^- &\overset{K_2}{\rightleftharpoons} H^+ + {}^-SCH_2CH(OH)CH_2S^- \end{aligned} \qquad (4.1)$$

runs are given in Table 4-2. The pH-volume data for these runs and a specimen plot of pH against volume of titrant added are given in Appendix II.

TABLE 4-2

pK_1 and pK_2 and their standard deviations of DTP at 30°.

	pK_1	s.d.	pK_2	s.d.
1	9.20	0.06	10.98	0.03
2	9.24	0.10	11.22	0.11
3	9.06	0.17	10.99	0.03
4	9.02	0.18	10.91	0.02
5	9.12	0.01	10.76	0.13
weighted mean*	9.13	0.09	10.96	0.17

* weighting factor = 1/s.d.

4-3 EXTINCTION COEFFICIENTS OF THE THREE ISOALLOXAZINES

The extinction coefficients were calculated from the optical density and concentration data using equation 3.7 in section 3-3.3 and the values for the three isoalloxazines are

given in Table 4-3.

TABLE 4-3

Extinction coefficients, $\varepsilon(M^{-1}cm^{-1})$, for isoalloxazines A, B and C in aqueous solution.

	λ_{max}(nm)	ε_{max}
A	260	46100
	303	19000
	456	16500
	477	16400
B	303	29000
	374	7700
	480	11500
	498	11500
C	264	36400
	341	8190
	434	8780

4-4 EQUILIBRIUM CONSTANTS FOR COMPLEX FORMATION

The measured fluorescence of the quenched isoalloxazine solutions is directly proportional to the concentration of the uncomplexed isoalloxazine since the fluorescence of the solutions are measured as a fraction, R, of the free isoalloxazine fluorescence. Thus the concentration of the complexed isoalloxazine is given by 1 - R. The equilibrium constant, K_e, (given by equation 3.9, section 3-3.4) was then calculated from equation 4.2.

$$K_e = \frac{1-R}{R} \frac{1}{[Q]} \qquad (4.2)$$

[Q] is the concentration of tryptophan.

The decrease in the fluorescence of the complexed isoalloxazine solutions with the concentrations of tryptophan used ranged from 30 to 70% relative to the unquenched isoalloxazine fluorescence. The fluorescence data and the equilibrium constants calculated for the three isoalloxazines are given in Table 4-4 with an estimation of the experimental error.

TABLE 4-4

Effect of tryptophan concentration on the fluorescence of isoalloxazines; and equilibrium constants (K_e) for isoalloxazines A, B and C at pH 6.34 and 23°.

	10^3[Q](M)	R	1-R	K_e
A:	5	0.640	0.360	113
	10	0.470	0.530	113
	15	0.377	0.623	110
	20	0.308	0.692	112
			mean	112 ± 3
B:	5	0.670	0.330	99
	10	0.506	0.494	98
	15	0.495	0.605	102
			mean	100 ± 3
C:	5	0.695	0.305	88
	10	0.540	0.460	85
	15	0.440	0.560	85
			mean	86 ± 3

4-5 REACTION OF ISOALLOXAZINES WITH SULPHITE ION

The reaction of isoalloxazines with sulphite ion was discussed in Section 1-4.2.2. A brief recapitulation is that sulphite adds reversibly to the N(5)-position of the isoalloxazine nucleus. Isoalloxazines unsubstituted at the 1-, 6- and 8-positions react further to yield a 6,8-disulphonated product after reoxidation of the reduced species involved in the reaction.

4-5.1 Reduction of Isoalloxazines A, B and C

Two successive reactions were observed for isoalloxazine C at high sulphite concentration as depicted in Figure 4. There was a rapid disappearance of isoalloxazine at 434nm followed by the slow appearance of the final product at 365nm. This clear definition of two stages to the reaction of C with sulphite was lost at lower concentrations of sulphite such that there appeared to be only one continuous stage (Figure 5).

Repetitive wavelength scans of the reactions of isoalloxazines A and B with sulphite indicated only the one stage to the reaction (Figures 6 and 7, respectively). However as with isoalloxazine C both of these isoalloxazines showed a dependence on the initial sulphite concentration in the amount of isoalloxazine reacting. For example, infinity readings showed that with 0.01M and 0.87M sulphite solutions 70% and 90%, respectively, of isoalloxazine C had disappeared; with 0.44M and 0.87M sulphite solutions the extent of the reaction with isoalloxazine A was 45% and

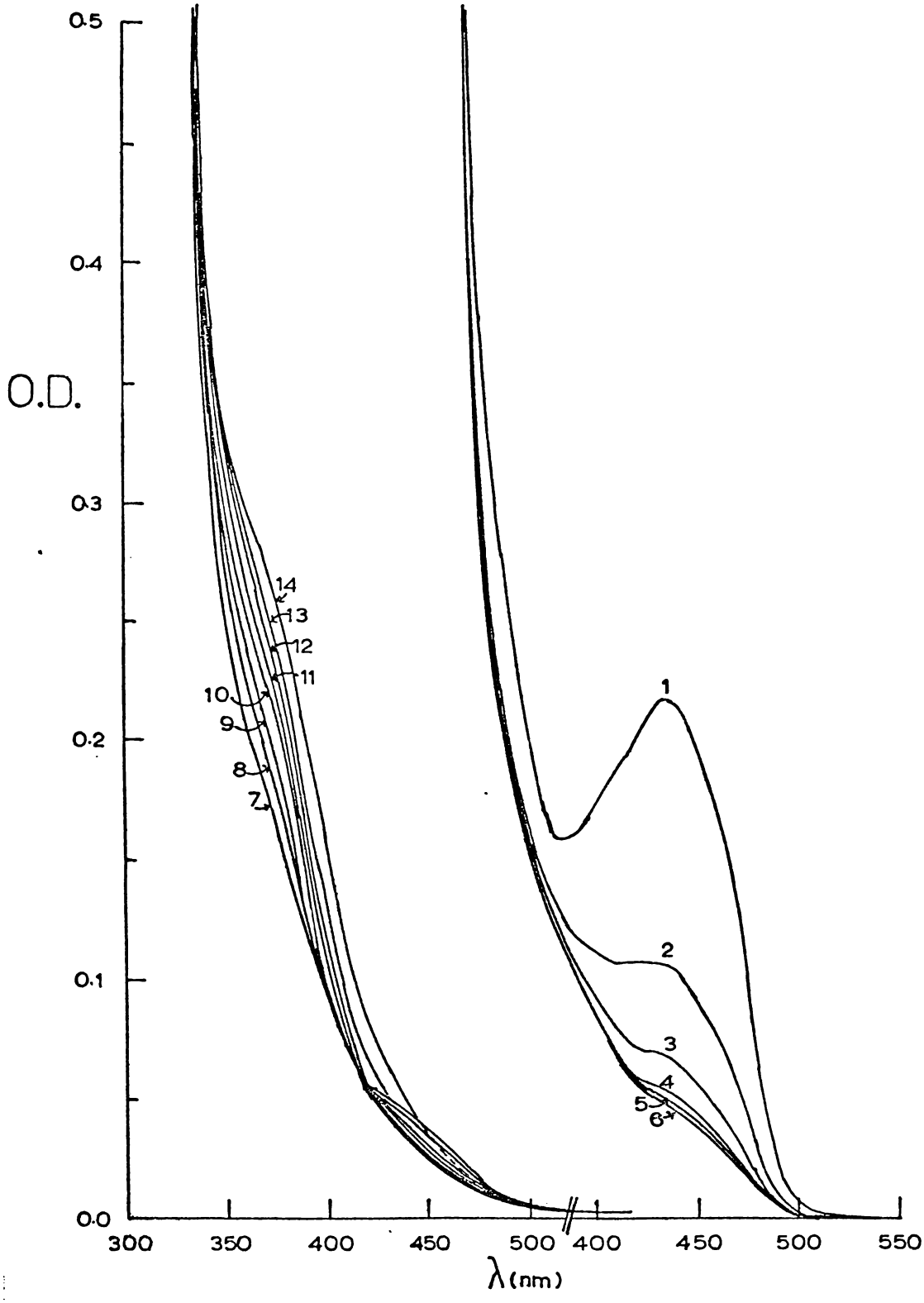


FIGURE 4: Repetitive wavelength scan of the course of the reaction of isoalloxazine C with a 0.14M solution of sulphite at pH 6.3 and 30°. Scan 1. $\frac{1}{4}$min; 2. 1; 3. $1\frac{3}{4}$; 4. $2\frac{1}{2}$; 5. $3\frac{1}{2}$; 6. 4; 7. 7; 8. 10; 9. 15; 10. 21; 11. 30; 12. 46; 13. 463min; 14 22hr 21min after mixing.

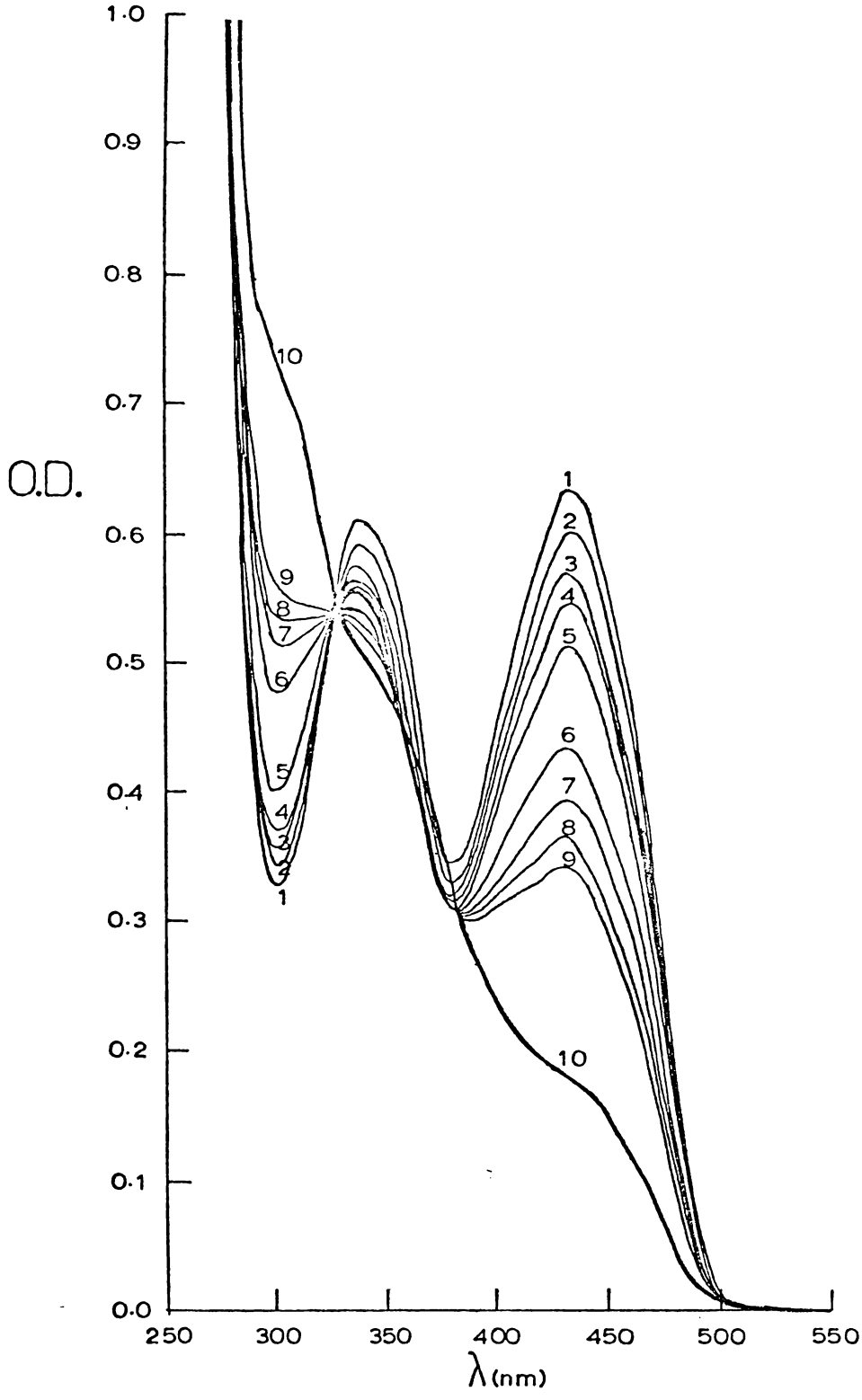


FIGURE 5: Repetitive wavelength scan of the course of the reaction of isoalloxazine C with a 0.01M solution of sulphite at pH 6.3 and 30°. Scan 1. ⅓min.; 2. 3; 3. 8; 4. 18; 5. 59; 6. 191; 7. 271; 8. 334; 9. 391min; 10. 18hr 37min after mixing of the reagents.

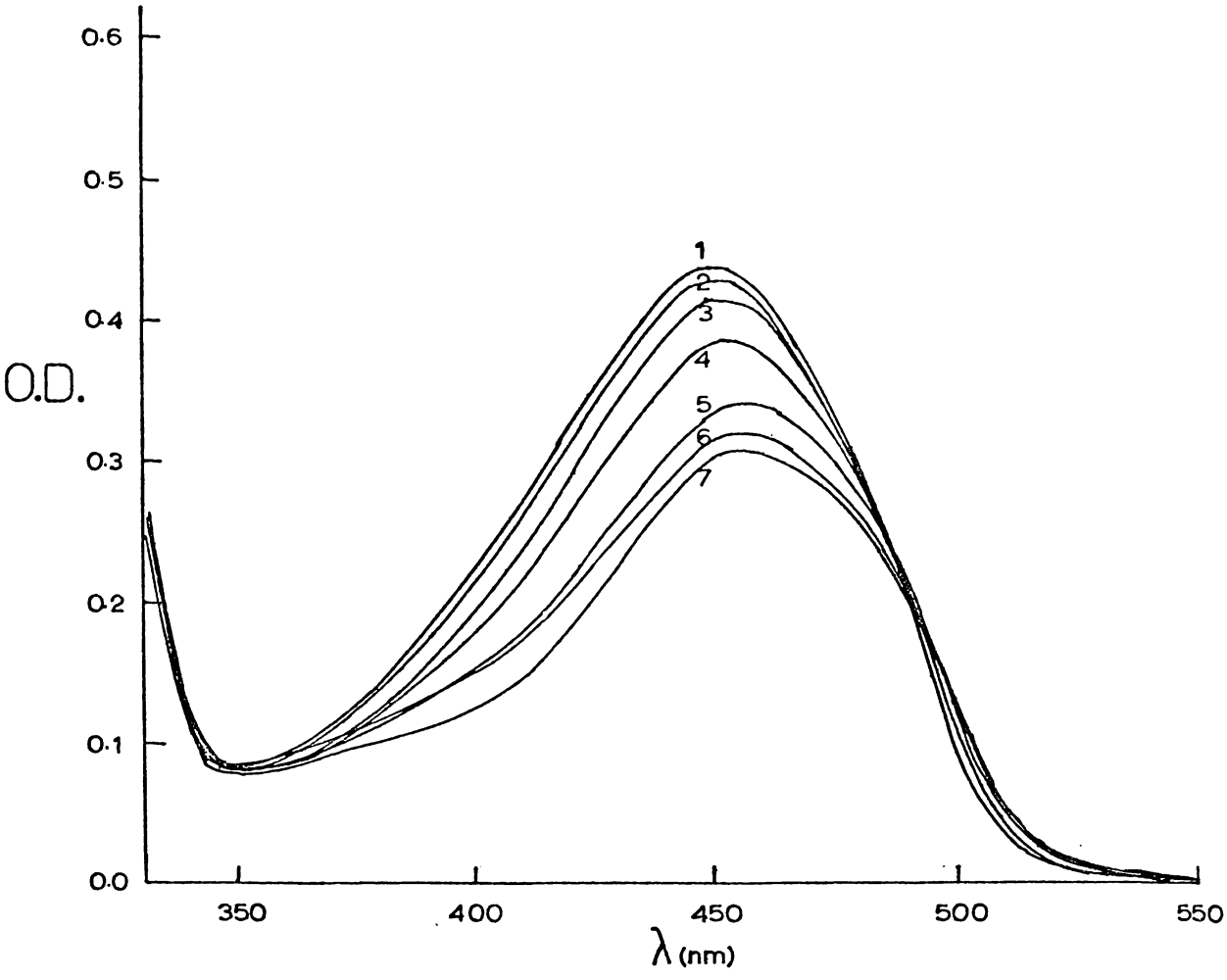


FIGURE 6: Repetitive wavelength scan of the course of the reaction of isoalloxazine A with a 0.87M solution of sulphite at pH 6.3 and 30°. Scan 1. ½min after mixing; 2. 10; 3. 28; 4. 71; 5. 156; 6. 214; 7. 269min.

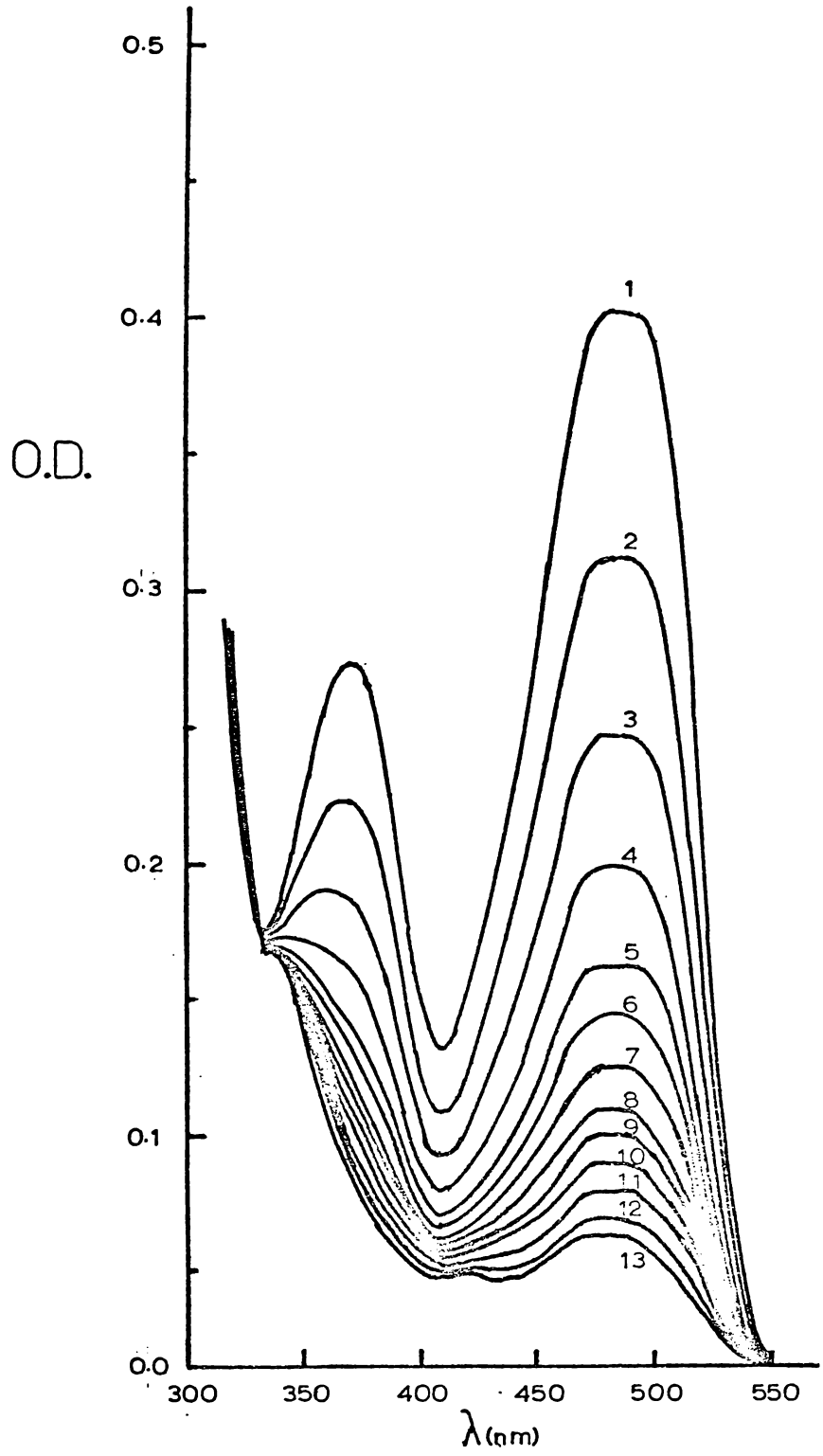


FIGURE 7: Repetitive wavelength scan of the course of the reaction of isoalloxazine B with a 0.01M solution of sulphite at pH 6.3 and 30°. Scan 1. 15sec after mixing; 2-11. 2 minute intervals; 12. 31min; 13. after approximately 20 half-lives.

55% respectively; and 86% and 98% of isoalloxazine B had reacted with 0.01M and 0.87M sulphite solutions respectively.

4-5.2 Reoxidation of the Reaction Solutions of the Three Isoalloxazines

The product from the reaction of C with sulphite is oxidized by the readmittance of air to the reaction solution to give an isoalloxazine which has a shift in the 340nm absorption as compared with isoalloxazine C (Figure 8). This new isoalloxazine then rapidly disappeared to give a product which had a different spectrum from both of the products from the first two anaerobic reactions (Figure 9).

Although both isoalloxazines A and B appear to react similarly with sulphite (e.g. only the one reaction was observed), the reactivity of the reduced species from each isoalloxazine with air differs markedly. Readmittance of air to the reaction solution of A yields an oxidized isoalloxazine with a change in λ_{max} of the long-wavelength absorption (Figure 10). The reaction of A with an aerobic sulphite solution produces the same change in the UV-visible spectrum, and there is also no further reaction occurring unlike the case with isoalloxazine C. The reaction solution of B, on the other hand, is totally insensitive to the presence of oxygen. The appearance of an isoalloxazine was not observed when air is readmitted to the anaerobic reaction solution of B and sulphite. When the sulphite is removed as SO_2 by drawing either air or oxygen-free nitrogen through the acidified solution, oxidized isoalloxazine B is regenerated. There are no changes in the wave-

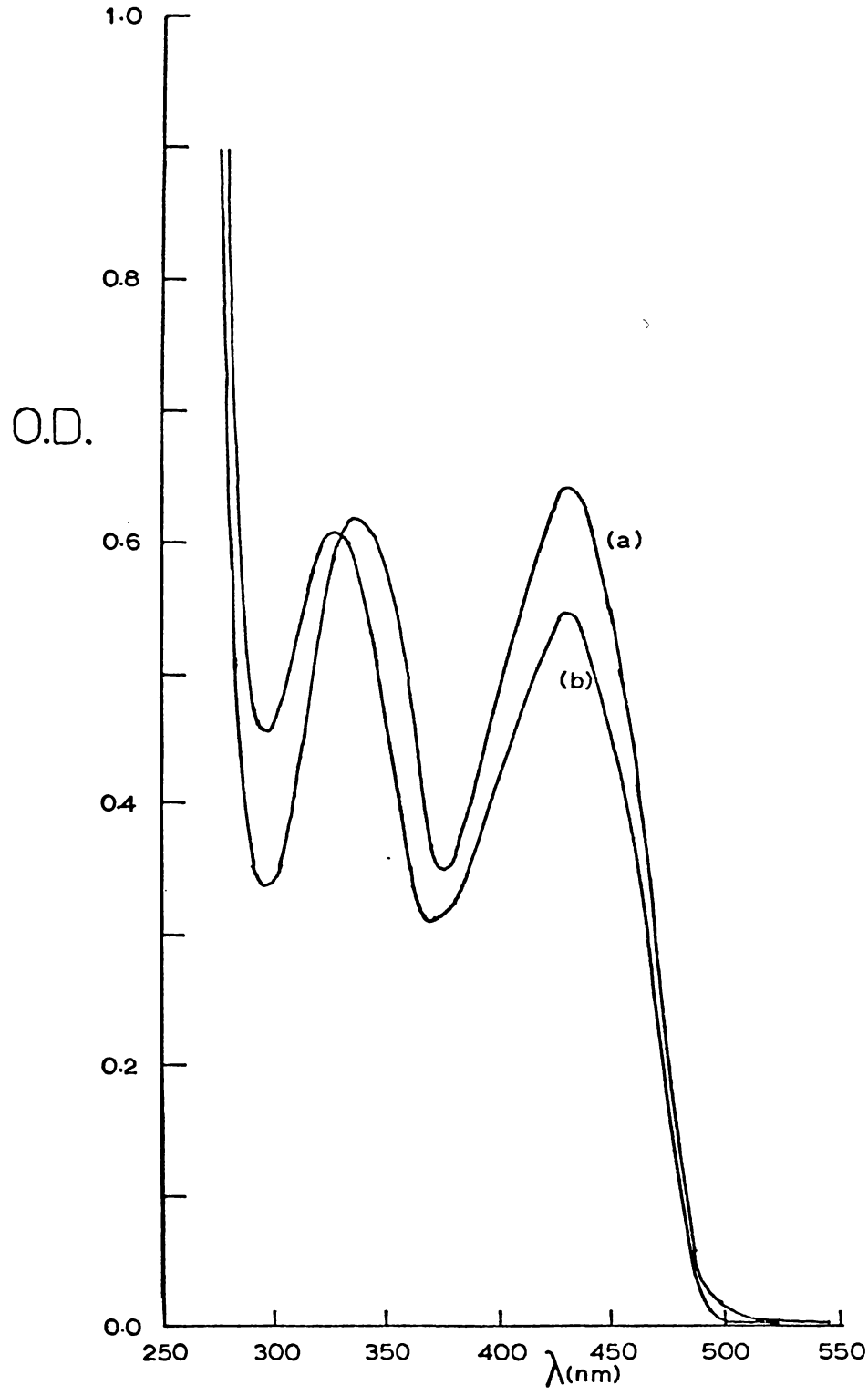


FIGURE 8: Comparison of the spectra of isoalloxazine C and the oxidized product (II) from the reaction of C with sulphite. Spectra (a) isoalloxazine C; (b) product II.

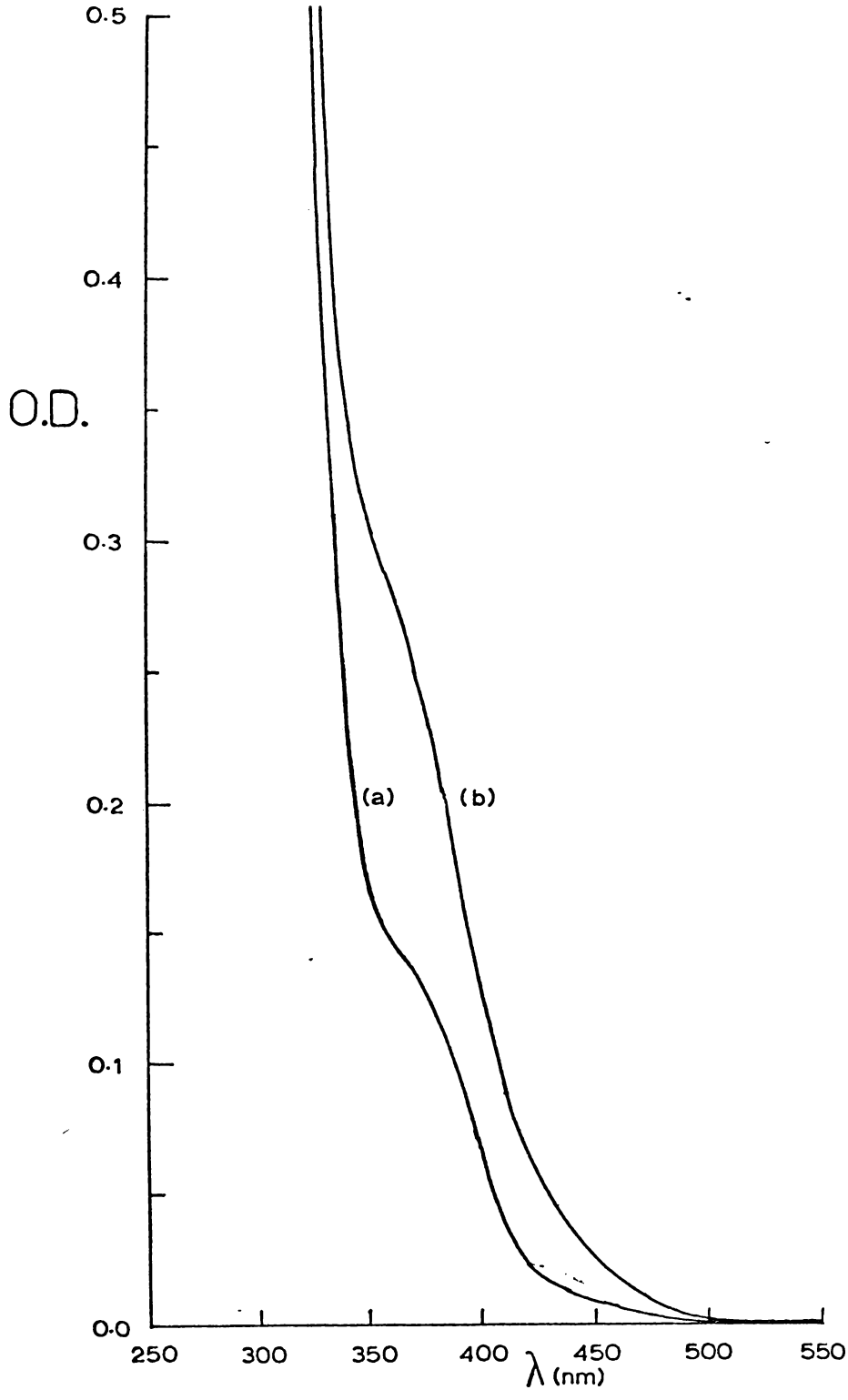


FIGURE 9: Comparison of the spectra of the reduced species from the anaerobic (structure II) and aerobic (III) reactions of isoalloxazine C with sulphite at pH 6.3 and 30°. Spectra (a) structure III; (b) II.

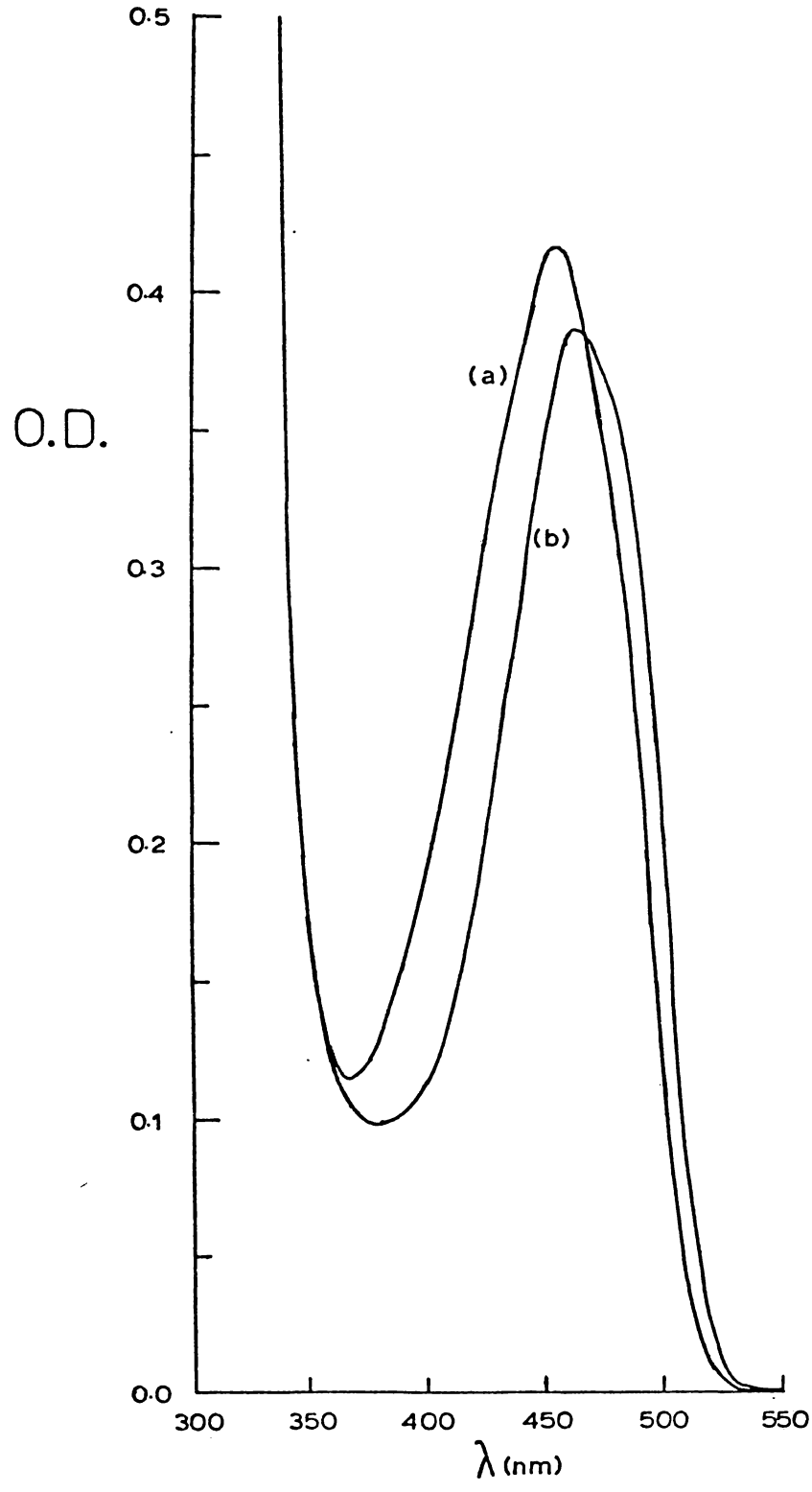


FIGURE 10: Comparison of the spectra of isoalloxazine A and the oxidized product (VI) from the reaction of A with sulphite. Spectra (a) isoalloxazine; (b) product VI.

lengths of any of the visible region absotptions (Figure 11). The implications of these observations are discussed in Section 4-5.4.

4-5.3 Kinetics of the Reaction of A, B and C with Sulphite

All three isoalloxazines followed first-order kinetics in the presence of excess sulphite. Typical examples of the first-order plots obtained for isoalloxazine B with sulphite are shown in Figure 12. A linear relationship was obtained between the observed rate constant, k_{obs}, and sulphite concentration (Figure 13). The rate law for the reaction of the isoalloxazines with excess sulphite is shown in equation 4.3.

$$-\frac{d[F]}{dt} = k_{obs}[F] \qquad (4.3)$$

$$k_{obs} = k_2[SO_3^{=}]$$

Therefore the slopes of the lines in the plots of k_{obs} against sulphite concentration (Figure 13) are the apparent second order rate constant, $k_2(app)$, for the three isoalloxazines. The $k_2(app)$ values are collected in Table 4-8.

A linear free-energy relationship has been reported for the reaction of a series of isoalloxazines (with no steric crowding at N(5)) with sulphite between the logarithm of $k_2(app)$ and the polarographic half-wave potential, $E_{\frac{1}{2}}$[19]. Isoalloxazines B and C, unlike A, do not have any steric crowding about the N(5) position and therefore one might expect a similar free-energy relationship to exist for these two isoalloxazines. Consequently it is interesting

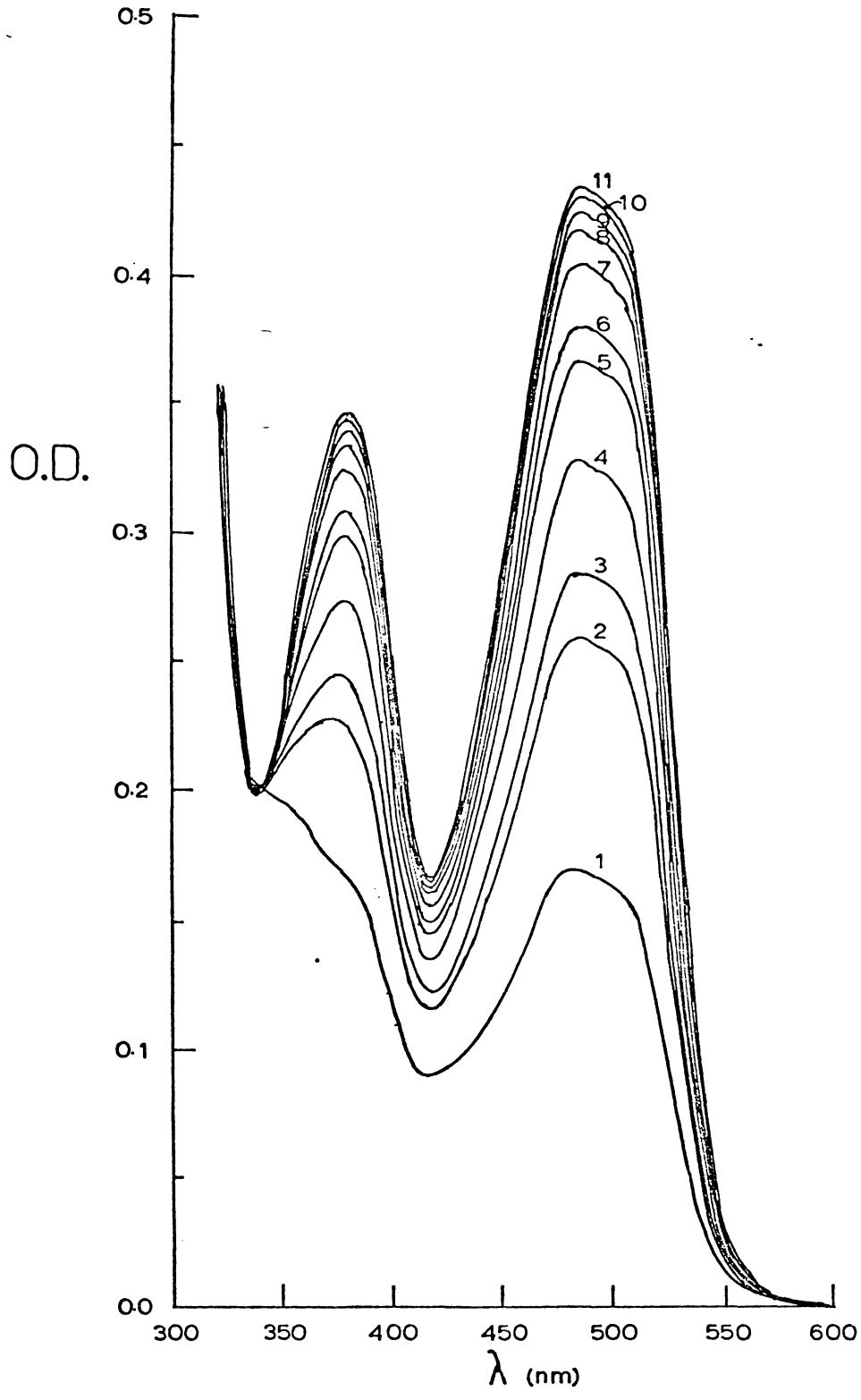


FIGURE 11: Repetitive wavelength scan of the regeneration of isoalloxazine B after the removal of sulphite. Scan 1. arbitary zero time; 2. 8½min after 1; 3. 30; 4. 48; 5. 62; 6. 71; 7. 101; 8. 120; 9. 140; 10. 181; 11. 220min.

TABLE 4-5

Effect of sulphite concentration (M) on the observed rate constant, k_{obs} (min^{-1}), for isoalloxazine A at pH 6.3 and 30°.

$[SO_3^{=}]$	$10^3 k_{obs}$	10^3 s.d.
0.44	2.22	0.04
0.87	4.70	0.07
0.87	4.87	0.08
0.90	5.41	0.30
0.90	4.56	0.17
0.90	6.77	1.63

TABLE 4-6

Effect of sulphite concentration (M) on the observed rate constant, k_{obs} (min^{-1}), for isoalloxazine B at pH 6.3 and 30°.

$[SO_3^{=}]$	k_{obs}	s.d.
0.01	0.187	0.003
	0.180	0.014
	0.148	0.006
0.10	0.572	0.012
	0.554	0.046
	0.521	0.032
0.87	3.64	0.04
	3.67	0.03
	4.03	0.10
0.90	4.01	0.03
	4.21	0.02
	4.16	0.04

TABLE 4-7

Effect of sulphite concentration (M) on the observed rate constant, k_{obs} (min^{-1}), for isoalloxazine C at pH 6.3 and 30°.

$[SO_3^{=}]$	k_{obs}	s.d.
0.01	0.0023	0.0004
0.10	0.068	0.008
0.90	0.629	0.006
	0.652	0.002
	0.638	0.002

TABLE 4-8

Apparent second order rate constants, $k_2(app)$ ($M^{-1}min^{-1}$), for isoalloxazines A, B and C with sulphite at pH 6.3 and 30°.

	$k_2(app)$	log k_2	$E_{\frac{1}{2}}(V)$
A	0.0055 ± 0.0007	-2.26 ± 0.29	-0.443
B	4.33 ± 0.12	0.637 ± 0.018	-0.261
C	0.715 ± 0.007	-0.146 ± 0.001	-0.356

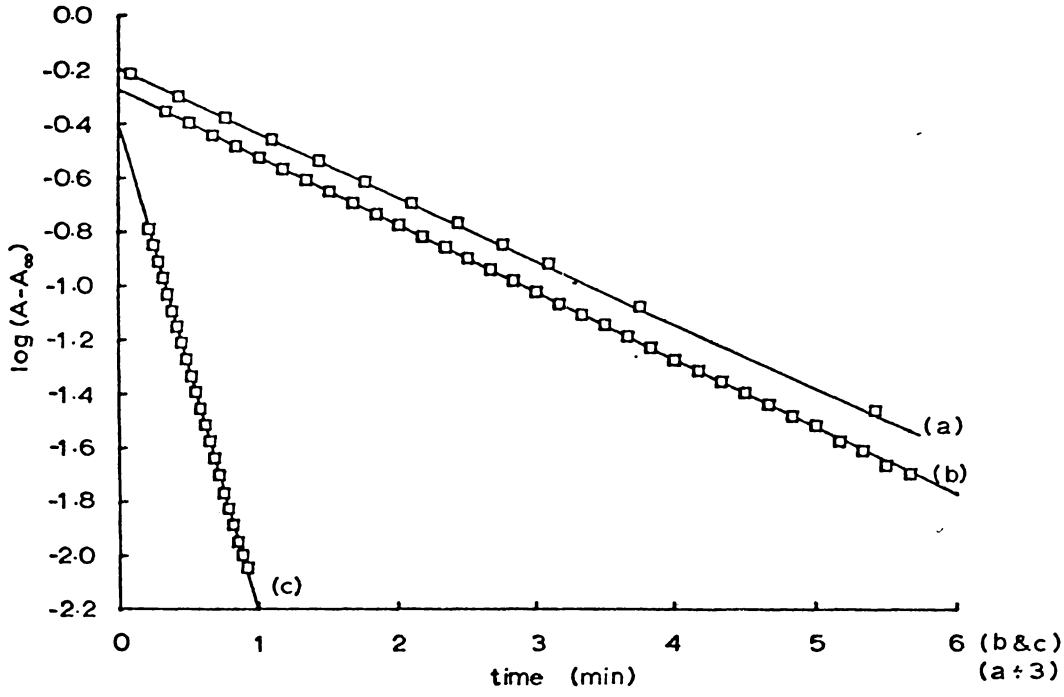


FIGURE 12: Typical examples of the first order plots obtained. These examples are for isoalloxazine B with sulphite ion at concentrations of (a) 0.01M; (b) 0.10M; (c) 0.90M at 30^{C} and pH 6.3.

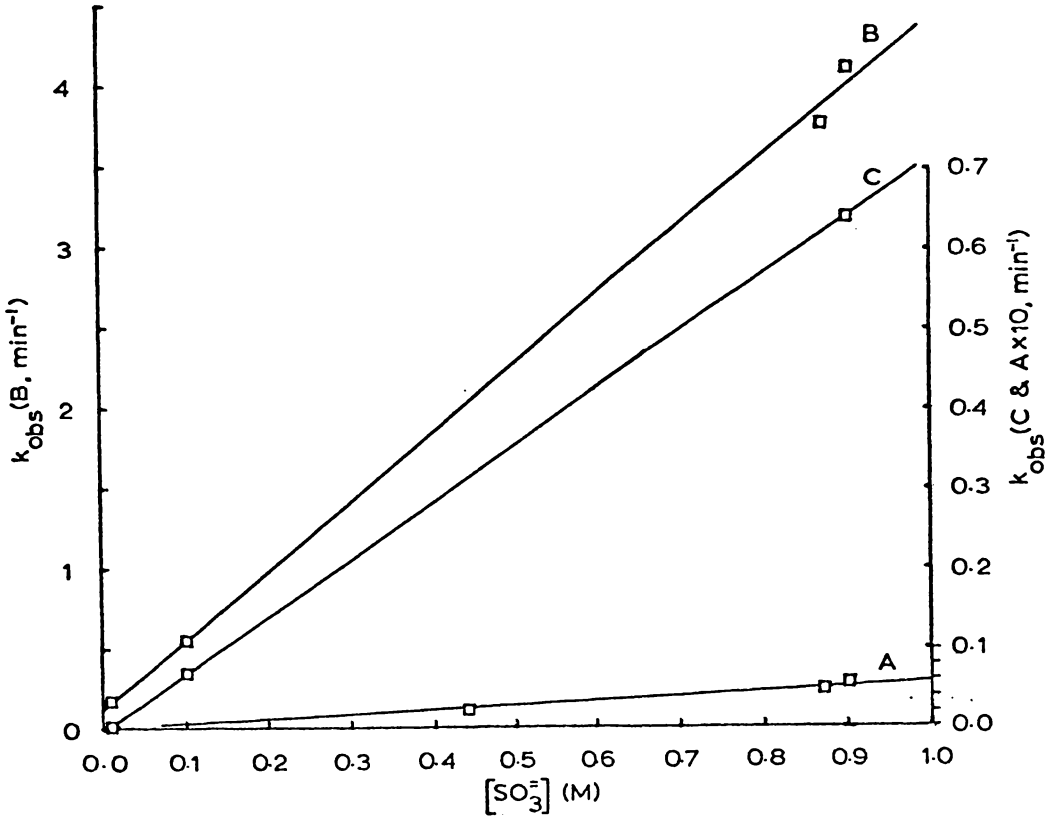


FIGURE 13: Effect of sulphite ion concentration on the observed rate constants of the three isoalloxazines A, B and C. These plots were used to obtain the apparent second order rate constants.

to draw a line through the points for B and C in a plot of logarithm of k_2(app) and $E_{\frac{1}{2}}$ for the purposes of comparing the rate constant of A with those of B and C (Figure 14). Although the values of k_2(app) follow the trend of the $E_{\frac{1}{2}}$ values for the three isoalloxazines, the point for A in Figure 14 falls below the line drawn through B and C by 1.48 logarithm units which corresponds to a factor of 30. The deviation of A from this line is greater than the experimental error of 0.29 in A, and thus the deviation may be regarded as a significant though small rate depression assuming that B and C define the normal free-energy relationship.

It was mentioned in the previous section that the reaction product of isoalloxazine B and sulphite was insensitive to oxygen. This fact is corroborated by the comparison of the values of k_{obs} determined for isoalloxazine B with a 0.90M sulphite solution in anaerobic and aerobic kinetic runs (Table 4-9).

TABLE 4-9

Comparison of observed rate constants, k_{obs} (min^{-1}), for isoalloxazine B with anaerobic and aerobic 0.90M sulphite solutions at pH 6.3 and 30°.

	anaerobic	aerobic
1	4.01 ± 0.03	3.89 ± 0.04
2	4.21 ± 0.02	4.10 ± 0.03
3	4.16 ± 0.04	4.13 ± 0.04
mean	4.13 ± 0.10	4.04 ± 0.13

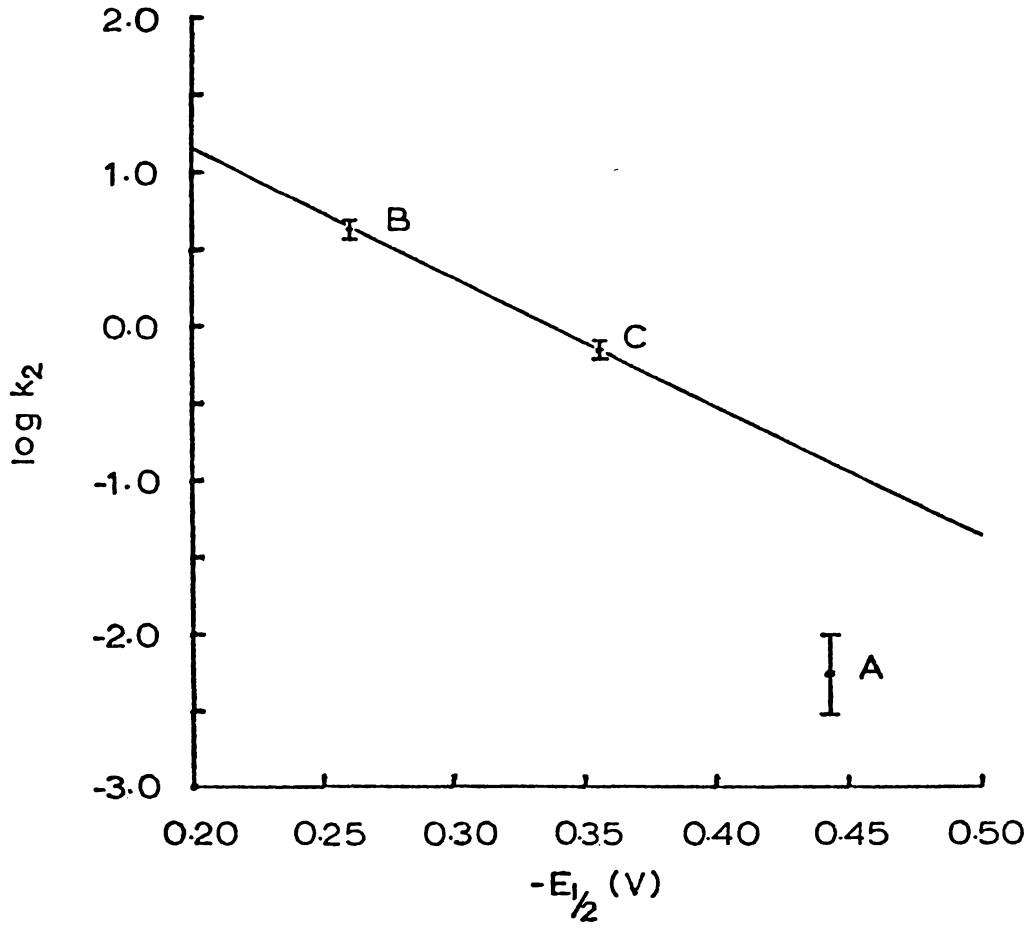


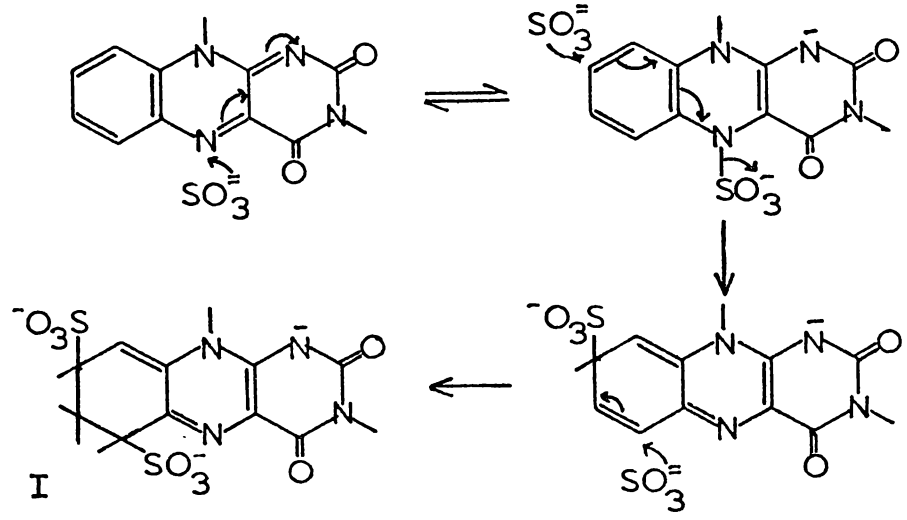
FIGURE 14: A plot of logarithm of $k_2(app)(SO_3^{=})$ against polarographic half-wave potential showing the rate depression for isoalloxazine A relative to the assumed linear free-energy relationship for isoalloxazines B and C.

The two sets of values agree within experimental error and therefore the reaction of isoalloxazine B with sulphite ion would appear to be unaffected by the presence of air.

4-5.4 Discussion and Conclusion of the Sulphite Reaction

The observation that the amount of isoalloxazine consumed in the reaction with sulphite is dependent on the initial sulphite ion concentration agrees with the conclusion of Hevesi and Bruice[34b] that the initial disappearance of isoalloxazine is an equilibrium process.

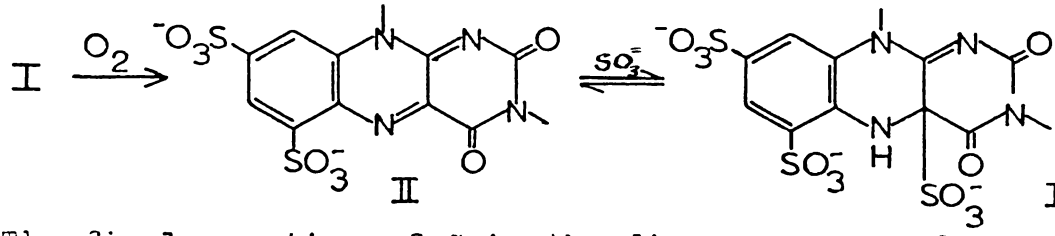
The two stages in the reaction of isoalloxazine C with sulphite mirrors the behaviour of 3-methyl-10-(2',6'-dimethylphenyl)isoalloxazine with sulphite observed by Hevesi and Bruice[34b]. Thus a similar interpretation may be used to explain the two stages in the reaction. The initial disappearance of the isoalloxazine is a reversible addition to the N(5)-position followed by attack at the 8-position with elimination of sulphite from the 5-position and further addition to the 6-position to give the slow appearance of the final product (I, equation 4.4).



(4.4)

The change in the wavelength of the 340nm absorption of

isoalloxazine C when air is readmitted to the reaction solution suggests that a new isoalloxazine is produced. Following on from equation 4.4, this new isoalloxazine would clearly be the 6,8-disulphonate derivative of C (II, equation 4.5).



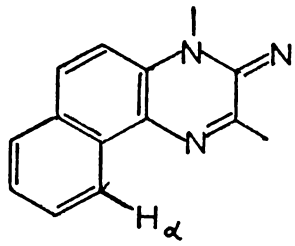
(4.5)

The final reaction of C is the disappearance of II; Hevesi and Bruice[34b] suggested that this is due to the formation of a 4a-addition product (III) as formation of the 5-sulphonate of II would involve electrostatic hindrance from the 6-sulphonate group. An additional factor is that the 6- and 8-sulphonate functions would promote 4a-addition by electron withdrawal. This step is in agreement with the observed difference between the spectra of the final products of the anaerobic and aerobic reactions of isoalloxazine C (Figure 9). These differences imply that a different type of reduced species is involved, i.e. in the anaerobic case structure I and in the aerobic case structure III.

The rate depression observed for the reaction of A with sulphite ion could imply that

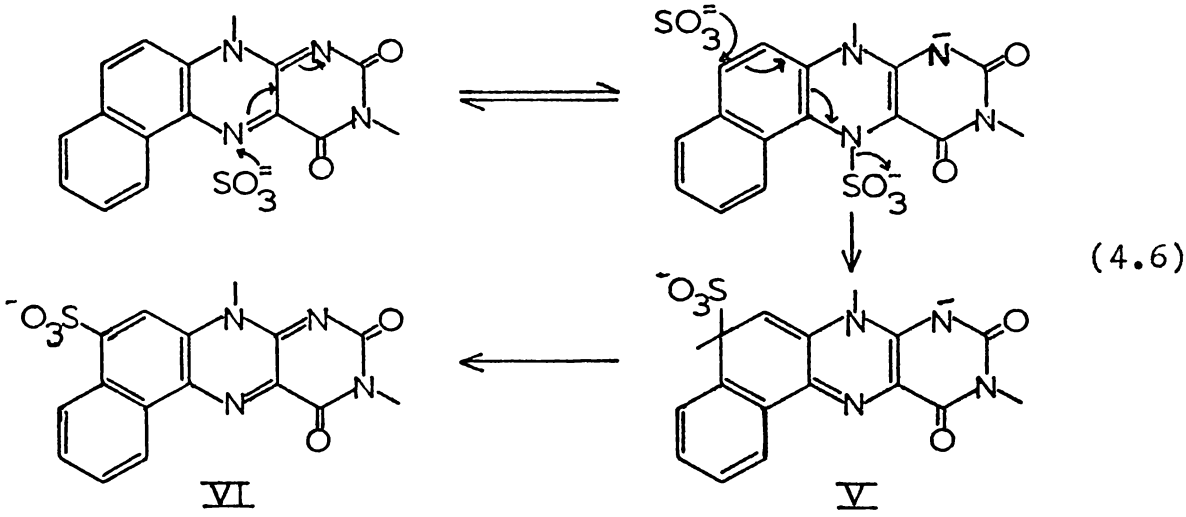
(a) a different site from that used in the case of B and C is involved in the attack of sulphite on A, or

(b) that the steric crowding by the α-hydrogen at the N(5)-position (IV) is slowing the reaction of A with sulphite.



IV

The latter suggestion would be a favoured reason if "5-addition involves strictly in-plane (of the isoalloxazine structure) attack" as Hemmerich states[10b]. Thus if the rate depression is caused by steric hindrance from the α-hydrogen of the naphthalene moiety (IV) then the change in the wavelength of the 460nm absorption of A on reoxidation of the reduced species resulting from the reaction with sulphite may be explained by the addition of a second sulphite ion to the 8-position as in the case of C, with expulsion of the 5-sulphonate group (V, equation 4.6) and subsequent oxidation of V by oxygen to give the 8-sulphonate derivative of A (VI).



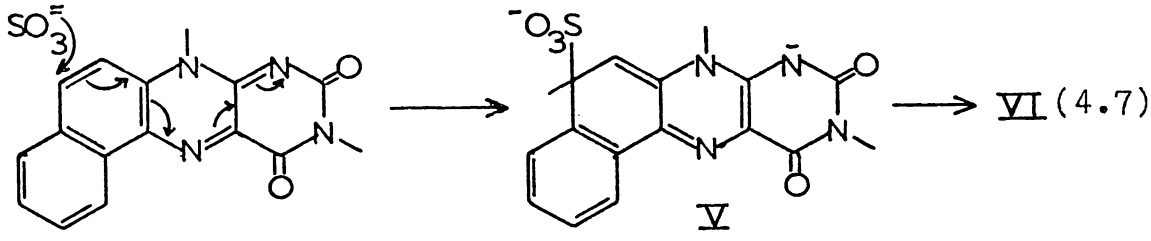
(4.6)

VI V

In this case then the probable reason why only the one reaction was observed for A with sulphite is that the slow rate of reaction obscures observation of the second reaction (as in the case for reaction of C at low sulphite concentration (Figure 5)). If steric effects are operating to hinder 5-addition, the first step of the reaction of A will be slowed relative to any subsequent attack at the 8-pos-

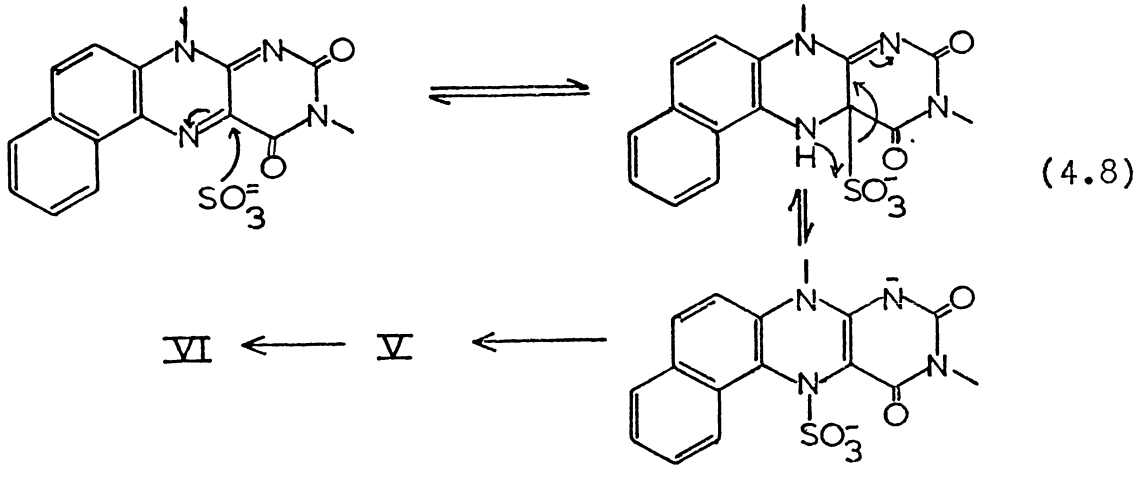
ition so that, unlike the case with C for which the first reaction is relatively very fast for high sulphite ion concentration, the two successive reaction may be indistinguishable.

However the rate depression may be caused by the attack of sulphite at a site other than the N(5)-position, for example, attack could occur at either the 4a- or 8-positions. Thus if such were the case in either situation A could not be expected to follow a free-energy relationship with B and C. In the latter case the 8-position is less electrophilic than the 5-position and consequently attack of the nucleophile might be expected to be slower at this position. Clearly, attack at the 8-position would produce V in a one step reaction (equation 4.7), which



would be consistent with the observation of the single stage in the reaction.

Formation of V via 4a-addition may be envisioned as in equation 4.8. Initial attack of sulphite occurring at the 4a-position, followed by isomerization to the 5-sul-



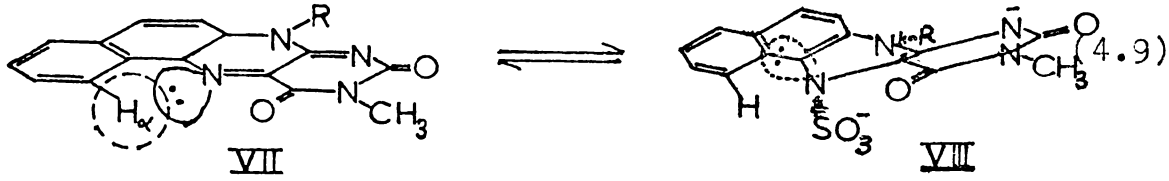
phonate with subsequent attack at the 8-position as in equation 4.6. Precedence for 4a-5 isomerism has been shown by Hevesi and Bruice[34b,38] who found that an equilibrium existed between the 4a- and 5-sulphonate adducts of 10-(2',6'-dimethylphenyl)-3-methylisoalloxazine-6,8-disulphonate. 4a-addition has been suggested as the initial reaction of the above isoalloxazine with sulphite since 5-addition would be electrostatically hindered by the 6-substituent. These consecutive reactions in equation 4.8 could also be indistinguishable from each other and hence could give rise to one observable reaction.

Since these three possibilities for the reaction of A with sulphite ion cannot be clearly determined from the data it will be of interest therefore to look at the possible reasons for the small size of the observed rate depression for A in terms of the three proposed mechanisms.

(a) As mentioned earlier Hemmerich[10b] has suggested that 5-addition is restricted to an in-plane approach. It is felt however that an out-of-plane approach by a nucleophile would be just as likely, if not more so, in the case of nucleophilic addition. In this situation an in-plane approach would involve interaction with the lone pair electrons on the 5-nitrogen and consequently would be less favourable than an out-of-plane approach which would not involve such interactions. In the case of isoalloxazine A, the steric crowding about the 5-position would also be less for an above-the-plane attack by sulphite, the amount of hindrance caused by the α-hydrogen (see IV) depending on the precise transition state geometry.

(b) In the ground state of isoalloxazine A (VII)

there will be a certain amount of steric strain between the lone pair electrons on N(5) and the coplanar α-hydrogen. As the sulphite ion adds to the N(5)-position the isoalloxazine molecule will change its configuration from the planar structure of the oxidized molecule to the 'butterfly' shape of the 1,5-dihydroisoalloxazine (VIII, equation 4.9)[10a]. The axis of this change in configuration occurs



VII VIII (4.9)

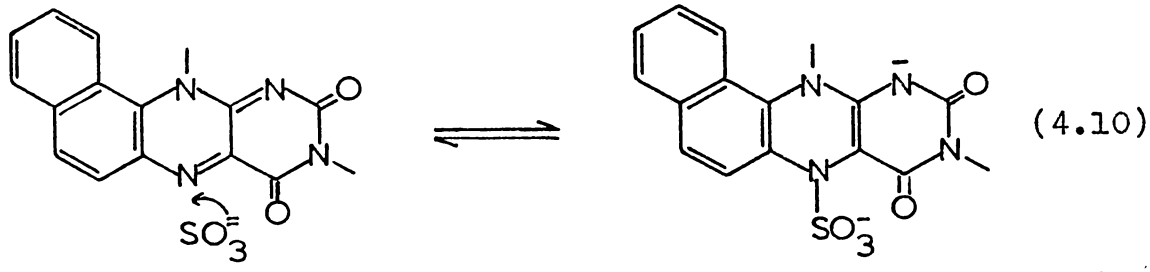
through the nitrogen atoms at the 5- and 10-positions. The effect of this change would be to release the strain between the N(5) lone pair electrons and the α-hydrogen at the expense of some crowding with the N-sulphonate substituent in the product. The release of the strain could give rise to a 'steric acceleration' effect which would have to be balanced against any decrease in the rate of reaction by steric crowding in the product.

(c) Assuming that no 5-addition at all occurs, the magnitude of the depression may just reflect the differences between the electrophilicity of the 8-position (or 4a-position) on the one hand and the 5-position on the other. The former two positions would not be subject to steric effects and thus the rate of reaction would depend on only the relative electrophilicity of the two positions, which would therefore necessarily be lower than that of the 5-position.

There is no compelling reason to suggest that the sulphite reaction mechanism of A is different from the

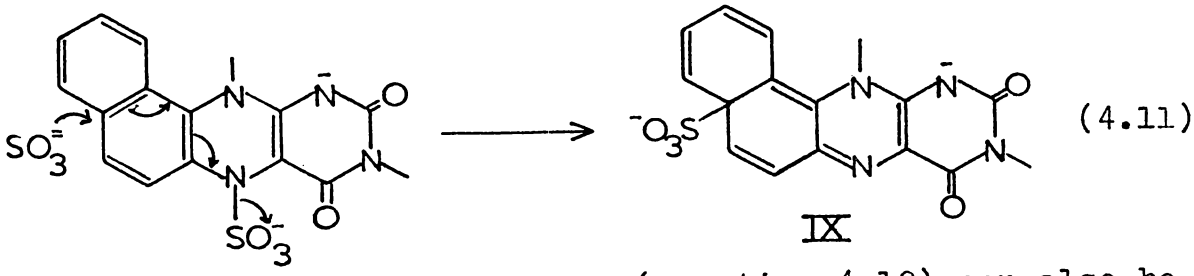
normal mechanism involving initial 5-addition, especially in view of the suggestion in (a), and the simplest interpretation is that the magnitude of the rate depression is due to either a balance between steric deceleration and acceleration effects or a weakly hindered out-of-plane approach of the nucleophile. With the evidence presented here these two possibilities cannot be readily distinguished from each other.

The observation of only the one reaction of isoalloxazine B with sulphite and the lack of any spectral changes in the reoxidized spectrum (Figure 11) suggests that the only stage in the reaction is a reversible addition of sulphite at the proposed reactive centre, the 5-nitrogen (equation 4.10).



(4.10)

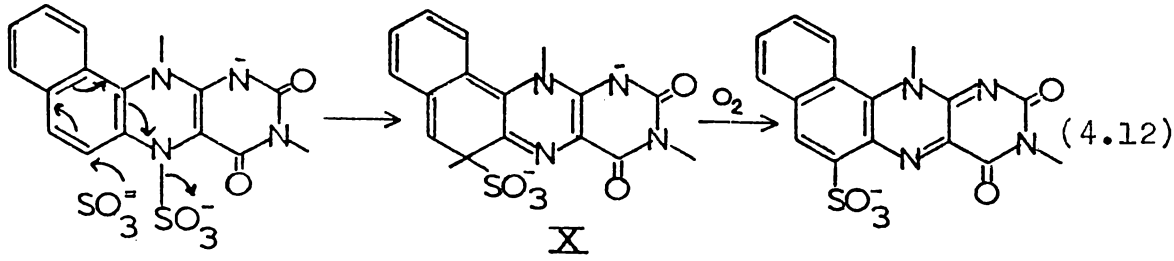
Further addition to the 8-position is highly unlikely since it would involve total loss of aromaticity in both benzenoid rings (IX, equation 4.11).



(4.11)

IX

Formation of the 6-sulphonate (equation 4.12) can also be eliminated since it would be electrostatically unfavourable because of the adjacent 5-sulphonate and since no spectral change was observed with the reappearance of an oxidized isoalloxazine. If 6-addition did occur the product (X)



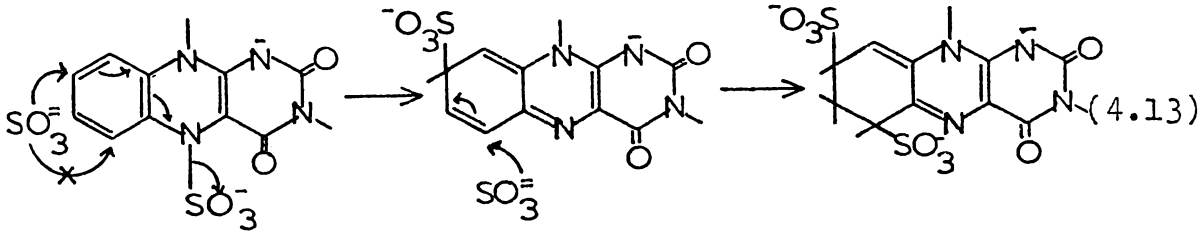
(4.12)

X

would be expected to be oxygen sensitive as with the case of the proposed 8-sulphonate of isoalloxazine A. Direct attack of sulphite at the 6-position in oxidized B may be eliminated for the same reasons. Consequently it may be concluded that the only reaction of isoalloxazine B with sulphite is an equilibrium addition to the N(5)-position as shown in equation 4.10.

Final conclusions which may be drawn about the reaction of isoalloxazines with sulphite generally, which can be deduced from the studies of the reactions of isoalloxazines A, B and C, are as follows:

(a) Hevesi and Bruice[34b] suggested that the second stage of the reaction of isoalloxazines (with free 1-, 6- and 8-positions) involves sulphite addition at the 8-position with expulsion of the 5-sulphonate substituent as $SO_3^=$ and then attack at the 6-position. From the behaviour of isoalloxazines A and B with sulphite it is concluded that this is in fact the correct sequence of reactions (equation 4.13); the former isoalloxazine appears to give



(4.13)

an 8-adduct but the latter does not give a 6-adduct. Thus 6-addition occurs only after 8-addition.

(b) The conclusion drawn in (a) suggests that the

addition of sulphite ion to the 6-position is simply addition of sulphite to an olefin and it is therefore not an inherent reaction of 5-addition compounds as shown by the lack of any such reaction of the sulphite-addition product of B in which the 6,7-double bond forms part of the benzenoid ring.

(c) The observation that the new isoalloxazine formed in the aerobic reaction of A with sulphite does not react further with sulphite (unlike C) implies that the 8-sulphonate function by itself causes insufficient activation of the 4a-position to promote further addition at this centre, whereas the presence of two sulphonate groups as in isoalloxazine C (at the 6- and 8-positions) is sufficiently activating to promote 4a-addition.

4-6 REACTIONS OF ISOALLOXAZINES A, B AND C WITH 1,3-DITHIO-2-PROPANOL

The formation of adducts by thiols at the 4a-, 5-, 6-, 8-, 9a- and 10a-positions of isoalloxazines was considered in section 1-4.2.2. In brief, kinetic evidence shows an intermediate adduct is formed in the reaction of dithiothreitol; the observation of general acid catalysis suggests that the 4a- is more likely than any of the other positions as the centre for this adduct formation; and of these other positions the 9a- and 10a-positions have been excluded as essential reaction centres.

4-6.1 Kinetics of the Reaction of A, B and C with DTP

The three isoalloxazines A, B and C followed first

order kinetics with large excess of 1,3-dithio-2-propanol (DTP) at the pH employed in the reaction (Figure 15). The observed rate constants, k_{obs}, obtained were found to be linearly related to the total thiol concentration (Figure 16). Therefore following on from the previously reported work[39,40] the rate law for this reaction is as shown in equation 4.14.

$$-\frac{d[F]}{dt} = k_2[F][DTP] \qquad (4.14)$$

Thus with the concentration of the dithiol held constant (in excess) the disappearance of the isoalloxazine is pseudo-first order and equation 4.14 reduces to equation 4.15

$$-\frac{d[F]}{dt} = k_{obs}[F] \qquad (4.15)$$

and $$k_{obs} = k_2[DTP].$$

Therefore the slope of the plot of the observed rate constant, k_{obs}, against the concentration of DTP will be the second order rate constant, k_2. The values of k_2 of the three isoalloxazines were derived from the above plots by least squares analysis and they are presented in Table 4-13.

From Table 4-13 it can be seen that the second order rate constants are in the order B > C > A and that they follow the order of their respective polarographic half-wave potentials, $E_{\frac{1}{2}}$. A linear free-energy relationship between the logarithm of k_2 and $E_{\frac{1}{2}}$ for the reaction of 1,4-butanedithiol with a series of isoalloxazines was observed by Bruice and coworkers[19]. The plot of logarithm of the second order rate constants obtained against the polarographic half-wave potential (Figure 17) establishes

TABLE 4-10

Effect of DTP concentration (M) on the observed rate constant, k_{obs} (min^{-1}), for isoalloxazine A at pH 9.2 and 30°.

10^2[DTP]	$10^2 k_{obs}$	10^2s.d.
0.96	1.62	0.01
1.73	2.60	0.04
1.73	2.55	0.04
1.73	2.54	0.04
3.20	6.01	0.11
3.40	4.82	0.11
3.62	6.58	0.21
3.67	5.61	0.20
3.76	5.63	0.12
7.26	12.34	0.26
7.41	10.77	0.26
7.49	13.84	0.25
7.62	11.98	0.14

TABLE 4-11

Effect of DTP concentration (M) on the observed rate constant, k_{obs} (min^{-1}), for isoalloxazine B at pH 9.2 and 30°.

10^3[DTP]	k_{obs}	s.d.
0.185	0.737	0.011
0.331	1.314	0.026
0.355	1.558	0.018
0.615	3.326	0.041
0.684	3.775	0.028
0.882	4.561	0.055
1.08	5.674	0.030
1.30	7.252	0.072
1.56	8.497	0.054
2.45	14.48	0.13

TABLET 4-12

Effect of DTP concentration (M) on the observed rate constant, k_{obs} (min^{-1}), for isoalloxazine C at pH 9.2 and 30°.

10^2[DTP]	k_{obs}	s.d.
0.656	1.488	0.003
0.705	1.616	0.011
0.717	1.628	0.006
0.735	1.549	0.042
0.735	1.537	0.008
1.37	3.304	0.009
1.45	3.555	0.059
1.49	3.411	0.023
2.87	6.962	0.029
2.95	7.112	0.041
2.99	7.122	0.059

TABLET 4-12

Effect of DTP concentration (M) on the observed rate constant, k_{obs} (min^{-1}), for isoalloxazine C at pH 9.2 and 30°.

10^2[DTP]	k_{obs}	s.d.
0.656	1.488	0.003
0.705	1.616	0.011
0.717	1.628	0.006
0.735	1.549	0.042
0.735	1.537	0.008
1.37	3.304	0.009
1.45	3.555	0.059
1.49	3.411	0.023
2.87	6.962	0.029
2.95	7.112	0.041
2.99	7.122	0.059

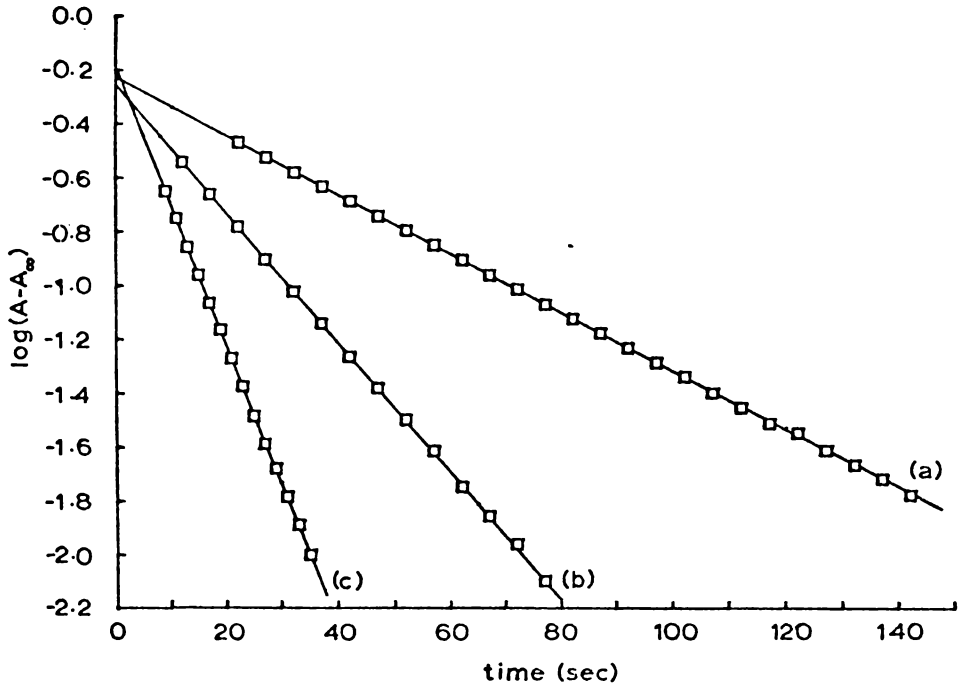
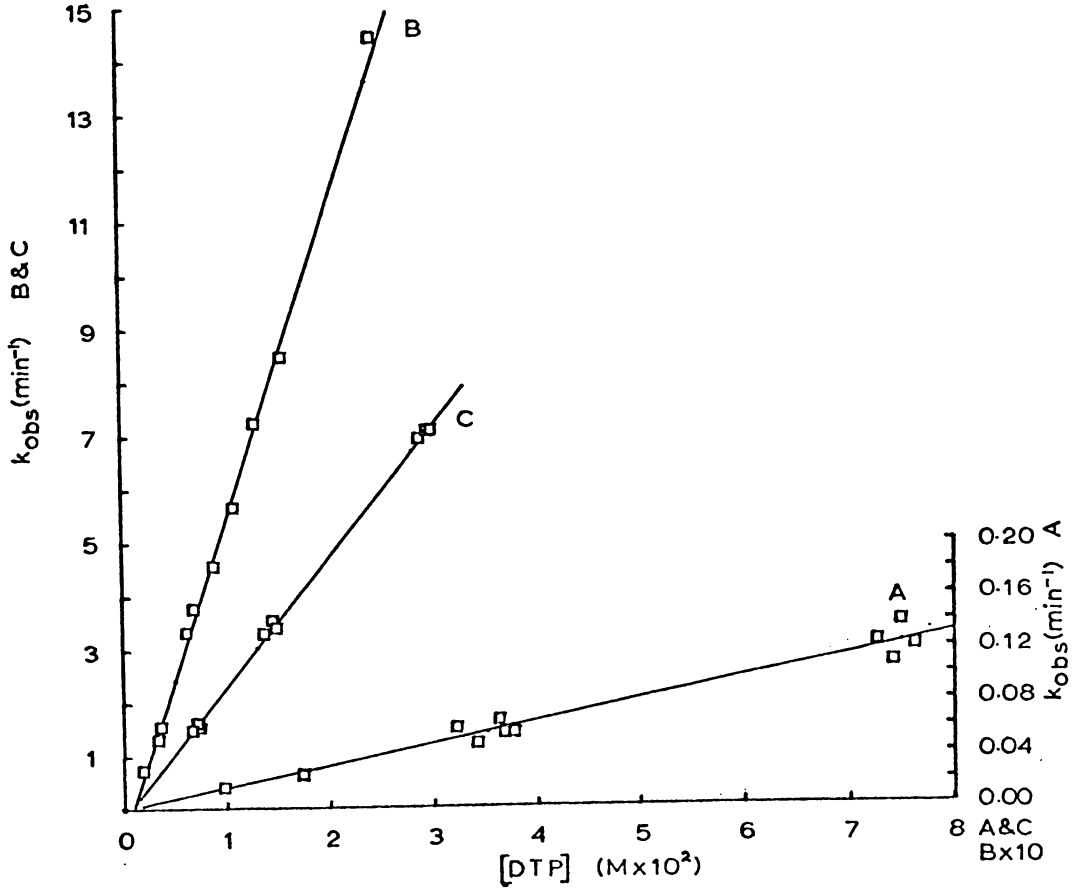


FIGURE 15: Typical examples of the first order plots obtained for the reaction of isoalloxazine C with 1,3-dithio-2-propanol at concentrations of (a) o.oo656M; (b) 0.00137M; (c) 0.0295M at pH 9.2 and 30°.

FIGURE 16: Effect of dithiol concentration on the observed rate constants of the three isoalloxazines A, B and C.



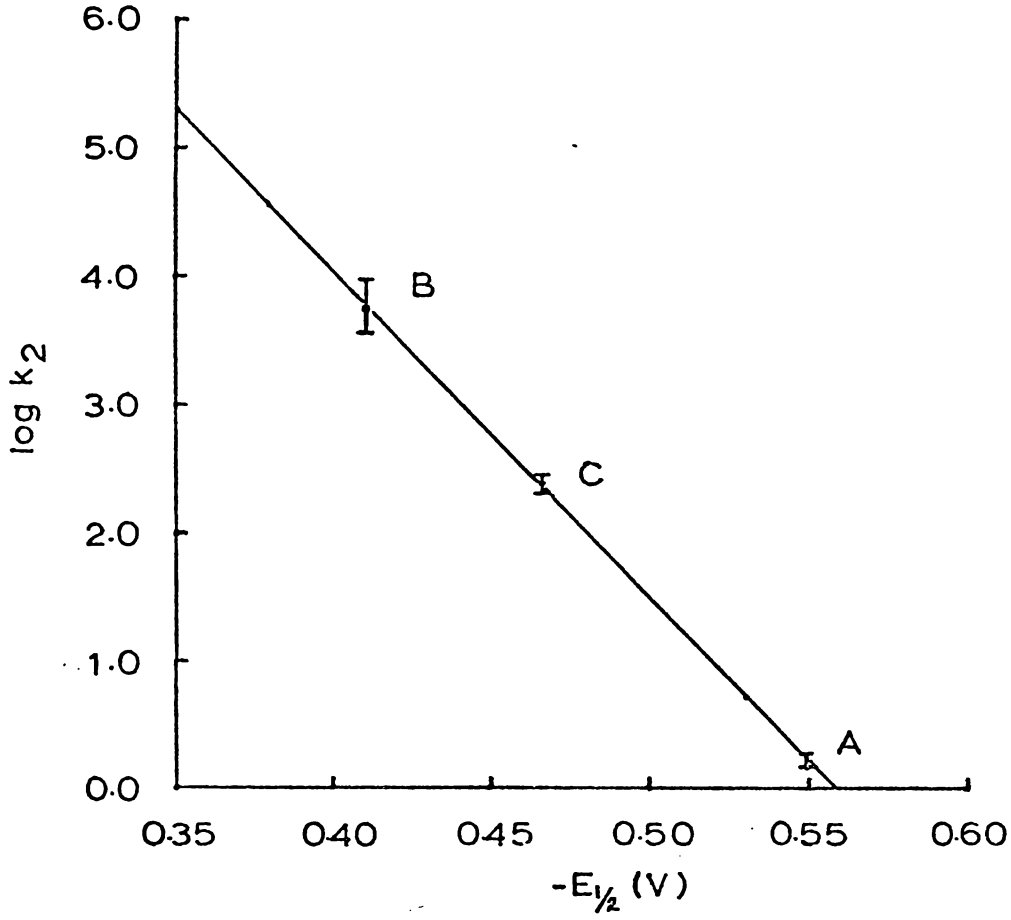


FIGURE 17: Linear free-energy correlation between the polarographic half-wave potentials and the logarithm of the second order rate constant for the reaction of the three isoalloxazines with DTP.

TABLE 4-13

Second order rate constants, k_2 ($M^{-1}min^{-1}$), for the reaction of isoalloxazines A, B and C with DTP at pH 9.2 and 30°.

	k_2*	log k_2	$E_{\frac{1}{2}}$(V)
A	1.67 ± 0.16	0.223 ± 0.021	-0.549
B	5920 ± 330	3.77 ± 0.21	-0.410
C	247 ± 5	2.39 ± 0.05	-0.466

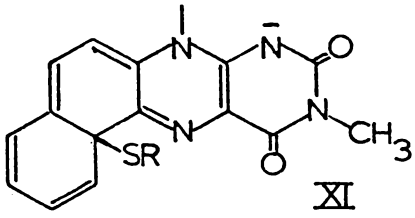
*These values were determined by least squares analysis of the lines of Figure 16; the errors were determined in the analysis and are adjusted to 90% confidence.

that a linear free-energy relationship exists for isoalloxazines A, B and C with slope of $26 \pm 1 V^{-1}$ (cf $32V^{-1}$ for 1,4-butanedithiol[19]).

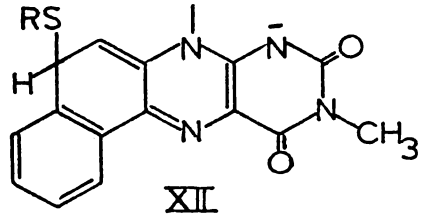
4-6.2 Discussion and Conclusion

The observation of the linear free-energy relationship for the three isoalloxazines suggests that there are no electronic or steric effects operating in isoalloxazines A and B which hinder the reaction of these two isoalloxazines with 1,3-dithio-2-propanol.

The possibility that the 6- and 8-positions on the isoalloxazines nucleus were sites for the formation of an adduct during thiol oxidation was considered in Section 1-4.2.2. From the structure of A and B, it can be seen that in A the 6-position is blocked while the 8-position is free, whereas in B the reverse situation exists with the 8-position blocked and the 6-position free. Addition of the thiol anion to the 6-position of A (XI) would result in the complete loss of aromaticity of the naphthalene moiety of A. This would be expected to be much less favourable than



XI



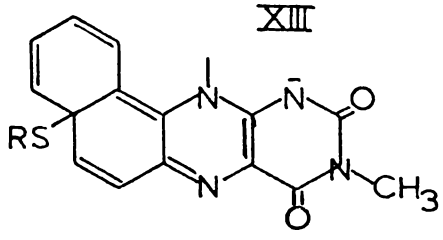
XII

addition to the 8-position in which case one of the benzene rings retains its aromaticity (XII). Consequently if 6-addition is obligatory for dithiol oxidation then a comparison of the 6-positions in isoalloxazines A, B and C shows that a rate depression from the linear free-energy relationship would therefore be expected for A. A similar

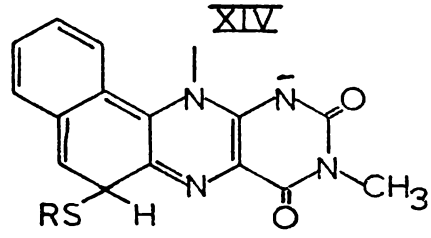
comparison of the 8-positions indicates that this would not be the case for A if 8-addition is involved in the reaction mechanism. The absence of any such depression in Figure 17 excludes essential formation of a 6-adduct for isoalloxazine A but not the possibility of an 8-adduct.

By analogy with the situation of the 6-position of A, similar arguments can be applied in the case of isoalloxazine B to exclude obligatory 8-adduct formation (XIII). Further the formation of a 6-adduct with B (XIV) cannot be excluded by analogy with the possible 8-addition in isoalloxazine A.

XIII



XIV



The conclusion to be drawn from the linear free-energy relationship is therefore that if there is a single essential site for adduct formation in the reaction of dithiols with isoalloxazines then it is neither the 6-position nor the 8-position. The possibility that both 6- and 8-addition can occur at similar rates can probably also be excluded on the basis that isoalloxazine A would react via XII, isoalloxazine B via XIV and therefore isoalloxazine C, with its 6- and 8-positions both free, would be expected on statistical grounds (since it will have two reaction centres rather than the one in A and B) to show a rate acceleration relative to A and B. It can be seen from Figure 17 that this is not the case, although the experimental error in the points for A, B and C could conceal small deviations but not a factor of logarithm of 2

(or 0.3) in the point for isoalloxazine C.

With the elimination of the 6- and 8-positions there remain only two electrophilic centres, the 4a- and 5-positions, for the formation of adducts with thiol anion, as the 9a- and 10a-positions have been eliminated in a previous study (see section 1-4.2.2). Considering the steric crowding present at the 5-position of A (see section 4-5.4) and the lack of a rate depression for A from the linear free-energy relationship (Figure 17) it might appear that 5-addition is inessential for the reduction of isoalloxazines by thiols. However, this premise assumes that only an in-plane approach[10b] of the thiol anion to N(5) occurs. It was felt that the formation of the 5-adduct cannot be excluded on this basis since an out-of-plane attack on the N(5)-position is a possibility. Consequently it might conceivably be only slightly hindered by the α-hydrogen, the amount depending on the geometry of the transition state. Therefore attack at the 4a- and 5-positions cannot be distinguished on the basis of these results although kinetic evidence presented by Loechler and Hollocher[39] favours the former site for the formation of an intermediate adduct on the redox reaction pathway of thiol oxidation.

4-7 REACTIONS OF ISOALLOXAZINES A, B AND C WITH NADH

The reaction of isoalloxazines with reduced nicotinamides was discussed in Section 1-4.2.1. Briefly, there is direct spectroscopic and kinetic evidence for complex formation in the case of the reaction with reduced N-methyl-

nicotinamide but not in the case if NADH; and bulky out-of-plane isoalloxazine substituents result in parallel rate depressions for reduced N-methylnicotinamide and NADH. These depressions are accommodated by a linear free-energy relationship between the logarithm of the rate constant and the logarithm of the equilibrium constant for complexing by tryptophan thus providing indirect evidence for essential face-to-face complexing of isoalloxazine and reduced nicotinamides on the reaction pathway in both cases.

4-7.1 Kinetics and Discussion of the Reaction of A, B and C with NADH

The reactions of the three isoalloxazines A, B and C with NADH appear to follow first order kinetics in both isoalloxazine as in Figure 18 and NADH (Figure 19) up to approximately 0.005M NADH. Above this concentration the pseudo-first order rate constants for A, B and C are depressed progressively from lines drawn through the initial points of plots of the observed rate constant, k_{obs}, against NADH concentration (Figure 19). This 'tailing off' of the k_{obs} values is probably due to a saturation effect caused by NADH. Such an effect is common in a reaction involving an equilibrium step followed by a rate limiting step as in equation 4.16.

$$X + Y \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} Z \xrightarrow{k_3} P \qquad (4.16)$$

Thus as the concentration of, say, Y increases the concentration of Z builds up such that the rate of reaction increases to a maximum dependent on the magnitude of the rate of breakdown of Z (k_3) to the products. Further

increases in Y do not proportionately increase the rate because if, for instance, 90% of X is already complexed by Y then by doubling the concentration of Y will not also double the concentration of Z as there is insufficient X for this to occur. Therefore the reaction will become independent of [Y] and it will tend towards zero order in Y as [Y] increases.

To test such a possibility Lineweaver-Burke plots[96] were drawn for all three isoalloxazines. These plots are described by a general equation given by equation 4.17,

$$\frac{1}{\nu} = \frac{K_d}{V}\frac{1}{a} + \frac{1}{V} \qquad (4.17)$$

where ν is the rate of reaction; V is the limiting rate as $a \to \infty$; and K_d is the value of a at $\frac{1}{2}V$. It may also be, in some cases, the dissociation constant of the proposed complex between X and Y (equation 4.16). Thus a plot of $1/\nu$ against $1/a$ will have a slope of K_d/V and an intercept with the y-axis of $1/V$. If saturation is occurring then the intercept on the x-axis is negative and it is equal to $1/K_d$.

Under the reaction conditions the rate law for the reaction of NADH with the isoalloxazines is described by equation 4.18.

$$\nu = -\frac{d[F]}{dt} = k_{obs}[F] \qquad (4.18)$$

This can be rearranged to give equation 4.19.

$$\frac{[F]}{\nu} = \frac{1}{k_{obs}} \qquad (4.19)$$

Linear relationships between k_{obs}^{-1} and $[NADH]^{-1}$ were

TABLE 4-14

Effect of NADH concentration (M) on the observed rate constant, k_{obs} (min^{-1}), for isoalloxazine A at pH 6.3 and 30°.

10^3[NADH]	$10^2 k_{obs}$	10^2s.d.
0.468	2.64	0.05
0.483	2.98	0.03
1.01	5.05	0.12
	5.92	0.05
	6.10	0.05
1.03	6.24	0.05
1.87	12.22	0.10
	10.81	0.13
	10.47	0.15
4.02	23.47	0.39
	22.81	0.26
10.0	40.86	0.37
14.8	61.47	0.46
20.3	71.97	0.35
	69.50	0.44
21.2	62.85	0.92
	64.55	0.72

TABLE 4-15

Effect of NADH concentration (M) on the observed rate constant, k_{obs} (min^{-1}), for isoalloxazine B at pH 6.3 and 30°.

10^3[NADH]	$10^2 k_{obs}$	10^2s.d.
0.132	0.35	0.01
1.02	2.17	0.02
1.08	2.44	0.04
3.85	7.45	0.03
	8.22	0.05
	7.77	0.13
5.06	10.52	0.05
6.58	12.64	0.11
	12.59	0.27
9.56	16.15	0.09
	14.06	0.48
10.72	16.57	0.32
	17.27	0.06
	17.12	0.25

TABLE 4-16

Effect of NADH concentration (M) on the observed rate constant, k_{obs} (min^{-1}), for isoalloxazine C at pH 6.3 and 30°.

10^3[NADH]	$10^2 k_{obs}$	10^2s.d.
0.996	4.82	0.16
1.03	4.81	0.02
2.05	10.14	0.06
	10.23	0.10
	10.18	0.09
4.06	20.10	0.11
	20.29	0.13
6.91	31.08	0.15
	29.91	0.39
	28.33	0.50
10.25	43.08	0.38
	44.58	0.40
	39.39	0.35
19.72	77.89	0.21
20.04	74.48	0.33
	82.01	0.84

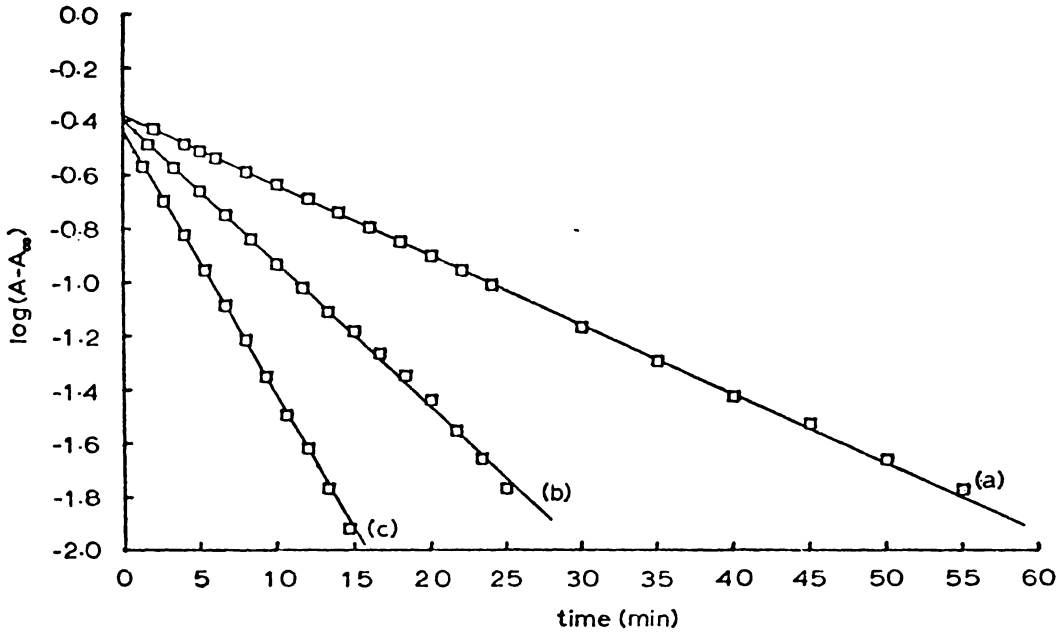


FIGURE 18: Typical examples of the first order plots obtained for isoalloxazine A with NADH at concentrations of (a) 0.00101M; (b) 0.00187M; 9c) 0.00402M at pH 6.3 and 30°.

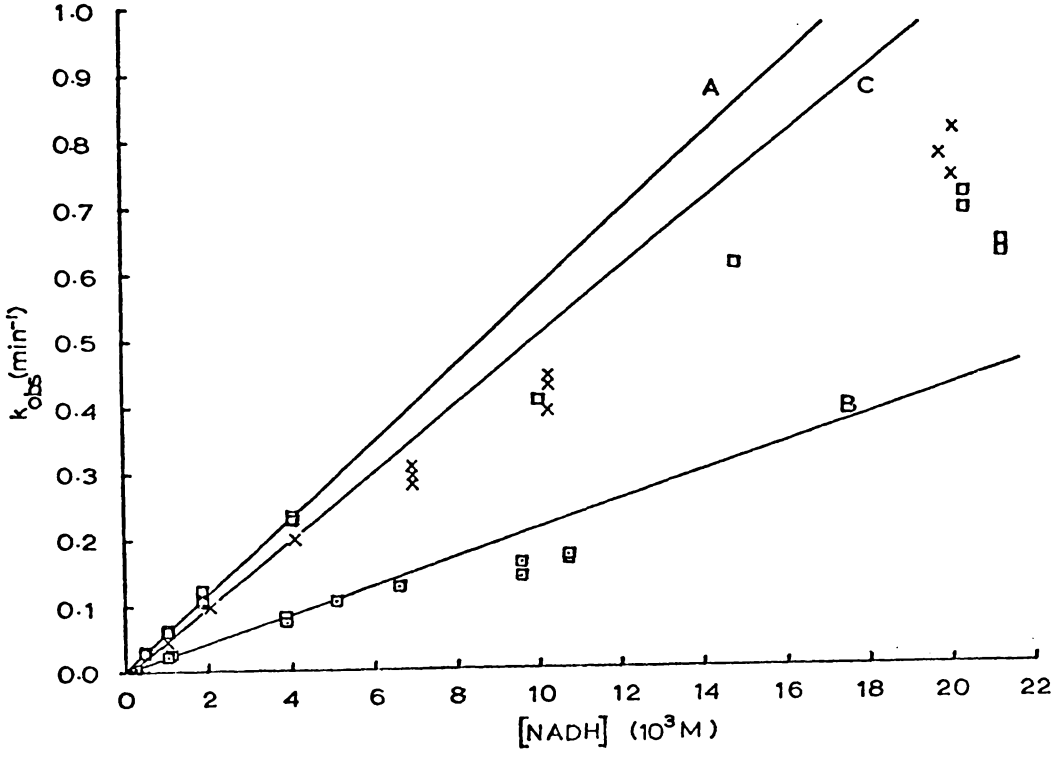


FIGURE 19: Effect of NADH concentration on the observed rate constants for the three isoalloxazines A, B and C showing the progressive deviation of the points as the concentration of NADH increases.

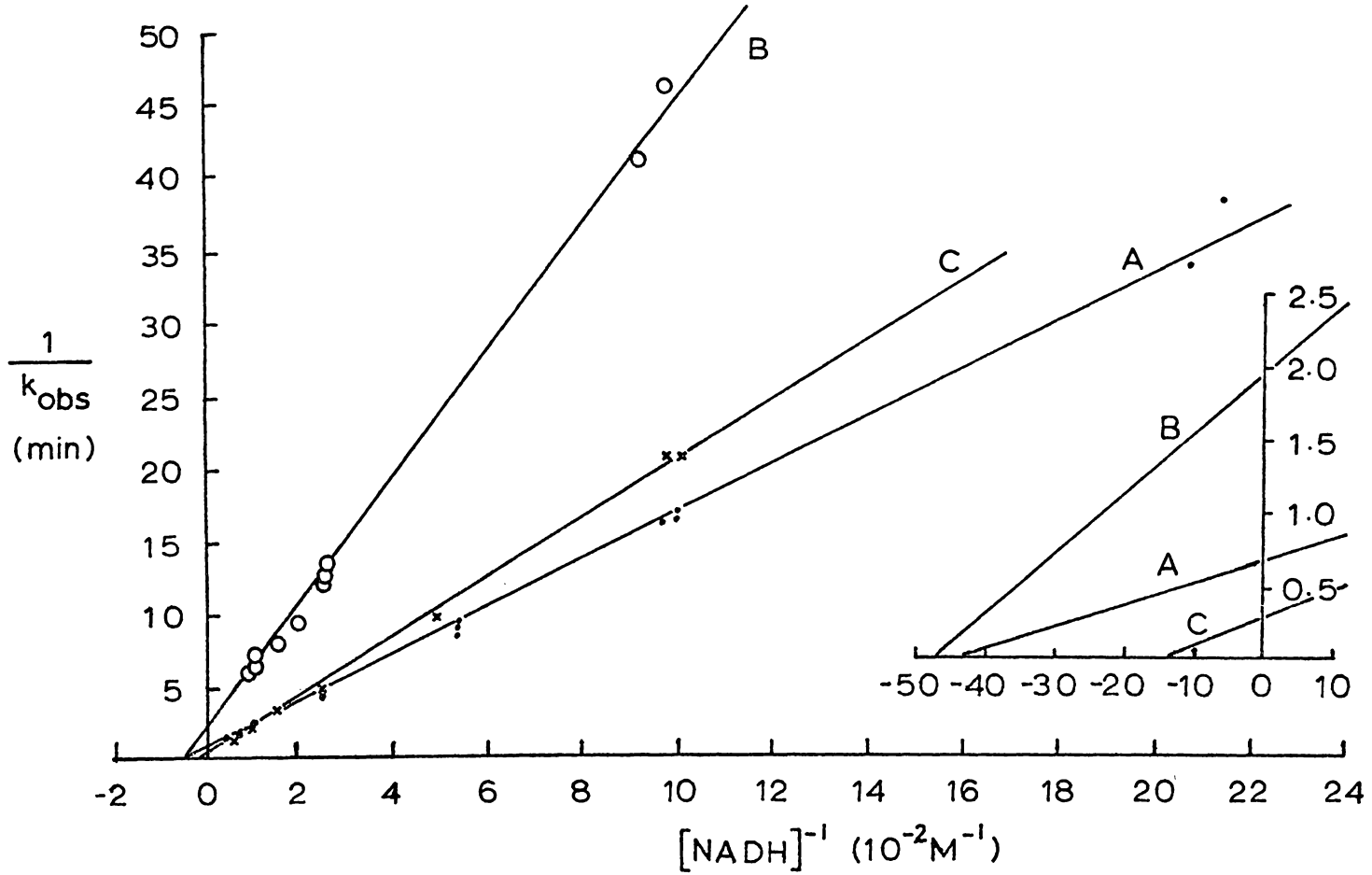


FIGURE 20: Linear correlations obtained for the Lineweaver-Burke plots for the reaction of the three isoalloxazines with NADH. Inset: an enlarged view of the area about the origin showing the negative intercepts of the lines with the x-axis.

observed for isoalloxazines A, B and C with negative intercepts on the x-axis (Figure 20). This therefore is direct evidence for preequilibrium complexing between NADH and each isoalloxazine. The parameters, V (or k_3) and K_d,

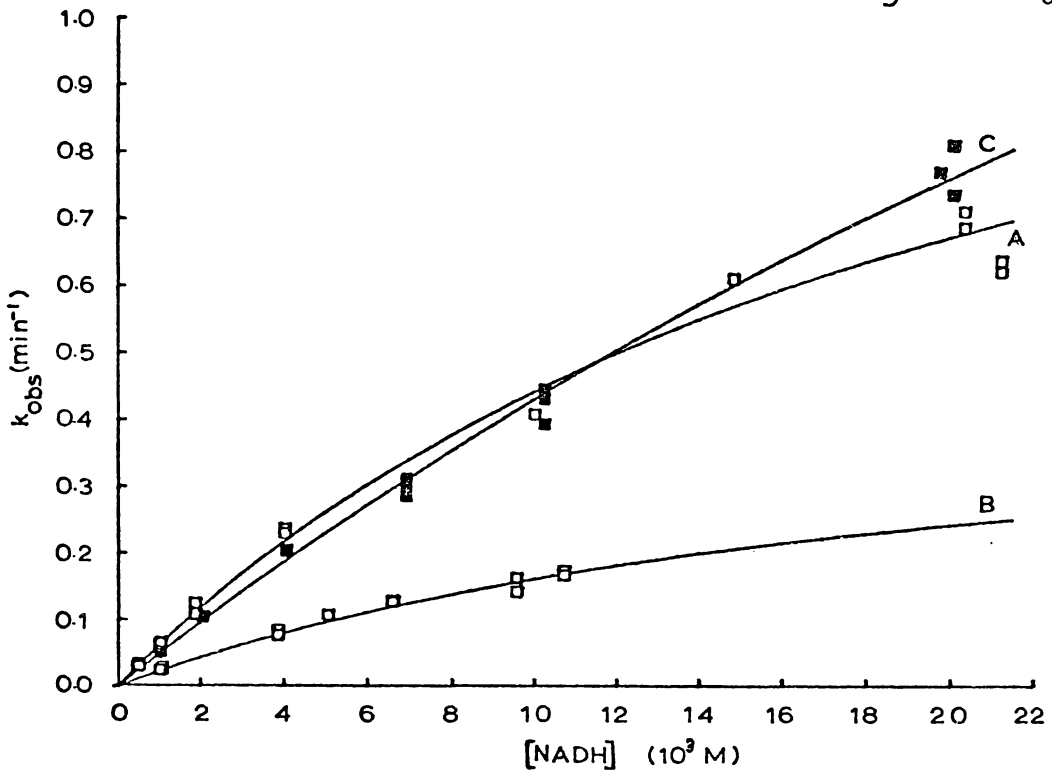


FIGURE 21: A plot of k_{obs}(NADH) against NADH concentration showing the fit of the experimental values with the curves generated by equation 4.20 using the values obtained for k_3 and K_d.

can be obtained directly from the Lineweaver-Burke plots but more reliable values can be obtained from a non-linear least squares treatment of equation 4.20 (which can be derived from the reaction in equation 4.16).

$$k_{obs} = \frac{k_3 [NADH]}{K_d + [NADH]} \qquad (4.20)$$

Details of this method are given in Appendix III. The values computed by this method are presented in Table 4-17 and their fit with the experimental data can be seen in Figure 21.

A rate equation can be derived for the formation of

TABLE 4-17

The Lineweaver-Burke parameters, K_d (M) and k_3 (min^{-1}), and the apparent second order rate constant, $k_2(app)$ ($M^{-1}min^{-1}$), for the reaction of A, B and C with NADH at pH 6.3 and 30°.

	K_d	k_3	k_3/K_d	$k_2(app)$
A	0.023 ± 0.002	1.47 ± 0.09	63 ± 9	58 ± 2
B	0.021 ± 0.003	0.51 ± 0.06	24 ± 6	19 ± 1
C	0.072 ± 0.009	3.56 ± 0.41	49 ± 12	50 ± 1

the reduced isoalloxazine from the reaction sequence shown in equation 4.21.

$$F + NADH \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} X \xrightarrow{k_3} FH^- + NAD^+ \qquad (4.21)$$

Therefore $$\frac{d[FH^-]}{dt} = k_3[X] \qquad (4.22)$$

and $$\frac{d[X]}{dt} = k_1[F][NADH] - k_{-1}[X] - k_3[X] \qquad (4.23)$$

Assuming that a steady state situation occurs for X, then

$$\frac{d[X]}{dt} = 0$$

Consequently $$[X] = \frac{k_1[F][NADH]}{k_{-1} + k_3} \qquad (4.24)$$

Substitution for [X] in equation 4.22 gives equation 4.25

$$\frac{d[FH^-]}{dt} = \frac{k_1 k_3}{k_{-1} + k_3}[F][NADH] \qquad (4.25)$$

The equilibrium constant for the formation of the complex between F and NADH is defined in equation 4.26.

$$K = k_1/k_{-1} \qquad (4.26)$$

The dissociation constant, K_d, of the complex is related to K by the equation $K = K_d^{-1}$. Therefore the apparent second order rate constant, k_2, is defined by equation 4.25 and is

$$k_2 = \frac{k_1 k_3}{k_{-1} + k_3} \qquad (4.27)$$

If $k_{-1} >> k_3$, that is, if X is formed in a rapid equilibrium with the reagents prior to breakdown, then equation 4.27 reduces to equation 4.28.

$$k_2 \simeq \frac{k_1 k_3}{k_{-1}} = \frac{k_3}{K_d} \qquad (4.28)$$

This is in fact the reciprocal of the slope of the Lineweaver-Burke plot and the agreement with the k_2(app) values determined from the early points of the k_{obs}-[NADH] plots (Figure 19) can be seen in Table 4-17, especially for C. The differences between the values obtained for A and B can be explained by the fact that the lines for A and B begin to curve to greater extent at much lower concentrations of NADH than for isoalloxazine C, and this curvature therefore will tend to lower the gradient of the line drawn through these points.

The decreased rate of reaction of isoalloxazine B is shown clearly by the k_2(app) values in Table 4-17 where it is seen that the rates of reaction vary in the order: A > C > B, although there is a difference from this sequence in the order of the k_3 values: C > A > B.

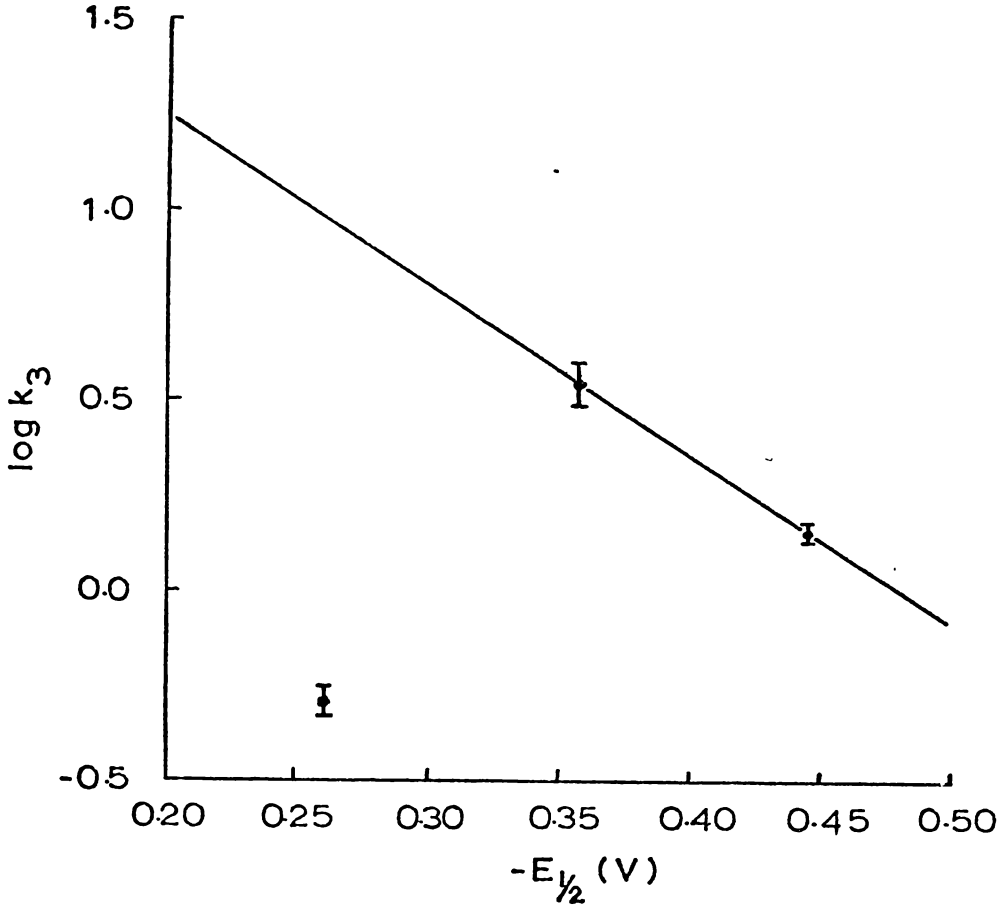


FIGURE 22: A plot of logarithm of k_3 (NADH) against polarographic half-wave potential showing the depression for isoalloxazine B below the line drawn through A and C.

The order of the k_2(app) values is the reverse order expected from the $E_{\frac{1}{2}}$ values and from the rates of reaction with 1,3-dithio-2-propanol; the k_3 values for A and C, however, follow the trend in $E_{\frac{1}{2}}$ and it is readily seen that the value for B is depressed from a line drawn through the points of A and C in a plot of logarithm of k_3 against $E_{\frac{1}{2}}$ as in Figure 22. In the NADH reaction isoalloxazine A is reduced three times faster than B (by comparison of k_2(app)) whereas in the dithiol reaction (section 4-6) B reacts more rapidly than isoalloxazine A by a factor greater than three thousand.

With a consideration of the evidence supporting the formation of pre-equilibrium complexes with reduced nicotinamides[19,26], and the supporting evidence of the results presented here, the rates of reaction of isoalloxazines A and B can be explained in terms of the orientation of the NADH moiety within the complex. Both A and B have an additional benzene ring attached to the basic isoalloxazine structure of C (see A and B, p. 35). This extra benzene ring is much closer to the supposed hydride acceptor site, the N(5)-position (see 1-4.2.1), in isoalloxazine A than it is in B. The complex formed with A might therefore have the NADH molecule advantageously closer to the N(5)-position, whereas with isoalloxazine B the nicotinamide moiety might be complexed further from the acceptor site.

Porter and coworkers[26] consider K_d, in the reaction of lumiflavin with N-methyl-1,4-dihydronicotinamide, to be a true dissociation constant of the complex formed since " the formation of the complex at a reduced nicotinamide concentration equal to K_d was too fast to be measured by the stopped flow method ". If this is true in the case of the three isoalloxazines then B has the largest association constant of the three (followed closely by A), but the reaction of B with NADH proceeds the least rapidly; that is, although the complex between NADH and the isoalloxazines A and B forms to approximately the same extent, the complex formed with B is much less productive than that of A. This then is confirmation that the orientation of the NADH within the complex is important, since B is inherently the more reactive isoalloxazine as shown by the $E_{\frac{1}{2}}$ values and the rates of reaction with dithiol.

The equilibrium constants (K_e) for complex formation between the three isoalloxazines and tryptophan were measured by fluorescence quenching (section 3-3.4). It was found that the equilibrium constants were in the order A > B > C (section 4-4). This confirms that isoalloxazines A and B form complexes with tryptophan more easily than isoalloxazine C does.

TABLE 4-18

Comparison of equilbrium constants, K_e and $1/K_d$ (M^{-1}), for complex formation of isoalloxazines A, B and C with tryptophan and NADH respectively.

	K_e	$1/K_d$
A	112	43.2
B	100	47.2
C	86	13.9

From Table 4-18, it can be seen that there is an overall correlation between these values and the values of the equilibrium constants for complex formation with NADH ($1/K_d$) derived from the Lineweaver-Burke analysis of the kinetic data in that the equilibrium constants for A and B in both cases are distinctly larger than those of isoalloxazine C. The difference in the magnitude of the values of K_e and $1/K_d$ for each isoalloxazine may suggest that the complexing of tryptophan responsible for fluorescence quenching may not involve the same part of the face of the isoalloxazine molecule as the NADH molecule and indeed the similarity of

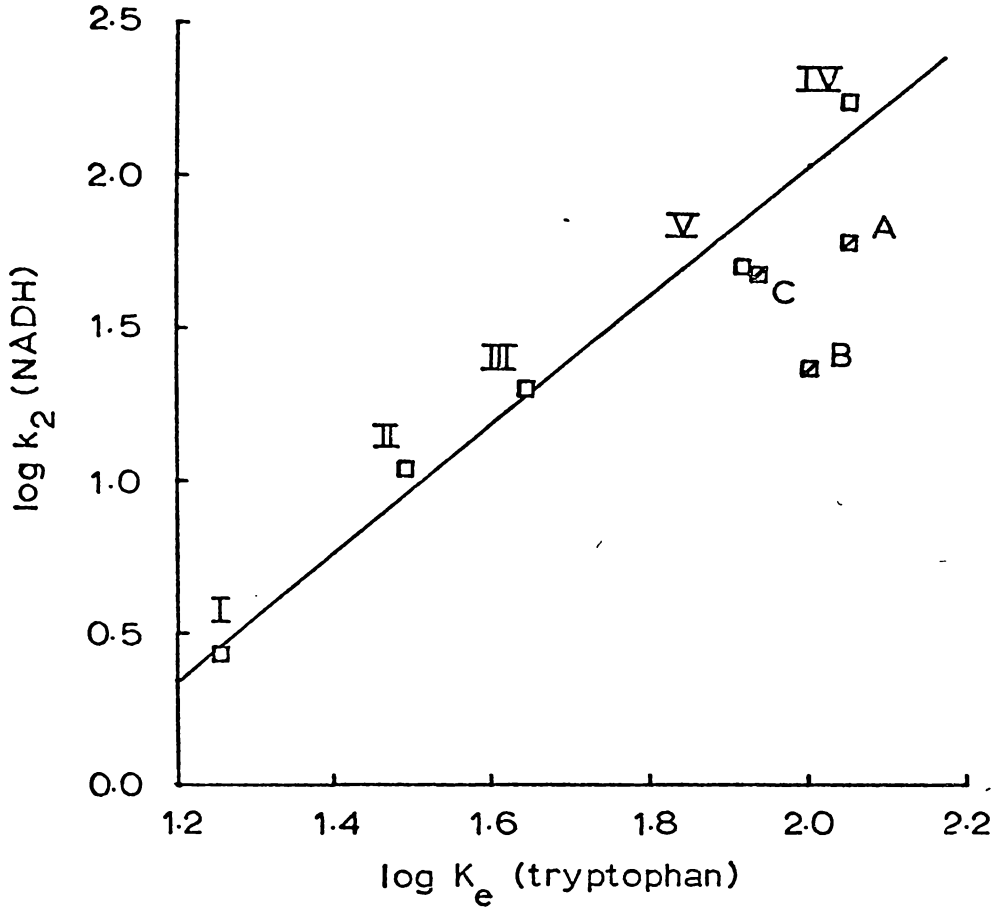


FIGURE 23: A plot of logarithm of k_2(NADH) against logarithm of K_e for the complexing of tryptophan showing the lack of any correlation for isoalloxazines A, B and C (cf line for isoalloxazines I-V from ref. 5).

the association constants, K_e, of the three isoalloxazines suggests even that tryptophan complexing might occur over the pteridine ring area which is common to all three isoalloxazines. Further, unlike the case for the reaction of another series of isoalloxazines with NADH[19], there is no linear relationship between the logarithm of k_2(NADH) and the logarithm of K_e for the complexing of A, B and C by tryptophan (Figure 23). This suggests that in this case the tryptophan complexes responsible for fluorescence quenching are not good models for these particular NADH-isoalloxazine pre-equilibrium complexes on the redox reaction pathway. This is consistent with the concept suggested earlier that complexing of NADH is assisted by

the additional benzenoid rings in A and B over a region of the isoalloxazine molecule where tryptophan complexing either does not occur or, if it does is less effective at quenching the isoalloxazine fluorescence. Since all oxidized flavins fluoresce, this is then clearly a function of the isoalloxazine nucleus and it is therefore a feasible supposition that if tryptophan were complexed partly over the extra benzenoid rings of isoalloxazines A and B then quenching by tryptophan would be expected to be less efficient. An understanding of the molecular orbitals in the fluorescence of isoalloxazines and a comprehensive study involving different quenching agents would obviously be needed to properly justify this interpretation.

4-7.2 Conclusions

In conclusion, the results obtained for the reaction of NADH with three isoalloxazines provide

(a) for the first time direct evidence for a complex with isoalloxazines on the redox reaction pathway; and

(b) evidence that the productivity of such a complex is determined critically by the orientation of the reduced nicotinamide ring with respect to the isoalloxazine hydrogen acceptor site.

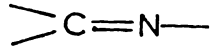
The latter point might obviously have considerable bearing on the orientation in which NADH and the flavin are held by the protein functional groups at the active site of such enzymes as NADH-dehydrogenase.

4-8 CONCLUSIONS FROM THE THREE REACTIONS OF ISOALLOXAZINES A, B AND C

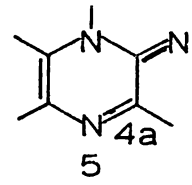
It has been suggested from kinetic evidence that sulphite attacks initially at the N(5) position on the isoalloxazine nucleus. A rate depression, albeit a small one, was observed for isoalloxazine A (with steric crowding at N(5)) when reacting with this nucleophile. By the same token, the lack of any such depression in the rate of reaction of isoalloxazine A with 1,3-dithio-2-propanol suggests that thiol oxidation does not occur through a 5-adduct. However, as mentioned earlier, this is based on the assumption that only in-plane attack occurs when the N(5) nitrogen is involved in adduct formation[10b]. It is considered, however, that the approach of the nucleophile to the N(5)-position need not be restricted to this single direction of attack; out-of-plane attack is just as likely, especially in the case of nucleophilic addition which would avoid unnecssary interaction between the approaching nucleophile and the lone pair electrons on the nitrogen at the 5-position. Thus on the basis of the kinetic data presented in section 4-6, addition of the thiol anion to the 5-position cannot be dismissed altogether. The linear free-energy relationship observed for 1,3-dithio-2-propanol (Figure 17) might then be explained in terms of a lower steric demand in the transition state for thiolate addition at N(5) than for sulphite addition. Differences in the size, polarizability and nucleophilicity of the thiolate and sulphite ions in relation to attack at an electrophilic nitrogen would certainly be expected to result

in different steric demands, but to our knowledge there have been no studies of such effects; nucleophilic addition to nitrogen is a rare reaction.

Consideration of the evidence presented in section 1-4.2.2 concerning the reaction of thiols with isoalloxazines and the knowledge that thiols are known to attack the carbon atom in imine structures (XVII)[92e,97] (c f the -N(5)=C(4a)< bond in isoalloxazines, XVIII) would



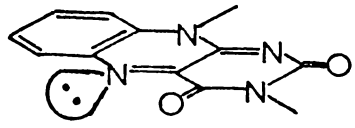
XVII



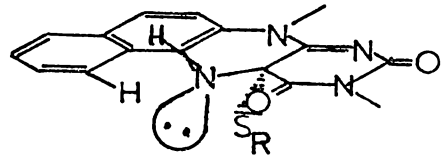
XVIII

suggest however that initial formation of a 5-addition compound on the redox reaction pathway of thiol oxidation is less likely than 4a-addition.

In section 4-5.4 it was concluded that the magnitude of the observed rate depression of isoalloxazine A in the reaction with sulphite was due to either a balance between steric deceleration and steric acceleration effects associated with an in-plane attack or an out-of-plane approach of the nucleophile to the 5-position. With consideration of the attack at the 4a-position by the dithiol the two possibilities above can now be distinguished. In the oxidized isoalloxazine molecule the carbon and nitrogen atoms at the 4a- and 5-positions respectively are sp^2 hybridized and the bonds to these atoms and the lone pair electrons on the nitrogen are coplanar (XIX). With the formation of the 4a-adduct the carbon and the nitrogen change to sp^3 hybridization. This has the effect, in isoalloxazine A, of altering the orientation of the lone



XIX



XX

pair electrons with respect to the α-hydrogen (see IV), and removes the lone pair electron orbital from the plane of the hydrogen atom. This consequently releases the strain described earlier (section 4-5.4) between the α-hydrogen and the lone pair electrons (XX). Thus if steric aceleration is a significant force in the reactions of isoalloxazine A it would also be expected to have an effect on the 4a-addition of dithiol to the isoalloxazine. The lack of any deviation within experimental error of the point for A from the linear free-energy relationship in Figure 17 for the dithiol suggests that this effect is not significant in the reactions of isoalloxazine A. In view of this it is concluded that the small size of the rate depression of A in the reaction with sulphite is due to an out-of-plane approach of the nucleophile

The observation of saturation kinetics with isoalloxazines A, B and C is the first to be observed with NADH itself, although a similar case was reported by Porter and coworkers[26] with lumiflavin and N-methyl-1,4-dihydronicotinamide. The latter being, however, of the order of a 100 times[19] more reactive than NADH itself as evident from the rates of isoalloxazine reduction. The observed order of the rate of reactions of the three isoalloxazines with NADH confirms, together with direct evidence, the suggestion that pre-equilibrium complexing occurs between reduced

nicotinamides and isoalloxazines, and also emphasizes the fact that the orientation of the NADH moiety within the complex is vitally important in the mechanism of the reaction.

APPENDIX I LEAST SQUARES ANALYSIS

The usual methods of analyzing kinetic data for a first order process usually involve the knowledge of either an initial or a final (infinity) reading. This value is then applied to every point as in the conventional first order plot of logarithm of $(A_\infty - A)$ versus time to determine the rate constant. Such methods allow no room for error in A_∞. Thus analytical methods that are independent of the initial and final readings are desirable. Several methods[98-100] have been devised for situations where both the initial and final readings are unknown. These procedures of Guggenheim[98], Kezdy[99] and Swinbourne[100] have the advantage of producing linear graphs with the minimum of calculation and they are therefore simple to apply. However, the disadvantages of these methods are the necessity for readings to be taken at constant time intervals and the requirement that for accuracy there be a fairly large time interval between the readings (at least one half-life[100]).

The non-linear method for unknown initial and final readings devised by Moore[101] eliminates the main disadvantages mentioned above. This procedure was used in computing the rate constants for this work and it is described below. The method has the added advantage of not requiring the readings to be taken at regular intervals and that the readings may be taken for any length of time. The initial and final values and the standard deviations of the three parameters are also determined with the rate constant.

For a first-order reaction it can be shown that

equation I.1 holds[102].

$$\ln (P_\infty - P)/(P_\infty - P_o) = -kt \qquad (I.1)$$

P is some physical property of the system which is related linearly to the concentration of the reactants and products. P_o and P_∞ are the values of the physical property when $t = 0$ and at the end of the reaction respectively; k is the rate constant of the reaction. Equation I.1 can be rearranged to give equation I.2,

$$P = P_\infty(1-e^{-kt}) + P_o e^{-kt} \qquad (I.1)$$

where the dependent variable is expressed in terms of the independent variable t. Equation I.2 can then be treated as a three parameter problem, that is, by letting k, P_∞ and P_o be unknowns. Solution of equation I.2 by the method of least squares must be done by an iterative procedure starting from approximate values of the unknown parameters[103]. The normal equations required for the solution are given in equation I.3[103].

$$\sum_{i=1}^{N} W_i \left(\frac{\partial P}{\partial k}\right)^2 \delta k + \sum_{i=1}^{N} W_i \left(\frac{\partial P}{\partial P_\infty}\right)\left(\frac{\partial P}{\partial k}\right)\delta P_\infty + \sum_{i=1}^{N} W_i \left(\frac{\partial P}{\partial P_o}\right)\left(\frac{\partial P}{\partial k}\right)\delta P_o = \sum_{i=1}^{N} W_i E_i \left(\frac{\partial P}{\partial k}\right) \qquad (I.3a)$$

$$\sum_{i=1}^{N} W_i \left(\frac{\partial P}{\partial k}\right)\left(\frac{\partial P}{\partial P_o}\right)\delta k + \sum_{i=1}^{N} W_i \left(\frac{\partial P}{\partial P_\infty}\right)^2 \delta P_\infty + \sum_{i=1}^{N} W_i \left(\frac{\partial P}{\partial P_o}\right)\left(\frac{\partial P}{\partial P_\infty}\right)\delta P_o = \sum_{i=1}^{N} W_i E_i \left(\frac{\partial P}{\partial P_\infty}\right) \qquad (I.3b)$$

$$\sum_{i=1}^{N} W_i \left(\frac{\partial P}{\partial k}\right)\left(\frac{\partial P}{\partial P}\right)\delta k + \sum_{i=1}^{N} W_i \left(\frac{\partial P}{\partial P_\infty}\right)\left(\frac{\partial P}{\partial P_o}\right)\delta P_\infty + \sum_{i=1}^{N} W_i \left(\frac{\partial P}{\partial P_o}\right)^2 \delta P_o = \sum_{i=1}^{N} W_i E_i \left(\frac{\partial P}{\partial P_o}\right) \qquad (I.3c)$$

The solution of these equations gives δk, δP_∞ and δP_o which are the estimated shifts in the approximate values of k, P_∞ and P_o respectively:

e.g. k(improved) = k(previous) + δk

W_i is a weighting factor given by (standard deviation of

observation, i)$^{-2}$. Unit weights can be used when the standard deviation is approximately the same for each observation. E_i is the difference between the ith value of P and the value calculated from equation I.2 using the estimated values of the three parameters. N is the total number of observations. The partial derivatives required to solve the normal equations are given by equation I.4.

$$\left(\frac{\partial P}{\partial k}\right)_{P_\infty, P_o} = t(P_\infty - P_o)e^{-kt} \qquad \text{(I.4a)}$$

$$\left(\frac{\partial P}{\partial P_\infty}\right)_{k, P_o} = 1 - e^{-kt} \qquad \text{(I.4b)}$$

$$\left(\frac{\partial P}{\partial P_o}\right)_{k, P_\infty} = e^{-kt} \qquad \text{(I.4c)}$$

The initial estimates for the three parameters do not need to be very accurate. P_o and P_∞ can be taken as the first and final observations and the rate constant can be estimated from an approximate value of $t_{\frac{1}{2}}$ from the relation $k = 0.693/t_{\frac{1}{2}}$.

The normal equations are solved by the inversion of the 3 x 3 matrix containing the coefficients of δk, δP_∞ and δP_o on the left hand side of the normal equations using an Algorithm of Busing and Leving[104] (proram SYMINV) and then the inverted matrix is multiplied by a third order vector comprising the right hand side of the normal equations (program MULT). This method is convenient as the diagonal elements of the inverted matrix are related to the standard deviations of the three parameters[105]. Thus the standard deviation, σ_i, is given by

$$\sigma_i = \sqrt{(b_{jj} \sum W_i E_i^2)/(N-3)}$$

where b_{jj} is the appropriate diagonal element of the inverted matrix.

Improvement of the least squares fit is shown by the convergence of the quantity $\sum W_i E_i^2$ to its minimum value. With unit weights a convenient test for convergence is when $\sum W_i E_i^2$ varies by less than 10^{-6} between successive cycles.

The computer programs given below are written in the Ortec Analytical Computer Language (ORACL) and they were run on a Digital PDP 11/05 computer. The program NONLIN calculates the coefficients of the normal equations and it is also the major control program for the other programs listed. SYMINV inverts the 3 x 3 matrix produced by NONLIN and this is then multiplied by a third order vector in MULT. Programs TABS and CAL deal with data entry and print-out of results respectively.

An example of the fit obtained with the experimental data is shown in Table A-1 and Figure 24 for the reaction of isoalloxazine C with a 0.00656M solution of DTP. The values of the parameters for this case are

$$k = 0.0248 \pm 0.0001 \text{ sec}^{-1}$$

$$P_\infty = 0.083$$

$$P_o = 0.675$$

$$W_i E_i^2 = 2.70 \times 10^{-6}.$$

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$$P_\infty = 0.083$$

$$P_o = 0.675$$

$$\sum W_i E_i^2 = 2.70 \times 10^{-6}.$$

TABLE A-1

Typical example of the fit obtained for the kinetic runs (isoalloxazine C with DTP (0.00656M)).

No.	Time	Abs(E)	Abs(T)	Diff.
1	22	0.426	0.426	0.000
2	27	0.386	0.386	0.000
3	32	0.350	0.351	0.001
4	37	0.320	0.319	0.001
5	42	0.292	0.292	0.000
6	47	0.267	0.268	0.001
7	52	0.246	0.246	0.000
8	57	0.227	0.227	0.000
9	62	0.210	0.210	0.000
10	67	0.194	0.195	0.001
11	72	0.182	0.182	0.000
12	77	0.170	0.171	0.001
13	82	0.160	0.160	0.000
14	87	0.151	0.151	0.000
15	92	0.143	0.143	0.000
16	97	0.136	0.136	0.000
17	102	0.130	0.130	0.000
18	107	0.124	0.125	0.001
19	112	0.119	0.120	0.001
20	117	0.115	0.116	0.001
21	122	0.112	0.112	0.000
22	127	0.108	0.108	0.000
23	132	0.105	0.105	0.000
24	137	0.102	0.103	0.001
25	142	0.100	0.100	0.000

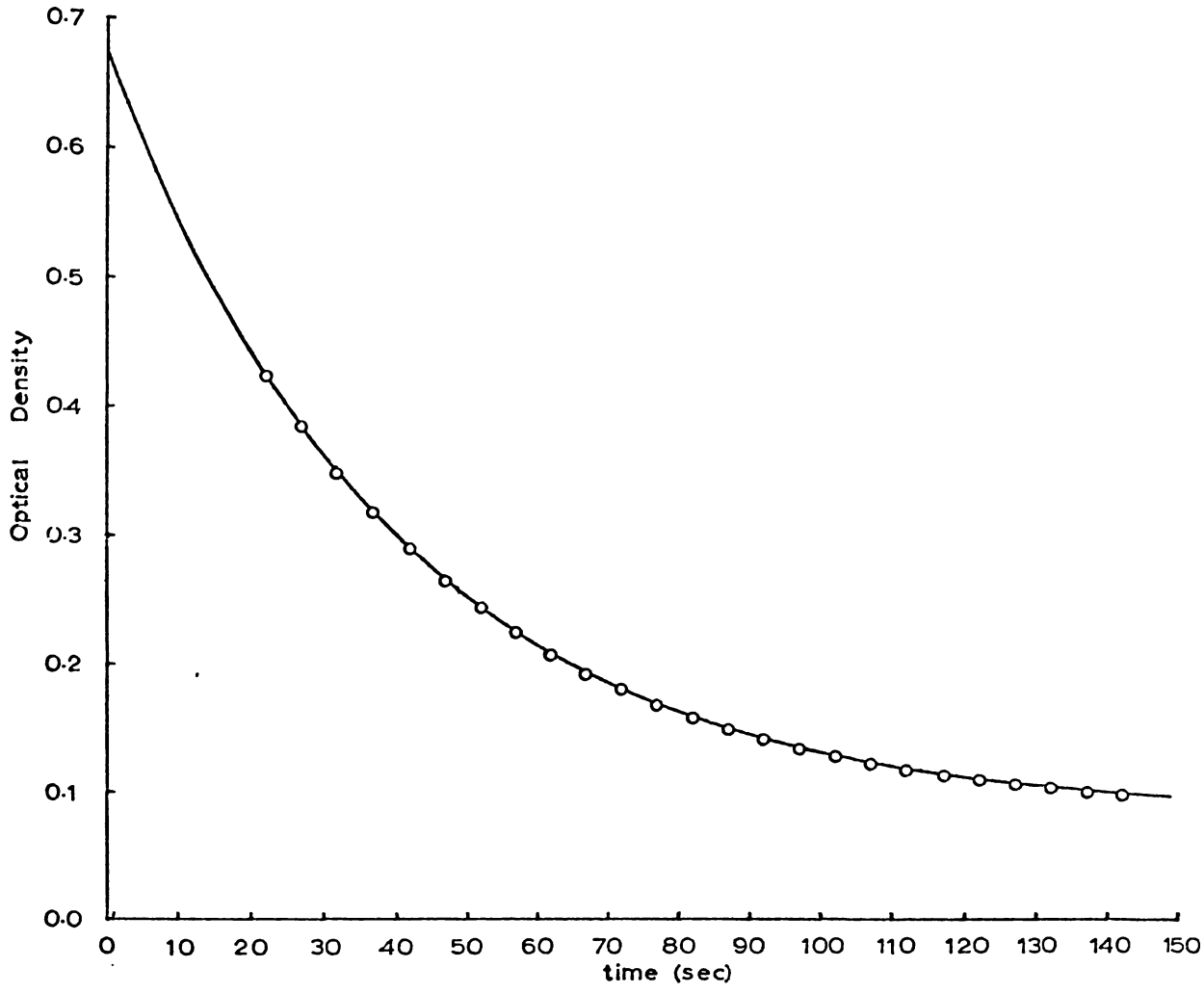

FIGURE 24: An example of a plot of optical density against time demonstrating the fit of the experimental points with the curve generated by equation I.1 using the values of k, P_∞ and P_o obtained by the non-linear least squares method.

NONLIN

```
    1 REMARK  NON-LINEAR LEAST SQUARES FIT FOR FIRST ORDER PROCESS
    2 REMARK  INITAL AND FINAL FINAL VALUES UNKNOWN
    3 REMARK  EQUATION IS OF THE FORM:  PI-P=(PI-PO)EXP(-KT)
    4 REMARK  DO NONLIN(NUMBER,K,PI,PO,F)
    5 COMMON Q;ERASE Q;INTEGER COMMON Q
   10 ARG (NUM,K,PI,PO,F)
   15 SET Q=NUM,W=1,NR=3
   20 IF(F).EQ.(0YES);GOTO 30
   22 COMMON TITLE;ERASE TITLE;STRING COMMON TITLE(75)
   25 TYPE !"TITLE"!;ASK TITLE
   28 DO TABS(Q)
   30 COMMON P,T,D,V,Y,A,B,G;ERASE V,Y,B,G
   35 DIMENS COMMON V(3),Y(3),B(3,3),G(2,3)
   40 SET V(1)=K,V(2)=PI,V(3)=PO,ES=0
   50 B(1,1)=0,B(1,2)=0,B(1,3)=0,B(2,2)=0,B(2,3)=0,B(3,3)=0
   55 Y(1)=0,Y(2)=0,Y(3)=),ES2=ES,ES=0
   60 FOR I=1,Q
   65 SET Z=EXP(-V(1)*T(I))
   70 B(1,1)=B(1,1)+W*(T(I)*(V(2)-V(3))*Z)^2
   72 B(1,2)=B(1,2)+W*T(I)*(V(2)-V(3))*Z*(1-Z)
   75 B(3,3)=B(3,3)+W*Z*Z;B(2,2)=B(2,2)+W*(1-Z)^2
   80 B(1,3)=B(1,3)+W*T(I)*(V(2)-V(3))*Z*Z
   82 B(2,3)=B(2,3)+W*(1-Z)*Z
   85 E=P(I)-V(2)*(1-Z)-V(3)*Z;ES=ES+W*E*E
   90 Y(1)=Y(1)+W*T(I)*E*(V(2)-V(3))*Z
   95 Y(2)=Y(2)+W*E*(1-Z);Y(3)=Y(3)+W*E*Z
  100 NEXT I
  105 SET B(2,1)=B(1,2),B(3,2)=B(2,3),B(3,1)=B(1,3)
  110 DO SYMINV(B,NR)
  115 DO MULT(A,Y,NR)
  120 FOR J=1,3
  125 G(1,J)=V(J)+D(J);V(J)=G(1,J)
  130 G(2,J)=SQRT((A(J,J)*ES)/(Q-3))
  135 NEXT J
  140 IF(ABS(ES2-ES)).GT.(10E-7);GOTO 50
  145 TYPE %,!!"   ES  =   ",ES,!
  150 DO CAL
```

MULT

```
  3 REMARK MULTIPLICATION OF NR ORDER VECTOR AND NR X NR MATRIX
  5 ARG (A,Z,NR)
 10 COMMON D; ERASE D; DIMENS COMMON D(NR)
 15 FOR J=1,NR
 20 D(J)=0
 25 FOR I=1,NR
 30 D(J)=D(J)+A(J,I)*Z(I)
 35 NEXT I
 40 NEXT J
 45 RETURN
```

SYMINV

```
  3 REMARK INVERSION OF NR X NR SYMETRICAL MATRIX
  5 ARG(B,NR)
 10 DIMENS W1(NR),W3(NR)
 11 COMMON A; ERASE A; DIMENS COMMON A(NR,NR)
 15 FOR I=1,NR; FOR J=1,NR; A(I,J)=B(I,J); NEXT J; NEXT I
 20 FOR I=1,NR
 25 FOR J=1,NR
 30 W1(J)=A(I,J)
 35 NEXT J
 40 IF(ABS(W 1(I))-.00001)45,50,50
 45 TYPE !"HELP!!"
 46 STOP
 50 SET D=1/W1(I)
 55 FOR J=1,NR
 60 W3(J)=-W1(J)*D
 65 NEXT J
 70 FOR J=1,NR
 75 FOR K=J,NR
 80 A(J,K)=A(J,K)+W1(J)*W3(K)
 85 A(K,J)=A(J,K)
 90 NEXT K
 92 NEXT J
 95 W3(I)=-D
100 FOR J=1,NR
105 A(I,J)=W3(J)
110 A(J,I)=W3(J)
115 NEXT J
120 NEXT I
125 FOR I=1,NR; FOR J=1,NR; A(I,J)=-A(I,J); NEXT J; NEXT I
130 RETURN
```

TABS

```
 1 REMARK  ENTRY OF DATA
 5 ARG (NUM)
10 COMMON P,T;ERASE P,T;DIMENS COMMON P(NUM),T(NUM)
15 TYPE !"ENTER"!
20 FOR I=1,NUM
25 ASK P(I),T(I)
30 NEXT I
32 TYPE !
35 RETURN
```

CAL

```
 1 REMARK  PRINT OUT OF RESULTS
 5 COMMON G,TITLE
10 TYPE !,TITLE,!!
15 TYPE "    K  =  ",%5.04,G(1,1)," + ",G(2,1),"    "
16 TYPE G(1,1)*60," + ",G(2,1)*60,!
20 TYPE "    PI =  ",%4.03,G(1,2)," + ",G(2,2),!
25 TYPE "    PO =  ",G(1,3)," + ",G(2,3),!!!!
30 RETURN
```

APPENDIX II CALCULATION OF pK_a VALUES OF 1,3-DITHIO-2-PROPANOL

The ionization constants of 2,3-dithio-1-propanol are separated by only 1.96 units of pK (pK_1 8.62, pK_2 10.58[106]) and they are therefore considered to be overlapping ionization processes[94]. A similar situation would be expected for the isomer, 1,3-dithio-2-propanol. Overlapping ionizations occur when a second proton is being titrated before ionization of the first has been completed. Such an occurrence will clearly lead to an indefinite endpoint of the first equivalent. When the ionization of the protons are overlapping the usual methods for the determination of pK, e.g. calculation of the individual concentrations of the ionic species involved, cannot be employed. Albert and Serjeant[94] have devised a computer program for calculating overlapping ionization constants. This program is based on an equation derived by Speakman[107]. This equation was rearranged by Albert and Serjeant to give equation II.1,

$$\frac{1}{K_1^m}\frac{\{H^+\}^2 F}{(2-F)} - K_2^m = \{H^+\}\frac{(1-F)}{(2-F)} \qquad \text{(II.1)}$$

where $\{H^+\}$ is the hydrogen ion activity; K_1^m and K_2^m are the mixed ionization constants (because both activity and concentration functions are used in the expression). The term F is defined by equation II.2.

$$F = \frac{C_A + [H^+] + [OH^-]}{C_t} \qquad \text{(II.2)}$$

C_A and C_t are the concentrations of K^+ added during the titration and the concentration of the substance being

titrated, respectively, and they are defined by equation II.3,

$$C_A = \frac{V_T\ N}{V + V_T} \qquad \text{and} \qquad C_t = \frac{10^3 W}{M(V + V_T)} \qquad \text{(II.3)}$$

where V and V_T are the initial volume and volume of titrant added respectively. The term N is the concentration of the titrant; W is the weight taken of the substance and M is its molecular weight.

The ionization process for a dibasic acid can be represented by equation II.4.

$$\begin{aligned} H_2A &\overset{K_1}{\rightleftharpoons} HA^- + H^+ \\ HA^- &\overset{K_2}{\rightleftharpoons} A^{-2} + H^+ \end{aligned} \qquad \text{(II.4)}$$

The thermodynamic ionization constants can be written as in equation II.5.

$$K_1^t = K_1^m f_{HA^-} \qquad \text{and} \qquad K_2^t = \frac{K_2^m f_{A^{-2}}}{f_{HA^-}} \qquad \text{(II.5)}$$

The activity functions are calculated from the Debye-Huckel equation (equation II.6),

$$-\log f_i = \frac{Az^2 \sqrt{I}}{1 + Ba_i \sqrt{I}} \qquad \text{(II.6)}$$

where A and B are constants which vary with the temperature and the dielectric constant of the solvent. The term a_i is an ionic size parameter and z is the charge on the ionic species. I is the ionic strength and this quantity cannot be calculated from the stoichiometric concentrations used. Thus estimations of I must be made to calculate the activity functions.

The activity functions may be evaluated by defining a

term FS. Thus at 30°, A and B have values of 0.5161 $mol^{-\frac{1}{2}}$ $l^{\frac{1}{2}}$ and $0.3301 \times 10^{8} cm^{-1} mol^{\frac{1}{2}} l^{\frac{1}{2}}$, respectively, and a_i has the value of 5×10^{-8} cm, then FS has the value

$$FS = \frac{\sqrt{I}}{1 + 1.65\sqrt{I}}$$

and then from equation II.6

$$f_i = \frac{1}{10^{Az^2FS}}$$

Therefore at 30° the values of f_{HA^-} and $f_{A^{-2}}$ are $10^{-0.5161FS}$ and $10^{-2.064\,FS}$ respectively. The thermodynamic values now become

$$K_1^t = \frac{K_1^m}{10^{0.5161FS}} \quad \text{and} \quad K_2^t = \frac{K_2^m}{10^{1.5479FS}}$$

Substitution of these values into equation II.1 gives

$$\frac{1}{K_1^t} \frac{\{H^+\}^2 F}{2-F} \frac{1}{10^{0.5161FS}} - K_2^t\, 10^{1.5479\,FS} = \{H^+\}\frac{1-F}{2-F} \qquad (II.7)$$

This equation, in conjunction with equation II.1, is used to calculate the thermodynamic ionization constants as described below.

1. Approximate values for K_1^m and K_2^m are obtained with the pH-volume data from equation II.1 by least squares analysis.

2. The concentration of the ionized species are evaluated using the approximate values of K_1^m and K_2^m in the following equations (II.8)

$$[HA^-] = \frac{K_1^m\{H^+\}}{D} C_t \quad \text{and} \quad [A^{-2}] = \frac{K_1^m K_2^m}{D} C_t \qquad (II.8)$$

where $D = \{H^+\}^2 + K_1^m\{H^+\} + K_1^m K_2^m$.

3. The values of $[HA^-]$ and $[A^{-2}]$ are then used to calculate the approximate ionic strength given by equation II.9,

$$I = \tfrac{1}{2}(C_A + [HA^-] + 4[A^{-2}] + 3\{H^+\}) \qquad (II.9)$$

and hence the first estimations of the activity functions.

4. The hydrogen ion activity is converted to the corresponding concentration by equation II.10.

$$[H^+] = \{H^+\}10^{\cdot 5161\,FS} \qquad (II.10)$$

This term is then used to calculate $[OH^-]$ and F (equation II.2) in which the concentrations rather than the activities must be used. The activity corrections for use in equation II.7 are also calculated.

5. Equations II.1 and II.8 are solved by least squares analysis, thereby obtaining values for the mixed and thermodynamic ionization constants. The refined values of K_1^m and K_2^m are used to recalculate $[HA^-]$ and $[A^{-2}]$; thus with $\{H^+\}$ an improved approximation of the ionic strength is calculated.

6. The values of the thermodynamic constants are further refined by repeating the calculations until successive values of the parameters are constant.

Table A-2 contains the pH-volume data for the five titrations of 1,3-dithio-2-propanol conducted and Figure 25 is a plot of pH against the volume of KOH added for run (e).

TABLE A-2

pH-volume data, pK_1 and pK_2 for the titration of 1,3-dithio-2-propanol by potassium hydroxide (1.2M), 30°.

	KOH(ml)	pH	pK_1	pK_2		KOH(ml)	pH	pK_1	pK_2
(a)	0.121	8.421	9.20		(b)	0.100	8.345	9.22	
	0.201	8.708	9.23			0.200	8.740	9.26	
	0.301	8.952	9.23			0.300	8.992	9.26	
	0.401	9.170	9.24			0.400	9.226	9.29	
	0.451	9.277	9.25			0.460	9.357	9.30	
	0.501	9.371	9.24			0.500	9.441	9.30	
	0.601	9.562	9.22			0.550	9.546	9.29	
	0.651	9.662	9.21			0.600	9.651	9.28	
	0.701	9.762	9.18			0.650	9.760	9.26	
	0.801	9.961	9.05			0.700	9.870	9.22	
	0.851	10.065		11.00		0.800	10.088	8.95	
	0.901	10.162		10.98		0.850	10.191		11.23
	0.951	10.260		10.97		0.910	10.312		11.17
	1.001	10.350		10.96		0.950	10.396		11.16
	1.101	10.524		10.95		1.000	10.499		11.15
	1.201	10.696		10.96		1.050	10.584		11.14
	1.251	10.781		10.97		1.100	10.676		11.14
	1.301	10.862		10.98		1.200	10.859		11.16
	1.401	11.029		11.00		1.300	11.037		11.20
	1.501	11.190		11.02		1.400	11.205		11.25
	1.601	11.333		11.03		1.500	11.360		11.31
						1.600	11.551		11.50

Table A-2 continued.

	KOH(ml)	pH	pK_1	pK_2		KOH(ml)	pH	pK_1	pK_2
(c)	0.100	8.222	9.10		(d)	0.100	8.190	9.07	
	0.150	8.448	9.12			0.160	8.449	9.09	
	0.200	8.163	9.13			0.200	8.591	9.11	
	0.250	8.749	9.13			0.250	8.722	9.11	
	0.300	8.870	9.14			0.300	8.843	9.11	
	0.400	9.091	9.15			0.400	9.062	9.12	
	0.450	9.198	9.15			0.450	9.166	9.12	
	0.500	9.299	9.15			0.500	9.268	9.11	
	0.550	9.400	9.13			0.550	9.366	9.10	
	0.600	9.503	9.11			0.600	9.464	9.07	
	0.650	9.610	9.08			0.650	9.570	9.04	
	0.700	9.717	9.01			0.700	9.673	8;99	
	0.760	9.847	8.85			0.760	9.800	8.79	
	0.800	9.931	8.53			0.800	9.881	8.46	
	0.850	10.036		11.03		0.860	10.009		10.96
	0.900	10.138		10.99		0.900	10.089		10.93
	1.000	10.330		10.96		1.000	10.279		10.89
	1.100	10.511		10.95		1.050	10.370		10.89
	1.150	10.600		10.95		1.100	10.460		10.89
	1.200	10.691		10.96		1.150	10.549		10.89
	1.250	10.779		10.97		1.200	10.637		10.89
	1.300	10.893		11.02		1.250	10.721		10.90
	1.350	10.949		10.99		1.300	10.809		10.91
	1.400	11.031		11.00		1.400	10.972		10.92
	1.450	11.111		11.01		1.460	11.070		10.92
	1.500	11.187		11.01		1.500	11.132		10.92
	1.600	11.321		11.00		1.600	11.272		10.90

Table A-2 continued.

	KOH(ml)*	pH	pK_1	pK_2
(e)	0.100	8.151	9.10	
	0.200	8.502	9.11	
	0.300	8.731	9.11	
	0.400	8.920	9.12	
	0.500	9.090	9.12	
	0.600	9.250	9.12	
	0.700	9.420	9.13	
	0.800	9.591	9.13	
	0.900	9.768	9.13	
	0.920	9.803	9.13	
	0.940	9.841	9.13	
	0.960	9.875		10.86
	0.980	9.910		10.86
	1.000	9.947		10.86
	1.100	10.110		10.84
	1.200	10.261		10.82
	1.300	10.405		10.81
	1.400	10.540		10.79
	1.500	10.677		10.78
	1.600	10.811		10.75
	1.700	10.946		10.70
	1.800	11.072		10.58
	1.860	11.148		10.45

*concentration of KOH 1.0M.

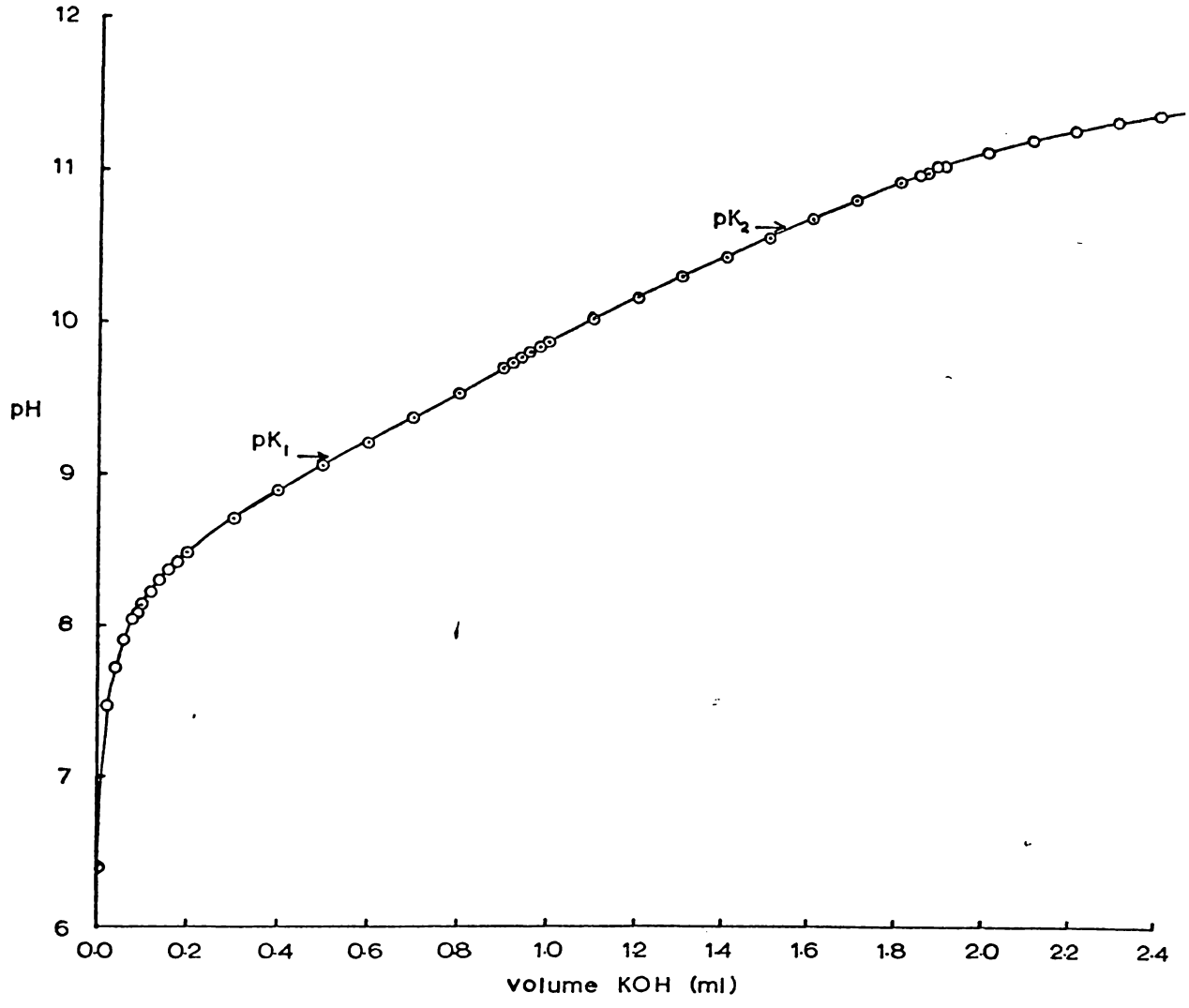

FIGURE 25: An example of a plot of pH against volume of potassium hydroxide added in the titration of 1,3-dithio-2-propanol. The points used in the analysis are designated by ⊙ and they are presented in Table A-2 part (e).

```
  5 REMARK  CALCULATES OVERLAPPING PK VALUES OF BIFUNCTIONAL
 10 REMARK  COMPOUNDS FROM POTENTIOMETRIC DATA AT ANY TEMPERATURE
 15 ASK !"NUMBER ",N," NUMBER POINTS IN FIRST EQUIVALENT ",K
 20 ASK !"MOLECULAR WEIGHT ",SL," WEIGHT ",WT," INITIAL VOLUME ",AL
 22 ASK !"CONC ACID ALLEL ",ACID," VOL ALLEL ",VOL
 25 ASK !"CONC TITRANT ",AC," TEMPERATURE ",T," PKW AT TEMP ",KW
 30 ASK !"PARAMETERS A & B AT TEMP ",D1,D2
 32 ASK !"K TYPE: NEG-ACID, 0-AMPHOLYTE, POS-BASE ",KTYPE
 35 ASK !"DATA IN ",VQ
 40 SET D2=D2*5*10^3
 45 COMMON PH,VA,NSUBS
 50 IF(VQ).NE.("DY")GOSUB 400
 55 DIMENS X(N),Y(N),H(N),OH(N),ACT(N),ACT1(N),ACT2(N)
 60 FOR I=1,N
 65 H(I)=10^(-PH(I));OH(I)=10^(KW+PH(I))
 70 ACT(I)=1;ACT1(I)=1;ACT2(I)=1
 75 NEXT I
 80 REMARK  SOLVE EQUATIONS BY LEAST SQUARES TO OBTAIN MIXED
 85 REMARK  CONSTANTS, CK1 & CK2, AND THERMODYNAMIC CONSTANTS,
 90 REMARK  TK1 & TK2.
 95 TK1=0,TK2=0
100 SUMX=0,SUMY=0,SUMXY=0,SUMX2=0,SUMA=0,SUMB=0,SUMAB=0,SUMA2=0
105 TK1A=TK1,TK2A=TK2
110 FOR I=1,N
115 F=((VA(I)*AC/(AL+VA(I)))+H(I)-OH(I))*SL*(AL+VA(I))/WT*1000
120 A=((10^(-PH(I)))^2)*F/(2-F);B=(10^(-PH(I)))*(1-F)/(2-F)
125 IF(KTYPE)130,135,140
130 X(I)=A/ACT2(I);Y(I)=B/ACT1(I);GOTO 145
135 X(I)=A;Y(I)=B/ACT(I);GOTO 145
140 X(I)=A*ACT2(I);Y(I)=B*ACT1(I)
145 SUMY=SUMY+Y(I);SUMX=SUMX+X(I);SUMA=SUMA+A;SUMB=SUMB+B
150 SUMXY=SUMXY+X(I)*Y(I);SUMX2=SUMX2+X(I)*X(I)
152 SUMAB=SUMAB+A*B;SUMA2=SUMA2+A*A
155 NEXT I
160 DM1=N*SUMX2-SUMX^2;DM2=N*SUMA2-SUMA^2
165 TK1=1/((N*SUMXY-SUMX*SUMY)/DM1)
170 CK1=1/((N*SUMAB-SUMA*SUMB)/DM2)
175 TK2=ABS((SUMX2*SUMY-SUMX*SUMXY)/DM1)
180 CK2=ABS((SUMA2*SUMB-SUMA*SUMAB)/DM2)
182 REMARK  IONIC STRENGTH AND ACTIVITY FUNCTIONS COMPUTED AT TEMP
185 FOR I=1,N
187 P=10^(-PH(I))
190 DM=(P^2+CK1*P+CK1*CK2)*SL*(AL+VA(I))/WT*1000
195 HA=CK1*P/DM;AA=CK1*CK2/DM;A2A=P^2/DM
197 CL=VOL*ACID*2/(AL+VA(I))
200 IF(KTYPE)205,210,215
205 ST=.5*((VA(I)*AC/(AL+VA(I)))+HA+4*AA)+1.5*H(I);GOTO 220
210 ST=.5*((VA(I)*AC/(AL+VA(I)))+CL+A2A+AA)+1.5*H(I);GOTO 220
215 ST=.5*((VA(I)*AC/(AL+VA(I)))+CL+A2A*4+HA)+1.5*H(I)
220 FS=SQRT(ST)/(1+D2*SQRT(ST))
225 ACT(I)=10^(D1*FS);ACT1(I)=10^(3*D1*FS)
230 ACT2(I)=10^(4*D1*FS);H(I)=P*ACT(I)
235 OH(I)=10^(-14)/H(I)
240 NEXT I
242 REMARK  CHECK CONVERGENCE OF VALUES OF CONSTANTS
245 EPA=ABS(TK1*10^(-5));EPB=ABS(TK2*10^(-5))
250 DIA=ABS(TK1A-TK1);DIB=ABS(TK2A-TK2)
255 IF(DIA).GT.(EPA);GOTO 100
260 IF(DIB).GT.(EPB);GOTO 100
262 REMARK  CALCULATION OF PK1 & PK2
265 SUM=0
270 TYPE !!!"SUBSTANCE:  ",NSUBS," = ",%6.03,SL,!
275 TYPE %3.01,"TEMPERATURE:  ",T,!!
280 TYPE "  TITRANT",!,AC,"M KOH"!"    (ML)     PH       PK1        PK2"!!
290 FOR I=1,K
295 PK=X(I)/(Y(I)+TK2);PK=LOG(1/PK)/LOG(10);SUM=SUM+PK
300 TYPE %5.03,VA(I),"  ",PH(I),"  ",PK,!
305 NEXT I
310 AV=SUM/K;SUM=0
315 FOR I=K+1,N
320 PK=X(I)/TK1-Y(I);PK=LOG(1/PK)/LOG(10);SUM=SUM+PK
325 TYPE VA(I),"  ",PH(I),"            ",PK,!
330 NEXT I
335 TYPE !"AVERAGE:   PK1 = ",AV,!"            PK2 = ",SUM/(N-K),!!!
400 REMARK  DATA ENTRY
405 ERASE PH,VA,NSUBS;DIMENS COMMON PH(N),VA(N)
410 STRING COMMON NSUBS(30)
415 ASK !"NAME OF COMPOUND:  ",NSUBS
420 TYPE !"ENTER VOL-PH DATA:"!
425 FOR I=1,N;ASK VA(I),PH(I);NEXT I;TYPE !
430 RETURN
```

APPENDIX III LEAST SQUARES ANALYSIS OF SATURATION KINETICS

This method has the same basis as the non-linear least squares program described in Appendix I. The equation used is also a non-linear function and it has the form as shown in equation III.1,

$$k_{obs} = \frac{k_3[NADH]}{K_d + [NADH]} \qquad (III.1)$$

where k_{obs} is the observed rate constant of the reaction between the isoalloxazine and NADH; k_3 and K_d are defined by the reaction sequence given in equation III.2.

$$F + NADH \rightleftharpoons (F\text{-}NADH) \xrightarrow{k_3} FH^- + NAD^+ \qquad (III.2)$$

k_3 is the rate constant for the breakdown of the complex between the isoalloxazine and NADH; and K is the equilibrium constant for the formation of the complex while $K_d = K^{-1}$ and is the dissociation constant of the complex.

Equation III.1 can be derived from the kinetic equations for the reaction in III.2, for which equations III.3 and III.4 can be written.

$$\frac{d[FH^-]}{dt} = k_3[F\text{-}NADH] \qquad (III.3)$$

$$\frac{d[F\text{-}NADH]}{dt} = k_1[F][NADH] - (k_{-1} + k_3)[F\text{-}NADH] \qquad (III.4)$$

where k_1 and k_{-1} are the forward and back rate constants of the equilibrium and $K = k_1/k_{-1}$.

The total isoalloxazine concentration is given by

$$[F]_o = [F] + [F\text{-}NADH] \qquad (III.5)$$

Assuming the steady state approximation for the complex

then $$\frac{d[F\text{-}NADH]}{dt} = 0$$

and therefore $$[F] = \frac{(k_{-1} + k_3)[NADH]}{k_1[NADH]} \qquad \text{(III.6)}$$

Substituting equation III.6 into III.5 gives

$$[F\text{-}NADH] = \frac{[F]_o[NADH]}{(k_{-1} + k_3)/k_1 + [NADH]} \qquad \text{(III.7)}$$

Finally, substitution of equation III.7 into III.3 gives

$$\frac{d[FH^-]}{dt} = \frac{k_3[F]_o[NADH]}{(k_{-1} + k_3)/k_1 + [NADH]} \qquad \text{(III.8)}$$

Under the reaction conditions the rate equation is

$$\frac{d[FH^-]}{dt} = k_{obs}[F] \qquad \text{(III.9)}$$

Thus comparison of III.8 and III.9 gives equation III.10.

$$k_{obs} = \frac{k_3[NADH]}{(k_{-1} + k_3)/k_1 + [NADH]} \qquad \text{(III.10)}$$

If $k_{-1} >> k_3$, i.e. the formation of the complex is very fast

then $$(k_{-1} + k_3)/k_1 \simeq k_{-1}/k_1 = K_d$$

and hence equation III.10 reduces to equation III.1.

The solution of equation III.1 is a two parameter problem with the unknowns being k_3 and K_d. By analogy with the three normal equations (I.3) in Appendix I there are therefore only two such equations required to solve equation III.1, and they are given in equation III.11.

$$\sum_{i=1}^{N} w_i \left(\frac{\partial k}{\partial k_3}\right)^2 \delta k_3 + \sum_{i=1}^{N} w_i \left(\frac{\partial k}{\partial K_d}\right)\left(\frac{\partial k}{\partial k_3}\right) \delta K_d = \sum_{i=1}^{N} w_i E_i \left(\frac{\partial k}{\partial k_3}\right) \qquad \text{(III.11a)}$$

$$\sum_{i=1}^{N} w_i \left(\frac{\partial k}{\partial k_3}\right)\left(\frac{\partial k}{\partial K_d}\right)\delta k_3 + \sum_{i=1}^{N} w_i \left(\frac{\partial k}{\partial K_d}\right)^2 \delta K_d = \sum_{i=1}^{N} w_i E_i \left(\frac{\partial k}{\partial K_d}\right) \quad \text{(III.11b)}$$

where k is equivalent to k_{obs}; δk_3 and δK_d are the calculated changes in the estimated values of k_3 and K_d respectively. The partial differential equations required to calculate the coefficients of δk_3 and δK_d are given in equation III.12.

$$\left(\frac{\partial k}{\partial k_3}\right)_{K_d} = \frac{[NADH]}{K_d + [NADH]} \quad \text{(III.12a)}$$

$$\left(\frac{\partial k}{\partial K_d}\right)_{k_3} = -\frac{k_3[NADH]}{(K_d + [NADH])^2} \quad \text{(III.12b)}$$

w_i is the weighting factor for the ith value of k_{obs} and it is given by (standard deviation of $k_i)^{-2}$. E_i is the same as in Appendix I.

The normal equations are then solved as described for the three parameter case in Appendix I to acheive the best values for k_3 and K_d. The fit of the experimental data with the theoretical curve can be seen in Figure 21 (section 4-7.1), the experimental values of k_{obs} are given in Tables 4-14, 4-15 and 4-16.

Given below is the printout of the programs used to calculate k_3 and K_d; SATKIN calculates the coefficients in the normal equations (III.11). The two sub-programs, SYMIN2 and MULT2, utilized with SATKIN are identical to those used in NONLIN in Appendix I (except that NR=2).

SATKIN

```
 10 REMARK  LEAST SQUARES ANALYSIS OF SATURATION KINETICS
 20 REMARK  TO SATKIN(N,KS,KD,F)
 30 REMARK  EQUATION IS OF FORM:  Y=AX/(B+X)
 40 ARG (N,KS,KD,F)
 50 SA=N
 60 IF(F).EQ.(0YES);GOTO 80
 70 DO ENTRY(SA)
 80 COMMON TIT,A,NA,X,W,Z,V,B;ERASE Z,V,B
 85 DIMENS COMMON B(2,2),V(2),Z(2)
 90 SET V(1)=KS,V(2)=KD,ES=0
100 B(1,1)=0,B(1,2)=0,B(2,2)=0,Z(1)=0,Z(2)=0,ES2=ES,ES=0
110 FOR I=1,N
115 P=V(2)+NA(I)
120 B(1,1)=B(1,1)+V(I)*((NA(I)/P)^2)
125 B(1,2)=B(1,2)-V(I)*NA(I)*NA(I)*V(1)/(P^3)
130 B(2,2)=B(2,2)+V(I)*((V(1)*NA(I)/(P*P))^2)
135 E=X(I)-V(1)*NA(I)/P
140 ES=ES+W(I)*E*E
150 Z(1)=Z(1)+V(I)*E*NA(I)/P;Z(2)=Z(2)-V(I)*E*V(1)*NA(I)/P*P
160 NEXT I
170 SET B(2,1)=B(1,2)
180 DO SYMIN2(B)
190 DO MULT2(A,Z)
200 FOR J=1,2
210 B(1,J)=V(J)+Z(J);V(J)=B(1,J)
220 B(2,J)=SQRT((A(J,J)*ES)/(N-2))
230 NEXT J
240 IF(ABS(ES2-ES)).GT.(10E-7);GOTO 100
250 TYPE !,%," ES = ",ES,!
260 RETURN
```

SAT

```
  5 CLOCK(1)
 10 ASK "NUMBER "N," KS ",KS," KD ",KD,!"DATA IN ? ",F,!
 15 ASK "PRINT OUT OF FULL RESUTS ? ",F2,!
 18 IF(F).EQ.(0YES);COMMON TIT,B;GOTO 30
 20 COMMON TIT,B;ERASE TIT;STRING COMMON TIT(75)
 25 TYPE !"ENTER TITLE "!;ASK TIT
 30 DO SATKIN(N,KS,KD,F)
 33 TYPE !!!,TIT
 35 IF(F2).NE.(0YES);GOTO 45
 40 DO CALC2(N);GOTO 55
 45 TYPE !!"  KS = ",B(1,1)," + -",B(2,1),!
 46 TYPE "  KD = ",B(1,2)," + -",B(2,2),!
 47 TYPE "  K  = ",1/B(1,2)," + -",B(2,2)/B(1,2)*B(1,2),!
 50 TYPE "  K2 = ",B(1,1)/B(1,2)," + -"
 52 TYPE (B(2,1)/B(1,1)+B(2,2)/B(1,2))*B(1,1)/B(1,2),!
 55 CLOCK(-1);TIME=INT(CLOCK(0)/50*50),TIMS=CLOCK(0)/50*50-TIME
 60 TYPE !" JOB : ",%4,TIME," MIN, ",%4.02,TIMS*50," SEC.",!
 65 TYPE !"**END**"!!
```

ENTRY

```
  5 REMARK DO ENTRY(SA)
 10 ARG (SA)
 20 COMMON NA,KO,W ERASE NA,KO,W DIMENS COMMON NA(SA),KO(SA),W(SA)
   25 TYPE !"W IS THE STANDARD DEVIATION IN KOBS"!
 30 TYPE !,"ENTER  (NAIM)  KOBS  W",!
 40 FOR I=1,SA
 50 ASK NA(I),KO(I),W(I)
 55 W(I)=1/(W(I)^2)
 60 NEXT I
 70 TYPE !
 80 RETURN
```

CALC2

```
 10 REMARK DO CALC2(NUMBER)
 20 ARG (N)
 30 COMMON TIT,B,NA,KO
 35 TYPE !!,"  POINT       (NAIM)          KOBS       IDEAL KOBS"!!
   40 FOR I=1,N
 50 RA=W(1)*NA(I)/(W(2)+NA(I))
 60 TYPE %4,I,"    ",%7.0C,NA(I),"    ",%7.05,KO(I),"    ",RA,!
   70 NEXT I
 80 TYPE !"    K3 = ",%6.04,B(1,1)," + -",B(2,1)
 90 TYPE !"    K1 = ",B(1,2)," + -",B(2,2)
 95 TYPE !"    KA = ",1/B(1,2)," + -",B(2,2)/B(1,2)*B(1,2)
  100 TYPE !"    K2 = ",B(1,1)/B(1,2)," + -"
105 TYPE (B(2,1)/B(1,1)+B(2,2)/B(1,2))*B(1,1)/B(1,2),!
  110 RETURN
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