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Reactivities of Alloxazines and Isoalloxazines

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The advances in the chemistry of alloxazines and isoalloxazines in the last 10-12 years have been reflected particularly in the study of their reactivities. The review deals with the reduction, oxidation, and nucleophilic and electrophilic substitution reactions, the reactions of exocyclic amino-groups, the methyl group in the 8-position of the flavine nucleus, and others. The reactivities of alloxazines and isoalloxazines are discussed with reference to quantum-chemical parameters. The bibliography includes 245 references.

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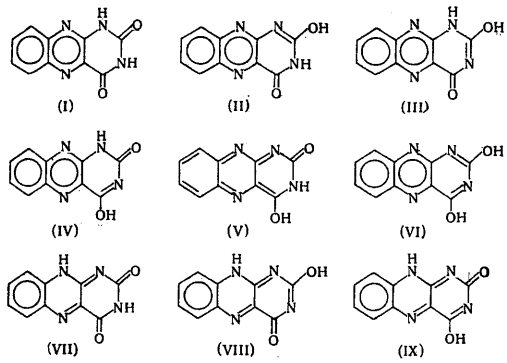
1. INTRODUCTION

Alloxazines and isoalloxazines—2,4-dioxo-1,2,3,4-tetrahydrobenzo[*g*]pteridines—constitute a heterocyclic series of compounds the natural representatives of which [riboflavin (vitamin B_2) and its coenzymes—riboflavin 5′-phosphate or flavin mononucleotide (FMN) and P^1-(riboflavin-5′)-P^2-(adenosine-5′)diphosphate or flavin-adenine dinucleotide (FAD)] combine with a specific protein and fulfil key electron transfer functions in metabolic processes.

However, for a long time after the discovery of riboflavin (1932), which proved to be the basic structural unit of the cofactors of flavin oxidation-reduction enzymatic systems, mainly the synthetic pathways of alloxazines and isoalloxazines and their analogues were investigated[1,2] using the complete flavin system as a basis for the discovery of its essential biological properties. The entire wide variety of the chemical characteristics of compounds of the alloxazine and isoalloxazine series has been demonstrated only in the last 10–12 years. Thus the electronic structures and steric configurations of the riboflavin molecule and of related compounds, the reactivities of ring nitrogen atoms, the capacity of the carbon atoms in the benzene ring for electrophilic substitution, the oxidation of alloxazines to *N*-oxides, the reactions of exocyclic amino-groups and of the methyl group in the 8-position of the flavin nucleus, photochemical reactions, spectroscopic properties of alloxazines and isoalloxazine molecules, etc. have been investigated; the results of the studies of the chemical properties have been interpreted in relation to the principal biochemical function of flavins. The combined studies on the reactivity and its dependence on the π-electronic parameters of the molecule led logically to the determination of the structure of a new flavin cofactor of succinate dehydrogenase—an enzyme which catalyses the dehydrogenation of succinic acid to fumaric acid in the key tricarboxylic acid metabolic energy cycle (the Krebs cycle)—and a new flavin cofactor of another enzyme—monoamine oxidase.

2. TAUTOMERIC FORMS OF ALLOXAZINE AND ISOALLOXAZINE

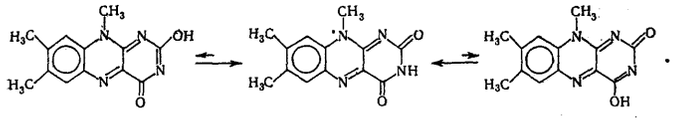
Alloxazine can have 6 possible lactam-lactim tautomeric forms (I)–(VI), while isoalloxazine has only 3 tautomeric forms (VII)–(IX) as a result of the fixation of the electronic configuration by the substitution of the hydrogen atom in the 10-position by an alkyl group:



The problem of the tautomeric forms of heterocyclic compounds is frequently solved by comparing their electronic spectra with those of model compounds having a known structure[3]. However, experimental studies of *N*- and *O*-alkyl derivatives of flavins[4-6] have shown that, owing to the insignificant differences between the absorption spectra, the study of the lactam–lactim tautomerism of such flavins by ultraviolet spectroscopy is impossible[7]. Calculation of the transition energies and oscillator strengths for the tautomeric forms (I)–(IX) by the Pariser–Parr–Pople self-consistent field (PPP SCF)

method also led to the conclusion that the transition from the lactam to the lactim form has little influence on the absorption spectra[8,9].

O-Alkyl, *S*-methyl, and deoxy-derivatives of lumiflavin differ markedly from lumiflavin itself in their basicity: they are fully protonated even in dilute acetic acid[5]. These compounds are relatively unstable and are readily hydrolysed, being easily converted into lumiflavin in the presence of weak oxidants. On this basis, it has been concluded that flavins exist in the lactam form[7] and have virtually no admixture of the hydroxyimino-forms[7,10]; the prototropic equilibrium is apparently fully displaced towards the lactam form[7]:



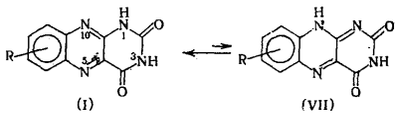
The infrared spectra of a large number of substituted alloxazines and isoalloxazines and their comparison with the spectra of compounds with fixed structures established that both alloxazines and isoalloxazines have the lactam structure in the solid state[11].

By comparing the calculated energies of the upper bonding and lower antibonding orbitals for all possible tautomeric forms of alloxazines and isoalloxazines, Nishimoto[9] showed that the electron affinity of the dioxo-forms is fairly high and that such compounds are effective oxidants. It has been suggested that in biological systems isoalloxazines are capable of tautomeric conversion into the enolic forms as a result of the formation of hydrogen bonds with proteins and thus behave as both electron donors and acceptors in biochemical reactions[9].

It is noteworthy that the π-electron energy of the 4-*C*-enolic form (IX) is higher by 4–5 eV than for the tautomeric 2-*C*-enolic form (VIII), i.e. form (IX) is energetically more favourable[12].

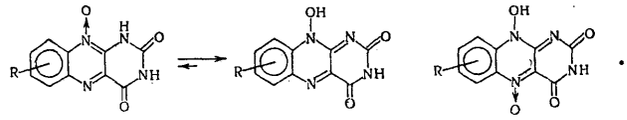
Berezovskii and Aksel'rod studied the possibility of the alloxazine and isoalloxazine tautomerism in many alloxazines[13,14], their 5-*N*-oxides[15], 10-*N*-oxides[13,14], and 5,10-di-*N*-oxides[15]. It was shown[13,14] that the difference between the structure of alloxazines and isoalloxazines, which depends on the $-N_{(5)}=C-C=N_{(10)}-$ (I) and $-N_{(5)}=C-C=N_{(1)}-$ (VII) chromophoric groups, can be established with reasonable certainty by ultraviolet and fluorescence spectroscopic methods.

Comparison of the ultraviolet, infrared, and fluorescence emission spectra of a number of substituted alloxazines with thoes of substances having fixed structures showed[11,13,16] that alloxazines as well as their 5-*N*-oxides (with the N→O bond)[15] have the alloxazine tautomeric structure of type (I). These data are consistent with the results of quantum-chemical calculations by the LCAO-MO method for a number of 7- and 8-substituted alloxazines and isoalloxazines, which indicate that the alloxazine structure (I) is energetically somewhate more favourable than the isoalloxazine structure (VII):[17]

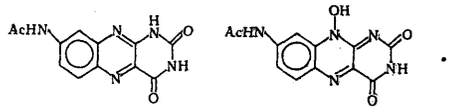


(I) (VII)

On the other hand, the *N*-hydroxy(isoalloxazine)-structure of type (VII) should be assigned to alloxazine 10-*N*-oxides and 5,10-di-*N*-oxides which lack substitutents at the 1-*N* atoms[13,15], in view of the similarity of the fluorescence and absorption spectra of such *N*-oxides with those of isoalloxazines (riboflavin and others):



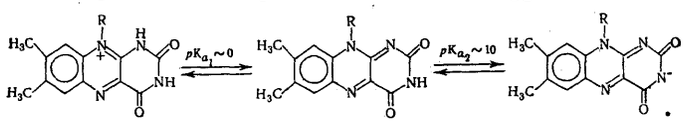
Comparison of the infrared spectra of 8-amino- and 8-dimethylaminoalloxazines established that amino-alloxazines exist in the amino- and not in the imino-form[11]. On the basis of a comparison of the absorption and fluorescence spectra with those of the 8-amino-1,3-dimethyl-alloxazine, which has a fixed structure, one may postulate that in alcoholic solutions 8-aminoalloxazines and their *N*-oxides have the alloxazine structure[14]. 8-Acylamino-alloxazines have the alloxazine structure of type (I) in alcoholic solutions and 8-acylaminoalloxazines *N*-oxides have the isoalloxazine structure of type (VII) with an N–OH group[14]:



Despite the existing extensive experimental results, hitherto no data are available on the interconversion of the tautomers and on the simultaneous presence of different tautomeric forms in solutions .

3. ACID–BASE PROPERTIES

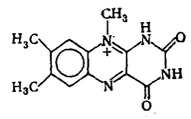
In the oxidised state flavin is weakly amphoteric; the following acid–base equilibrium has been suggested[18]:



The ionisation constants of riboflavin, 2′,3′,4′,5′-tetra-acetylriboflavin, riboflavin 5′-phosphate, and FAD have been determined from measurements of oxidation–reduction potentials[19], fluorescence[20,21] and absorption[19,22] intensities, and also by acid–base titration[23–25]. The following values have been obtained for riboflavin: pK_{a1} = 0,[26] −0.2,[19] and 0.1;[22] pK_{a2} = 9.5,[19] 10.2,[20] 9.95,[24] and 10.[23] The acid dissociation constants of alloxazine and its derivatives with substitutents in the benzene ring have been determined spectrophotometrically (by the method of Geissman and Davis); it was found that the pK_a for these compounds are satisfactorily consistent with the inductive effects of the substituents, the pK_a increasing in the following sequence[22]:

$$8\text{-Cl} < \text{H} < 7.8\text{-di-CH}_3 < 8\text{-NH}_2 .$$

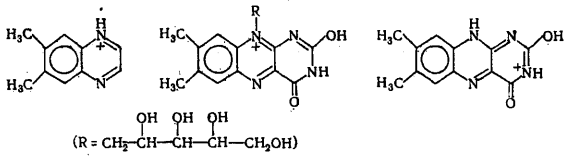
X-Ray diffraction analysis of the structures of riboflavin hydrobromide[27,28] and 10-methylisoalloxazine[29–32] established that in the crystalline state the flavin is protonated at 1-N and the cation has the following structure:



Protonation of the flavin in sulphuric acid results in a considerable hypsochromic shift of the long-wavelength

absorption band and a bathochromic shift of the band in the near ultraviolet, so that they merge into a single band at 390 nm.[7] Comparison of the ultraviolet spectrum of the unsubstituted flavinium cation with those of model alkylflavinium derivatives led Dudley et al.[7] to the conclusion that in aqueous solutions flavins are protonated at 1-N. This agrees with calculations[8,33] according to which the maximum π-electron density is localised at 1-N.

However, later calculations by the self-consistent field (SCF) method[12,34-37], the extended Hückel method[36,38], and also by the simple LCAO-MO method[17] of the distribution of electron density in substituted alloxazines and isoalloxazines established that the highest electron density is concentrated at the oxygen atoms of the carbonyl groups. It is noteworthy that the basicity of atoms of different chemical types is determined not only by electron density but also by the energy of formation of the X—H bond[38]. The similarity of the magnetic circular dichroism spectra of flavinium and 6,7-dimethylquinoxalinium cations has also been used as an argument in support of the proposed structure of the cation[39]; this structure does not conflict with the protonation of riboflavin at the oxygen of the $C_{(2)}$—O carbonyl group:

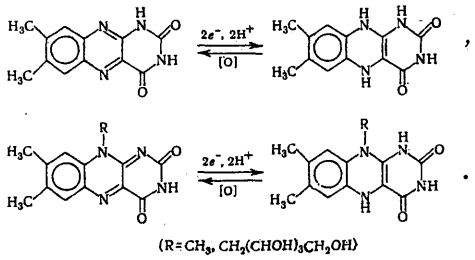


The structure of the monocations of alloxazines and isoalloxazines protonated at the oxygen atom in the 2-position has been proposed on the basis of the disappearance of one of the carbonyl absorption bands (1650 cm^{-1}) in 50% sulphuric acid[40]. The conclusion that alloxazines and isoalloxazines are *O*-protonated agrees with the available data on the protonation of lumazines—substances which are very similar to flavins[41].

Infrared spectroscopy established that, regardless of the nature of the cations, metal salts of flavins have the lactim structure in the solid state[11].

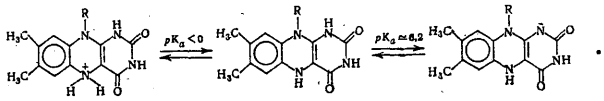
4. REDUCTION

Under aerobic conditions, both alloxazines and isoalloxazines exist in the fully oxidised form, since their oxidation-reduction potentials (for example, −0.21 V for riboflavin) are much lower than the potential of atmospheric oxygen (+0.82 V).[42] Certain reducing agents (sodium hydrosulphite, zinc in acetic or hydrochloric acid, hydrogen in the presence of the palladium or platinum catalyst, etc.) are capable of reducing these compounds under anaerobic conditions to the non-fluourscent 5,10- and 1,5-dihydro-forms[43-47]. In the presence of oxygen, the fully or partly reduced alloxazines and isoalloxazines are reoxidised:



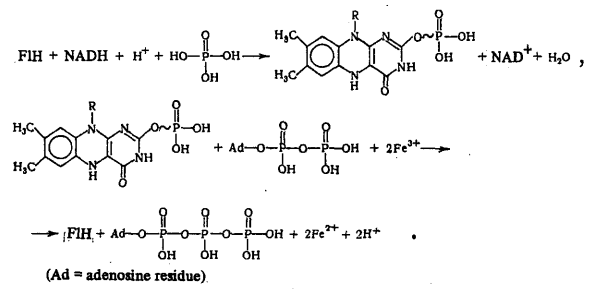
(R = CH_3, $CH_2(CHOH)_3CH_2OH$)

In dilute aqueous solutions the reduced flavins are colourless and in concentrated solutions they are orange-brown[48]. An equation has been formulated for the acid–base equilibria of flavins in the reduced form[18]:



Comparison of the ultraviolet spectra of 1,5-dihydrolumiflavin and its *NN*- and *OO*-dialkyl derivatives established that 1,5-dihydroflavins have the lactam structure, which has also been confirmed by the infrared spectra of a 5-acyl-1,5-dihydrolumiflavin and 1,5-dihydroriboflavin in chloroform[4,7]. However, in contrast to flavins, the conversion of 1,5-dihydroflavins into the lactim form does not involve a considerable energy increase, in agreement with the ease of alkylation at the oxygen atom[7].

It has been suggested that oxidative phosphorylation processes involving flavins (FlH) yield phosphate esters of the lactim form of 1,5-dihydroflavin, which transfer the phosphate group to ADP with formation of ATP:[49,50]

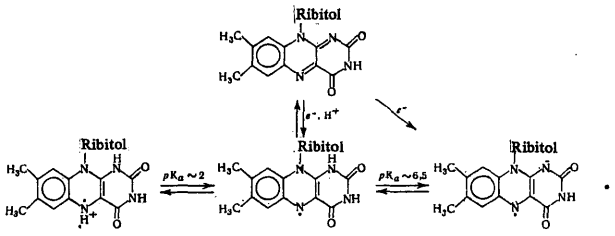


(Ad = adenosine residue)

Since alkylation at 3-N does not affect the acidity of 1,5-dihydroflavin (pK_a = 6,[18] 7.2 [51]), it is believed that the dissociable proton is at 1-N.

On the basis of the analysis of the absorption spectra at different pH, a non-polar molecular structure folded along the $N_{(5)}$—$N_{(10)}$ axis and having the "butterfly wings" conformation has been proposed for 1,5-dihydroflavin[7,10,18]. This non-planar structure of 1,5-dihydroflavins agrees satisfactorily with calculations by the Hückel[52] and self-consistent field[34] methods and has been reliably established by the X-ray diffraction analysis of 5-acetyl-3,7,8,10-tetramethyl-5,10-dihydroisoalloxazine[53] and also 5-acetyl-9-bromo-1,3,7,8,10-pentamethyl-1,5-dihydroisoalloxazine[54,55]; the planes of the benzene and pyrimidine rings are inclined at an angle of 140–160°.[53,56]

In contrast to the two-electron reduction of alloxazines and isoalloxazines discussed above, one-electron reduction of flavins, for example riboflavin, leads to the formation of free radicals—flavosemiquinones[19]:



However, one cannot be certain whether the flavosemiquinones are intermediates[57] in the two-electron reduction or whether they are formed in the interaction between the oxidised and reduced forms of flavins[58]:

$FlH + FlH_3 \rightleftharpoons FlH_2$ (green chloroflavin),

which is more likely, since flavosemiquinones exist in solution in equilibrium with other forms [7,59,60]; at pH 8–9 the concentration of radicals is minimal and the equilibrium is displaced towards the oxidised and reduced forms [61,62]. Fairly stable dimeric complexes are formed from products corresponding to different degrees of reduction [63,64]:

$FlH + Fl\dot{H}_2$ (green verdoflavin),

$Fl\dot{H}_2 + FlH_3$ (carmine red rhodoflavin hydrochloride).

Numerous studies have been made on the one- and two-electron reduction reactions of flavins and on the determination of the structure of flavin radicals [51,65–70].

The reduction of flavins in the dark takes place with only a few electron-donating substrates: NADH, NADPH, and their analogues [71–73], dihydrolipoic acid [74,75]; *cis*- and *trans*-dihydrophthalic acids and their esters [76], and mercaptans [77]. The reaction of flavins (FlH) with NADH and NADPH and with their analogues is of second order and that with dihydrolipoic acid is of first order; the reduction proceeds via a two-electron mechanism [73], rapid dissociation of one of the thiol groups preceding the reduction process. The reduction by dihydrophthalic acids and their esters is a second order reaction of pH > 9. [76] Flavins react with mercaptans according to the mechanism [73,77]

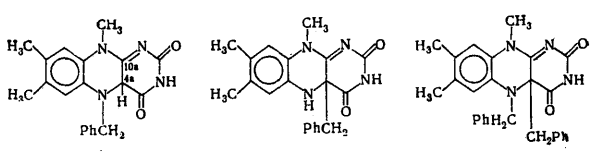
$$2RSH + FlH \rightarrow RSSR + FlH_3 .$$

Under anaerobic conditions, flavins are readily reduced photochemically by many substances which behave as electron donors: ethylenediaminetetra-acetic acid (EDTA) and aminoacids—cysteine, methionine, proline, histidine, etc. [78–83]

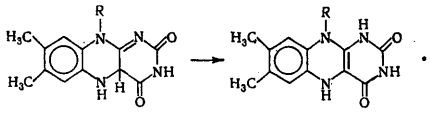
The hypothesis that water may function as the electron donor in the electrochemical reduction of flavins [84–87] appeared to be controversial [88,89], but certain experimental data obtained in recent years by manometric and mass-spectrometric methods indicate that water is decomposed in photochemical reactions involving riboflavin or lumiflavin [90–92]. Probably photoexcited flavin reacts with an external electron donor in the triplet state [93–95]. The ribityl side chain promotes the transition from the first excited singlet state of flavin to the triplet state [96,97]. In the absence of an external electron donor, its role is assumed by the ribityl side chain (in riboflavin), which is cleaved in this process [95].

It has been suggested that the direct cleavage of riboflavin to lumichrome can also proceed via the singlet excited state [98]. Different aspects of the nature of the first excited triplet and singlet states have been investigated [99–106].

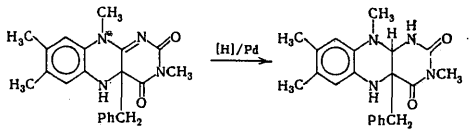
Apart from the reduction of alloxazines and isoalloxazines involving the nitrogen atom in the 1- and 5-positions, other types of reduction have been studied. Hemmerich and coworkers carried out the photochemical reduction of lumiflavin by phenylacetate ions and isolated mono- and di-benzyl derivatives of 4a,5-dihydrolumiflavin [82,107]:



It is possible that 4a,5-dihydroflavins of this kind are formed in biochemical reactions under the influence of hydride ions, for example, in the dehydrogenation of succinic acid to fumaric acid in the presence of succinate dehydrogenase [108]. It is also possible that in biochemical reactions the 4a,5-dihydroflavin is subsequently converted into the 1,5-dihydroflavin as a result of an intramolecular proton shift [108]:

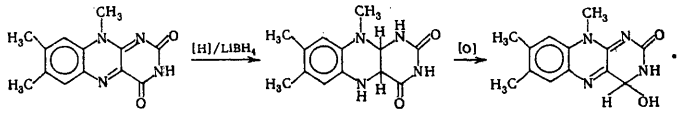


Catalytic hydrogenation of 4a-benzyl-3-methyl-4a,5-dihydrolumiflavin leads to further reduction and the formation of 4a-benzyl-3-methyl-1,4a,5,10a-tetrahydrolumiflavin [82]:



On reduction with sodium borohydride, flavinium salts are also converted into 1,4a,5,10a-tetrahydro-derivatives [109].

The reduction of isoalloxazines, for example, lumiflavins with lithium borohydride takes place via two pathways: under anaerobic conditions, 1,5-dihydrolumiflavin is formed (see above), i.e. the usual reduction product, while in the presence of atmospheric oxygen the reaction leads to the formation of 3,4-dihydrolumiflavin, which is resistant to reoxidation in the crystalline state and in solution in organic solvents, but undergoes photochemical oxidation to lumiflavin [110]. Possibly the initial product of this reaction is 1,4a,5,10a-tetrahydrolumiflavin, which is then partly oxidised and rearranges to 3,4-dihydrolumiflavin [110]:



The reduction by lithium borohydride involving the carbonyl group in the 4-position has also been observed for alloxazines, for example 1,3,7,8-tetramethylalloxazine, which is converted into the 3,4-dihydro-derivative [111].

It is remarkable that D- and L-aminoacid oxidases reduced with lithium borohydride do not lose their biocatalytic activity, which suggests that the dehydrogenation of the substrate does not involve the 4-position of the flavin prosthetic group [112].

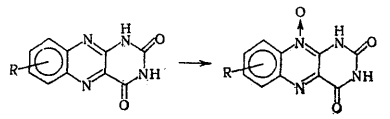
Under severe catalytic hydrogenation conditions, the benzene ring in the isoalloxazine molecule is reduced with formation of an octahydroisoalloxazine [113].

5. OXIDATION

Isoalloxazines, including riboflavin, are resistant to oxidising agents, even when these are as strong as nitric acid, hydrogen peroxide, and bromine, but are oxidised by chromic acid and potassium permanganate [1,2]. When riboflavin is oxidised with lead tetra-acetate or periodic acid, the side chain between adjacent hydroxy-groups is cleaved and 7,8-dimethyl-10-formylmethylisoalloxazine is formed [114,115]; the same compound is obtained on photolysis.

Petering and Giessen [116,117] oxidised 8-chloroalloxazine with 30% hydrogen peroxide in 88% formic acid and ascribed the structure of 8-chloroalloxazine 5,10-di-*N*-oxide to the product [116]. Later Berezovskii and Aksel'rod

showed[118,119] that in the oxidation of the 8-chloroalloxazine, alloxazine, 7-methylalloxazine, 7,8-dimethylalloxazine (lumichrome), 1,3-dimethylalloxazine, and others with 30% hydrogen peroxide in 88% formic acid, peracetic acid, and monopersulphuric acid, only the corresponding alloxazine mono-*N*-oxides can be identified unambiguously at the instant of formation:



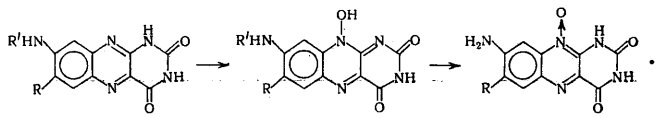
Alloxazine *N*-oxides have the 10-*N*-oxide structure[118], since the oxidation of the nitrogen atoms in the 1- and 3-positions of the pyrimidine ring and in the 5-position of the pyrazine ring in the molecule is hindered by the oxo-groups in the 2- and 4-positions.

It is noteworthy that the unambiguous course of the oxidation of the pyrazine ring of 8-chloroalloxazine agrees with the fact that the pyrazine ring of 6-chloroquinoxaline also yields mainly the mono-*N*-oxide on oxidation[120]. Alkaline cleavage of 8-chloroalloxazine *N*-oxide followed by decarboxylation yielded the corresponding 7-chloro-2-hydroquinoline 1-*N*-oxide[118].

The formation of 10-mono-*N*-oxides is consistent with calculations according to which the excess electronic charge at the nitrogen atom in the 5-position of the alloxazine rings is much lower than at the nitrogen atom in the 10-position[17,121].

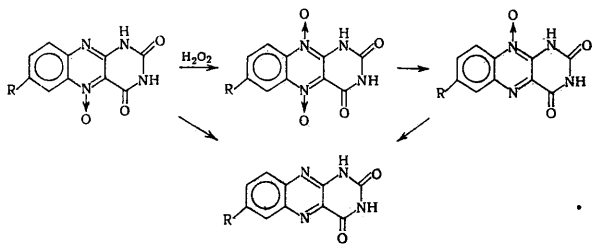
It is striking that derivatives with a nitro-group in the 9-position (for example 7,9-dinitroalloxazine and 6,9-dinitrolumichrome) are not oxidised with hydrogen peroxide and do not give rise to *N*-oxides[122]. On the other hand, 7-methyl-6-nitroalloxazine is oxidised with formation of the 10-*N*-oxide[122].

Aminoalloxazines could not be oxidised unambiguously with hydrogen peroxide to give the corresponding 10-*N*-oxides. This was achieved via the acyl derivatives of 8-aminoalloxazines. The corresponding *N*-oxides of amino- and acylamino-alloxazines were synthesised in this way[14]:



(R = H, CH_3; R' = CH_3CO, C_6H_5CO, $CH_3C_6H_4SO_2$)

The 5-*N*-oxides of alloxazine, 7-chloro-, 7-methyl-, and 1,3-dimethylalloxazine, obtained by Goldner's method[123-125] in which the corresponding nitrosouracils are cyclised, were oxidised with hydrogen peroxide in formic acid at 40°C to the corresponding alloxazine 5,10-di-*N*-oxides[15]:

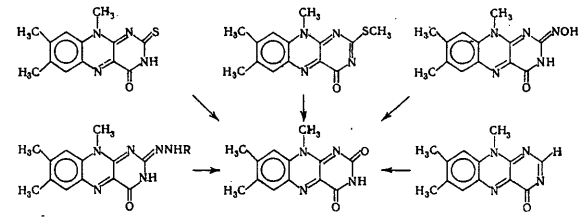


A characteristic feature of alloxazine 5,10-di-*N*-oxides is the non-equivalent stabilities of the *N*-oxide groups, which corresponds to the different basicities of the nitrogen atoms in the 5- and 10-positions of the alloxazine rings.

Thus, on heating 7-methylalloxazine 5,10-di-*N*-oxide in formic acid, the oxygen atom and the hydrogen atom in the 5-position are split off and 7-methylalloxazine 10-*N*-oxide is formed[15]. Under the same conditions, alloxazine 5-*N*-oxides are deoxygenated with formation of non-oxidised alloxazines.

The $N_{(10)} \rightarrow O$ bond is much stronger than $N_{(5)} \rightarrow O$, but prolonged boiling of alloxazine 10-mono-*N*-oxides in formic acid leads to complete deoxygenation with formation of alloxazines[15].

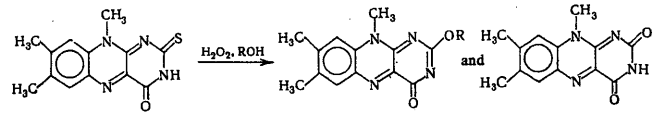
Whereas isoalloxazines, for example lumiflavin, are resistant to the action of hydrogen peroxide and other oxidants, the 2- and 4-derivatives (2- and 4-thiolumiflavins, thio- and imino-ethers, oximes, hydrazones, semicarbazones, and also 2- and 4-deoxy-derivatives) can be readily oxidised with atmospheric oxygen or dilute hydrogen peroxide to the flavin oxo-form[5,6,126-130]:



(R = H, COC_5H_4N, $CSNH_2$, $C_6H_4NO_2$)

2-Iminolumiflavin, which is not oxidsed by weak oxidising agents, is an exception[126].

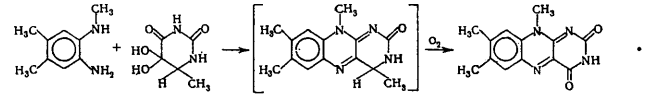
It is noteworthy that, when hydrogen peroxide is allowed to react with 2-thiolumiflavin in alcohol, the product is not 2-deoxylumiflavin, postulated previously[128], but a mixture of 2-*O*-alkyllumiflavin and lumiflavin, which can be readily separated[6]:



2- and 4-Deoxylumiflavins are unstable and are oxidised to lumiflavin at the instant of formation[127]. However, when nitrous acid, which behaves as a "radical-scavenger",[131] is employed, it is possible to obtain a stable 2-deoxylumiflavin in the form of the perchlorate[6]. It can be converted into lumiflavin by photochemical oxidation[6].

4-Deoxyalloxazine and 4-deoxylumiflavin, obtained by the condensation of the corresponding diamines with isodialuric acid, are even less stable than the 2-deoxy-isomers; they exist in the form of hydrates and at the instant of formation are oxidised by atmospheric oxygen to alloxazine and lumiflavin respectively[132].

In exactly the same way the condensation of *o*-diamines with 6-methylisodialuric acid yields lumiflavin as the sole reaction product. It is interesting to note that in this case the C–C bond is readily cleaved (at room temperature)[132]:



The introduction of nucleophilic substitutents into the benzene ring of the isoalloxazine molecule enhances the resistance of 2-deoxyisoalloxazines and 2-thioisoalloxazines to oxidation. Thus 8-acetamide-2-deoxynorlumiflavin is not oxidised by atmospheric oxygen or even weak oxidising agents[133].

In contrast to 2-thiolumiflavin, 8-amino-2-thionorlumiflavin is not appreciably susceptible to the action of atmospheric oxygen. Only the reaction with hydrogen peroxide results in a slow formation of the 2-oxo-group and the liberation of sulphur [133].

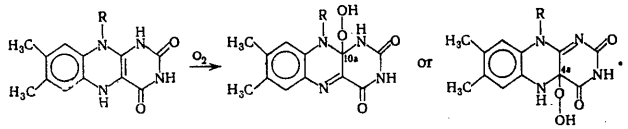
In contrast to flavins (FlH), 1,5-dihydroflavin (FlH_3) are readily oxidised even by atmospheric oxygen with the possible involvement in the reaction with oxygen of the free radicals $F\dot{I}H_2$ which are always present in a small amount in the reduced flavin [134]:

$$FlH + FlH_3 \rightleftharpoons 2F\dot{l}H_2,$$
$$2F\dot{l}H_2 + O_2 \rightleftharpoons 2FlH + H_2O_2.$$

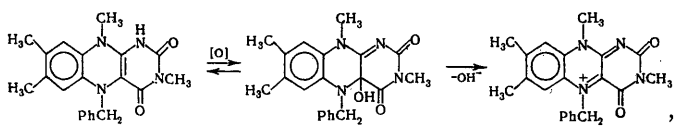
However, it is more likely that 1,5-dihydroflavins rather than the free radicals (flavosemiquinones) react with oxygen [56,135]. A spectrophotometric study of the kinetics of their oxidation established that the rate of reaction increases with the formation of the oxidised flavin and therefore the reaction may be regarded as autocatalytic [134]. Since 1,5-dihydroflavin is usually present in a non-coplanar conformation [7], it probably reacts with oxygen after transition to a planar vibrationally excited state as a result of the formation of a π-complex between the oxidised and reduced flavins [56]. Alkylation of 1,5-dihydroflavin at the nitrogen atom in the 1-position stabilises the 1-coplanar state, hinders the conformational transition of the molecule to the planar state, and sharply increases its resistance to oxidation by atmospheric oxygen [7,136].

In flavoproteins the coplanar structure of the dihydroflavin may arise as a result of the formation of a complex with adenine or tryptophan [56].

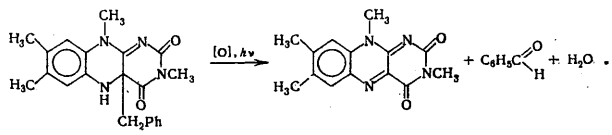
In the oxidation of 1,5-dihydroflavins and 5,10-dihydroalloxazines, the intermediates are hydroperoxides with the hydroperoxy-group in the 10a- [137-140] or 4a-position [140], the 4a-position being apparently more reactive [82,107,141]:



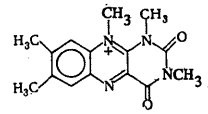
5,10-Dihydroalloxazines react with their hydroperoxides to form 10a-hydroxy-1,10a-dihydroalloxazines [142]. Whereas 5-benzyl-3-methyl-1,5-dihydrolumiflavin is reversibly oxidised in the dark via the intermediate formation of a free radical to a carbinol base, which at pH < 4 splits off a hydroxide ion and is converted into a quaternary 5-benzyl-3-methyl-lumiflavinium salt:



its isomer, 4a-benzyl-3-methyl-4a-5-dihydrolumiflavin, undergoes an irreversible photochemical oxidation with simultaneous cleavage of the C—C bond and the formation of 3-methyl-lumiflavin [82,107]:

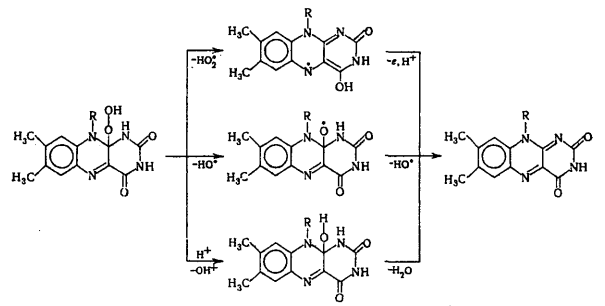


Quaternary flavinium salts are formed by the oxidation of 1,3-dialkyl-1,5-dihydrolumiflavins and their 5-acetyl derivatives with nitrous acid [136]:



O-Alkyl derivatives of 5-acetyl-1,5-dihydrolumiflavin react in the same way. The planarity of the dihydroflavin molecule in the oxidation reaction may be achieved by the intermediate formation of a free radical [136].

The prosthetic group of flavoproteins of various types has a specific protein environment and this probably determines the nature of electron transfer from the reduced form of the flavin either directly to oxygen (oxidases) or to an intermediate one-electron acceptor (dehydrogenases) and is reflected in their behaviour in the reaction with sulphite [140]. Thus oxidases form with sulphite an adduct via the nitrogen atom in the 5-position in the flavin, while dehydrogenases do not react with sulphite [140,143]. The differences between the reactions of flavoproteins of different types (dehydrogenases and oxidases) may be related also to the subsequent behaviour of dihydroflavin hydroperoxides, which can be cleaved in different ways [140]:



A similar series of reactions may be expected for the adduct inolving the 4a-position [140].

In accordance with the mode of cleavage of dihydroflavin hydroperoxides, dehydrogenases react predominantly with formation of HO_2 and an intermediate flavin radical [140]; it has been established that the majority of enzymes of this type form free Flavin radicals on reaction with oxygen, which then rapidly interact with one-electron acceptors [144,145]. A characteristic feature of the dihydro-forms of oxidases is reaction involving the formation of flavins and hydrogen peroxide [146].

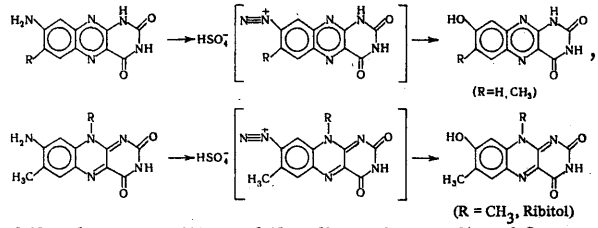
6. NUCLEOPHILIC SUBSTITUTION

8-Chloroalloxazine does not react with aliphatic and aromatic amines, pyridine, and morpholine on heating [147]. 8-Halogeno-substituted isoalloxazines behave differently—they readily react with nucleophilic agents such as monoethanolamine, diethanolamine, piperidine, and morpholine on heating in the presence of their excess or on carrying out the reaction in dimethylformamide (DMF) solution [147,148]. 8-Hydroxyethylaminonorlumiflavin was obtained in this way from 8-chloronorlumiflavin [148].

The lesser reactivity of 8-chloro-7,10-dimethylisoalloxazine compared with 8-chloro-10-phenylisoalloxazine can be accounted for by the influence of the electron-donating CH_3 group in the 7-position [147]. The chlorine atom in the 8-position of the isoalloxazine ring is also substituted

by a methoxy-group. Thus 8-chloronorlumiflavin reacts with sodium methoxide to form 8-methoxynorlumiflavin[148].

8-Hydroxyalloxazines and hydroxy-analogues of natural flavins—8-hydroxynorlumiflavin and 8-hydroxynor-riboflavin[149,150]—have been synthesised by the diazotisation of sulphuric acid solutions of 8-aminoalloxazines, their 9-azo-derivatives, and also 8-aminoisoalloxazines with subsequent hydrolytic decomposition of the diazonium salts[149,150]:



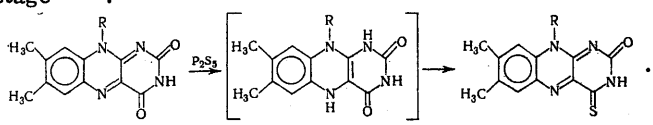
(R=H, CH_3)

(R = CH_3, Ribitol)

If the decomposition of the diazonium salts of 8-aminoisoalloxazines is carried out under the conditions of the Sandmeyer reaction, the corresponding 8-halogenoisoalloxazines are formed. 8-Chloronorlumiflavin[133], 8-chloronor-riboflavin, and 8-bromonorlumiflavin were obtained in this way[151]. It was shown that dimethyl sulphoxide promotes the nucleophilic substitution of the diazo-group in the 8-position of isoalloxazine by a halogen[151].

The reaction of alloxazines and isoalloxazines with nucleophilic agents usually does not entail substitution at the 2-C and 4-C carbon atoms of the pryimidine ring.

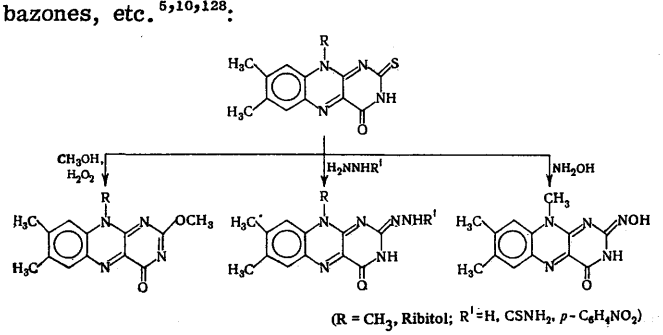
The introduction of sulphur into the 4-position of the isoalloxazine rings in the reaction of lumiflavin and 2′, 3′, 4′, 5′-tetra-acetylriboflavin with phosphorus pentasulphide in pyridine with formation of 4-thiolumiflavin[127] and 2′, 3′, 4′, 5′-tetra-acetyl-4-thioriboflavin[152] constitutes an exception. In this case there is probably initial reduction by sulphide and then substitution in the 1,5-dihydroflavin stage[10,127]:



(R = CH_3, $CH_2(CHOAc)_3CH_2OAc$)

A similar selective substitution for sulphur takes place also in the purine series[153]. The 2-oxogroup is resistant to the action of phosphorus pentasulphide.

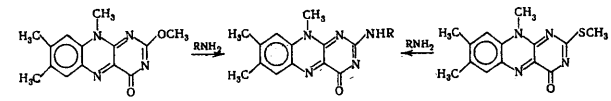
Nucleophilic substitution in the 2- and 4-positions of flavins occurs only with their derivatives: 2- and 4-thioflavins and *O*- and *S*-alkyl derivatives. 2-Thiolumiflavin and 2-thioriboflavin readily react with nucleophilic agents (the alkoxy-group, hydrazine hydrate, *p*-nitrophenylhydrazine, semicarbazide, thiosemicarbazide, and hydroxylamine) with formation of *O*-alkyl derivatives of oximes, hydrazones, semicarbazones, thiosemicarbazones, etc.[5,10,128]:



(R = CH_3, Ribitol; R′=H, $CSNH_2$, *p*-$C_6H_4NO_2$)

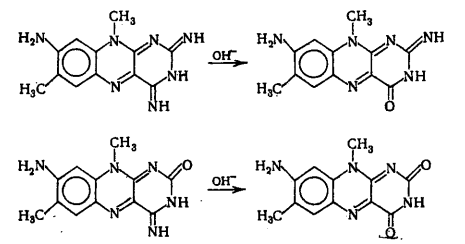
Similar reactions have also been described for 4-thiolumiflavin[5].

2-*O*-Alkyl derivatives and also 2- and 4-thioethers react with ammonia and amines (aniline, monoethanolamine, morpholine, and others) with formation of imines and iminoethers[5,10]:

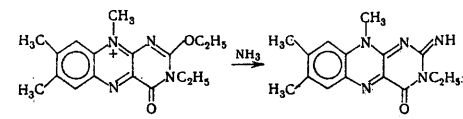


(R=H, C_6H_5, CH_2CH_2OH, $(CH_2)_3N(CH_3)_2$)

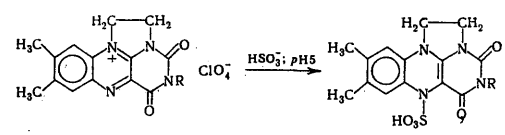
The imino-group in the 4-position is hydrolysed selectively under mild conditions. Thus 8-aminonorlumiflavin-2,4-di-imine is converted after only a brief treatment with warm alkali into the 3-monoimine, which is much more resistant to alkali; the 4-monoimine readily gives rise to the dioxo-compound under similar conditions[133]:



When nitrogen-containing heterocycles are converted into quaternary salts, the attack by nucleophilic agents is facilitated, as happens for nucleophilic substitution of pyridinium, pyrylium, thiapyrylium, and other cations[154]. Dudley and Hemmerich showed that 2-*O*,3-*N*-dialkyllumiflavinium salts react with ammonia via a simple addition–elimination mechanism with formation of lumiflavinimine[136]:



When sodium bisulphite is allowed to react with flavinium salts having substituted nitrogen atoms in the 1- and 3-positions, the nucleophilic bisulphite ion adds to the 5-position with formation of a dihydroflavin-sulphite adduct[155]:



The sulphite adduct of the 1,3,7,8,10-pentamethylisoalloxazinium salt is less stable and was not obtained in a pure form, but in this case too the sulphite anion probably adds to the 5-position[155].

It has been established that sulphite ions form adducts with the flavin prosthetic groups of many flavoproteins: glucose oxidase[143], D- and L-aminoacid oxidases, hydroxynitrile lyase, lactate oxidase, and glycolate oxidase[140], i.e. with enzymes of the oxidase type which transfer hydrogen directly to molecular oxygen. Flavin dehydrogenases, which transfer hydrogen to a specific acceptor, do not react with the sulphite anion[140]. This is an interesting case of correlation between the chemical reactivity of the flavin prosthetic group of flavoproteins of different types and their biochemical functions.

7. ELECTROPHILIC SUBSTITUTION IN THE BENZENE RING

Electrophilic substitution of the benzene ring in compounds of the alloxazine and isoalloxazine series was unknown until recently. Direct introduction of various substitutents gives rise to many possibilities for the structural modification of the molecules of riboflavin and related compounds, since the possibility of obtaining benzene ring-substituted alloxazines and isoalloxazines is greatly restricted by the limitations of the direct syntheses[1,2].

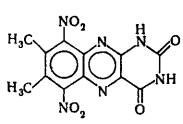
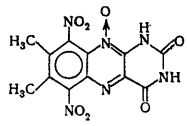
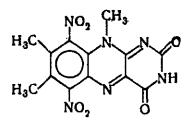
It has been established by LCAO-MO calculations[17] that the introduction of electron-donating substituents (CH_3, Cl, OH, NH_2, etc.) into the 7- and 8-positions of the alloxazine and isoalloxazine rings give rise to changes in the distribution of electron density (q_π) and peripheral electron density (f_π) mainly in the benzene component of the molecule, the type of distribution of q_π and f_π being largely similar for alloxazines and isoalloxazines.

Quantum-chemical calculations show that when either the static "isolated molecule" approximation (active centre characterised by the maximum value of q_π) or the dynamic approximation assuming localisation (the localisation energy LE is a minimum at the active centre) is employed[156] for alloxazine, lumichrome, riboflavin, and 8-amino- and 8-hydroxy-alloxazines, the electrophilic attack may be directed primarily to the carbon atom in the 9-position or to the 6-position in 7-methylalloxazine. Apart from the 9-position, the 7-position is reactive in alloxazine, 8-aminoalloxazine, and 8-hydroxyalloxazine and the 6-position is reactive in lumichrome and riboflavin[17] (see Table). These conclusions are fully consistent with the experimental data.

Berezovskii and Aksel'rod studied the nitration of alloxazines[122,157], their 10-*N*-oxides[122], and isoalloxazines[122] under conditions such that the reaction was known to have an electrophilic mechanism[158].

In the nitration of alloxazine with fuming nitric acid in the presence of concentrated sulphuric acid at 20°C, the product is a mixture of 7- and 9-mononitro-derivatives in proportions of 2:3. Nitration of alloxazine under severe conditions—with fuming nitric acid in 25% oleum at 70°C—leads to 7,9-dinitroalloxazine, which is formed also when the 7- and 9-mononitro-compounds are nitrated under the same conditions. In accordance with the directing effect of the first nitro-group, the second nitro-group substitutes the hydrogen atom in the benzene ring in the meta-position[157]. Nitration of 7-methylalloxazine and its 10-*N*-oxide with nitrating mixture at 20°C yields only the 6-nitro-derivatives[122]. Selective nitration in the 6-position is consistent with the lower localisation energy for 7-methylalloxazine (see Table) compared with the 8-position.

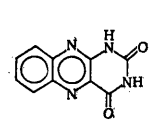
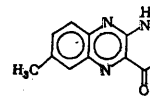
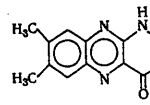
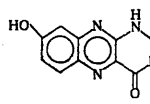
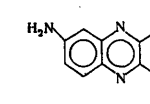
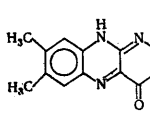
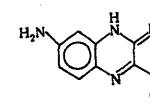
Nitration of 7,8-dimethylalloxazine, its *N*-oxide, and lumiflavin with fuming nitric acid in the presence of concentrated sulphuric acid leads unambiguously to the formation of the 6,9-dinitro-compounds[122]:

Under these conditions, mononitro-derivatives could not be obtained. Possibly the methyl groups in the 7- and 8-positions greatly enhance the nucleophilic properties of the 6- and 9-positions, which become equivalent and the nitration involves them both at the same rate. Otherwise the reaction would have terminated at the stage corresponding to the formation of the mononitro-compound (the 6- or 9-nitro-derivative) which is incapable of undergoing further nitration. Indeed it was found that 7,8,10-trimethyl-9-nitroisoalloxazine (obtained by direct synthesis) remains unchanged under the conditions of the nitration reaction[122].

McCormick[159] achieved the bromination and chlorination of lumiflavin and 2′,3′,4′,5′-tetra-acetylriboflavin in the 9-position by treatment with *N*-halogenosuccinimide in trichloroacetic acid in the presence of catalytic amounts

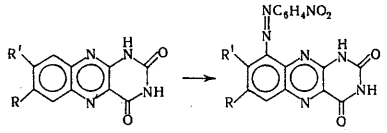
The activity indices of the 7- and 8-substituted alloxazines and isoalloxazines.

Compound	Site of substitution	q_π	f_π	LE
	6	+0.012	0.163	2.347
	7	−0.002	0.223	2.449
	8	+0.021	0.012	2.548
	9	−0.013	0.293	2.277
	6	−0.019	0.212	2.247
	8	+0.007	0.002	2.494
	9	−0.011	0.263	2.282
	6	−0.017	0.216	2.384
	9	−0.042	0.276	2.271
	6	+0.014	0.181	2.358
	7	−0.013	0.191	2.383
	9	−0.045	0.353	2.164
	6	+0.017	0.118	2.372
	7	−0.033	0.013	2.278
	9	−0.106	0.594	1.980
	6	−0.002	0.044	2.417
	9	−0.051	0.071	2.321
	6	+0.029	0.032	2.413
	7	−0.048	0.210	2.234
	9	−0.110	0.011	2.042

q_π = excess π-electron density; f_π = peripheral π-electron density; LE = localisation energy.

of benzoyl peroxide. Possibly the flavin reacts with the halogen in a protonated form via an electrophilic mechanism [159], but, since benzoyl peroxide is used as a catalyst, the possibility of a radical mechanism cannot be ruled out.

Berezovskii and Tul'chinskaya and coworkers studied the azo-coupling of 8-amino- and 8-hydroxy-alloxazines with diazotised aromatic amines [150]. When 8-amino-alloxazines were coupled with phenyl- and *p*-nitrophenyl-diazonium chlorides in aqueous DMF at pH 2–3, 8-amino-9-arylazoalloxazines were obtained and in the azo-coupling of 8-hydroxyalloxazines in a weakly alkaline medium the products were 9-arylazo-8-hydroxyalloxazines:



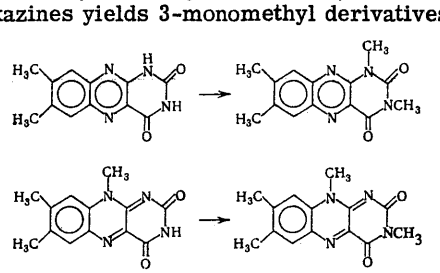
(R=H, CH_3; R'=OH, NH_2)

The finding that the azo-coupling of 8-amino and 8-hydroxy-alloxazines involves the 9-position of the alloxazine ring and not the 7-position is consistent with the calculated reactivity indices (q_π, f_π, LE; Table).

The attempt to carry out the azo-coupling of 8-amino-norlumiflavin and 8-aminonor-riboflavin with *p*-nitrophenyldiazonium chloride was unsuccessful, which, apart from steric factors, may be accounted for also by a reduced reactivity of the 9-position compared with 8-aminoalloxazine (a decrease of the peripheral electron density and a higher localisation energy; Table).

8. ALKYLATION

Alloxazines and isoalloxazines are alkylated in both oxidised and reduced forms. Alkylation of alloxazines with methyl iodide [44,160], diazomethane [161], or dimethyl sulphate [162] in DMF in the presence of potassium carbonate [4] involves the ring nitrogen atoms of the pyrimidine ring and leads to 1,3-dimethyl derivatives, while methylation of isoalloxazines yields 3-monomethyl derivatives [4,160,161]:

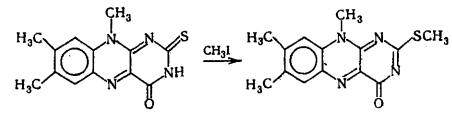


2′,3′,4′,5′-Tetra-acetylriboflavin is alkylated similarly with formation of, for example, the 3-methyl, 3-ethyl, and 3-benzyl tetra-*O*-acetylriboflavins [44]. 8-Aminonor-lumiflavin is methylated by methyl iodide in an alkaline medium in the 3-position with 8-amino-3-methylnorlumi-flavin as the product [133].

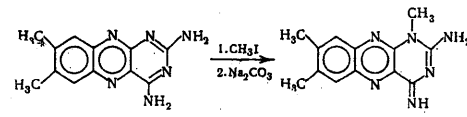
Like isoalloxazines, alloxazine *N*-oxides are not alkylated at the nitrogen atom in the 1-position. When alloxazine 10-*N*-oxide was methylated with methyl iodide or dimethyl sulphate in an alkaline medium, only 3-methyl-alloxazine 10-*N*-oxide was obtained [13].

Compounds with various substituents at 1-N and 3-N can be obtained by further alkylation of monoalkyl-substituted alloxazines [111,160].

When 2- and 4-thiolumiflavins are treated with alkylating agents (methyl iodide in chloroform), they are alkylated to the corresponding thioethers and the reaction with the 2-thio-derivative takes place also without a basic catalyst [5], for example:



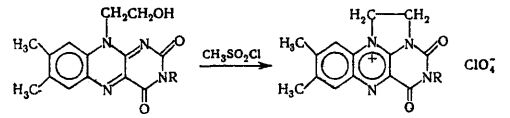
On the other hand, 2,4-diamino-7,8-dimethylbenzo[*g*]-pteridine is methylated by methyl iodide in boiling cellosolve with formation of a quaternary salt, which on treatment with aqueous sodium carbonate solution is converted into the 1-*N*-methyl derivative [163]:



Berezovskii and coworkers studied the alkylation of 8-aminoalloxazine and 8-aminoisoalloxazine with ethylene oxide [164]. Hydroxyethylation of an aqueous suspension of sodium derivatives of 8-aminoalloxazine, 8-aminonorlumi-chrome, and 8-dimethylaminoalloxazine yielded on heating 3-(2′-hydroxyethyl)-8-(2′-hydroxyethylamino)alloxazine, 3-(2′-hydroxyethyl)-8-(2′-hydroxyethylamino)norlumi-chrome, and 3-(2′-hydroxyethyl)-8-dimethylaminoallox-azine respectively, the product obtained from 8-aminonor-lumiflavin being 3-(2′-hydroxyethyl)-8-aminonorlumiflavin.

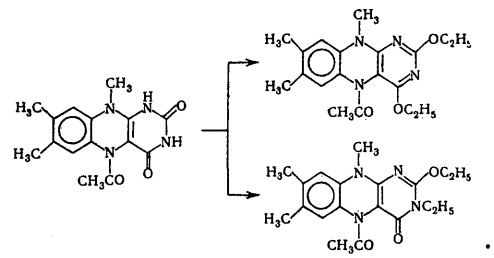
3-(2′-Hydroxyethyl)-8-(2′-hydroxyethylamino)alloxazine is readily dealkylated by 70% sulphuric acid with formation of 8-aminoalloxazine [164].

It has been established for 10-(2′-hydroxyethyl)-7,8-dimethylalloxazine [155] that isoalloxazines can undergo intramolecular alkylation in the 1-position in the presence of methanesulphonyl chloride, DMF, and potassium carbonate with formation of quaternary flavinium salts:



A similar intramolecular alkylation at the 1-N-position has been observed also for 10-formylmethyl-7,8-dimethyl-isoalloxazine [7,26].

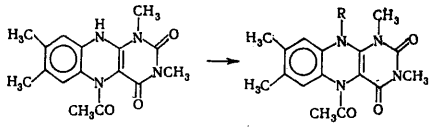
In contrast to flavins, 1,5-dihydroflavin reacts with electrophilic agents at 2-O, 4-O, 3-N, and 5-N. Owing to the high sensitivity of the dihydro-form to oxygen, stabilised 5-acetyl-1,5-dihydro-compounds were employed [4]; they are readily alkylated with formation of a mixture of 2-O, 4-O, and 2-O, 3-N-dialkyl derivatives; the nitrogen atom in the 1-position of dihydroalloxazines is not alkylated [4]:



The finding that 5-acetyl-1,5-dihydrolumiflavin is not alkylated at the 1-nitrogen atom can be accounted for by the enhanced electron density at the carbonyl oxygen in the $C_{(2)}$—O^{136} group and not by the steric hindrance due to the substituent in the 10-position [4].

5,10-Dihydroalloxazines behave differently. Thus alkylation of 5-acetyl-5,10-dihydroalloxazine in DMF in the presence of potassium carbonate yields a complex mixture of products of alkylation at 1-N, 3-N, or 10-N which contains, after acid hydrolysis and oxidation, 1- and 3-monomethylalloxazines, 1,3-dimethylalloxazine, and a small amount of 3,10-dimethylisoalloxazine [4].

If one begins with 5-acetyl-1,3-dimethyl-5,10-dihydroalloxazine, 10-alkylation takes place with formation of 5-acetyl-10-alkyl-1,3-dimethyl-1,5-dihydroisoalloxazines [136]:

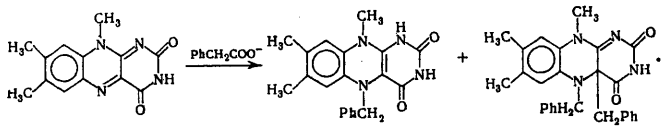


(R = CH_3, C_2H_5, $CH(CH_3)_2$, $CH_2COOC_2H_5$, $CH_2C_6H_5$)

The course of the further conversion of this compound into an oxidised form of isoalloxazine is unknown.

1,3,10-Trialkyl-1,5-dihydroflavins are alkylated in the 5-position by alkyl halides in DMF in the presence of potassium carbonate [136].

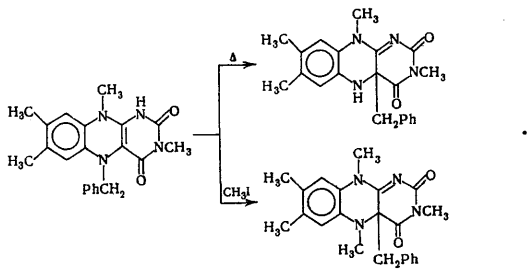
Hemmerich and coworkers studied the reductive photochemical alkylation of flavins on treatment with phenylacetate ions which are decarboxylated in this process [141], the residue formed entering into the 5- or 4a-position with formation of mono- and di-benzyldihydro-derivatives [82,107,141]:



Isolumiflavin (6,7,10-trimethylisoalloxazine) gives only the 4a-benzyldihydro-derivatives, evidently owing to steric hindrance [82].

The reductive photochemical alkylation of 5-benzyl-lumiflavin on treatment with cyclopentadiene, thiolane, and dialkyl sulphides also involves the 4a- or 5-position [165,166].

On fusion (without access of air), 5-benzyl-1,5-dihydrolumiflavin rearranges to 4a-benzyl-4a,5-dihydro-lumiflavin [82]. A similar rearrangement occurs also in the methylation of 5-benzyl-1,5-dihydrolumiflavin with methyl iodide, the rearrangement being accompanied by methylation in the 5-position [82]. Under the same conditions, the isomeric 4a-benzyl-4a,5-dihydrolumiflavin is not alkylated in the 5-position:

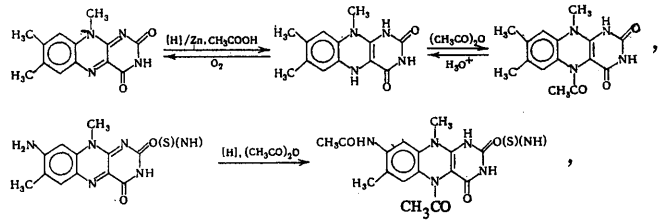


Flavin radicals alkylated in the 5-position were obtained by alkylating a mixture of oxidised and reduced flavin with alkyl halides in the presence of triethylamine [70].

9. ACYLATION

In contrast to alkylation, acylation of alloxazines and isoalloxazines takes place selectively only with their dihydro-derivatives at the 5-nitrogen atom and leads to the formation of 5-acyl-5,10-dihydroalloxazines and 5-acyl-1,5-dihydroisoalloxazines and their derivatives [127]. 5,10-Dihydroalloxazines and 1,5-dihydroisoalloxazines, which are unstable and sensitive to atmospheric oxygen, are thereby stabilised [4].

The acylation reaction is carried out simultaneously with reduction by adding zinc dust to a suspension of the alloxazine or isoalloxazine in a 1:1 mixture of acetic acid and an organic acid anhydride. Hemmerich and coworkers obtained in this way 5-acetyl-1,5-dihydrolumiflavin [127,133], 8-acetamido-5-acetyl-1,5-dihydronorlumiflavin, and its 2-thio- and 2-imino-analogues:



as well as 5-acetyl-5,10-dihydroalloxazine, its 1,3-dimethyl derivative [4], 5-acetyl-1,5-dihydro-2-thiolumiflavin [127], 2′,3′,4′,5′,5-penta-acetyl-1,5-dihydroriboflavin [4,167], etc.

On reductive acetylation, quaternary flavinium salts can be converted into the corresponding 5-acetyl-1,5-dihydro-derivatives [155].

The acetyl group in the 5-position inhibits reoxidation but at 50°C and in the presence of atmospheric oxygen it is readily hydrolysed by hydrochloric acid [4,127,133]. Simultaneous reduction and formylation with zinc dust and formic acid are achieved similarly and result in the formation of 5-formyl-5,10-dihydroalloxazine, 5-formyl-1,5-dihydrolumiflavin [4], and 4a-benzyl-5-formyl-4a,5-dihydrolumiflavin [82].

When riboflavin is treated with chlorocarbonic ester in an alkaline solution and is simultaneously reduced with sodium hydrosulphite, only the 5-position is acylated, the primary and secondary hydroxyls in the ribityl chain not being involved; the reaction leads to the formation of 5-ethoxycarbonyl-1,5-dihydroriboflavin [167]. However, when riboflavin is treated with benzoyl chloride in alkaline solution with simultaneous reduction, the product is 2′,3′,4′,5′,5-pentabenzoyl-1,5-dihydroriboflavin [167].

Brustein and Hemmerich showed that, when 3-methyl-lumiflavin reacts with pyruvic acid under aerobic conditions under illumination, reductive acetylation takes place with formation of 5-acetyl-3-methyl-1,5-dihydrolumiflavin [168]. Under anaerobic conditions, photochemical reduction predominates, leading to the formation of 3-methyl-1,5-dihydrolumiflavin [168].

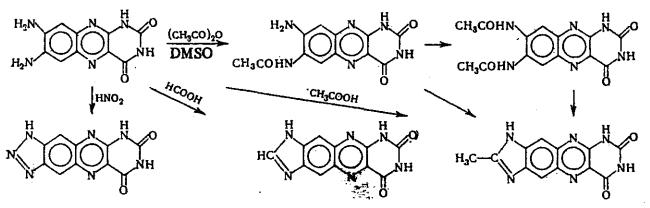
A similar reductive acylation reaction is observed in biological catalysis by flavoproteins, which are reduced by pyruvate with formation of the corresponding adduct [169]. Possibly the first stage of the flavin-dependent dehydrogenation is such "group transfer", in contrast to "species transfer" (hydride ion transfer) known previously [168]. This hypothesis is confirmed by the finding that certain flavoproteins do not exhibit oxidation-reduction properties [170], for example hydroxynitrile lyase.

10. REACTION OF AMINO-SUBSTITUENTS

The amino-groups in the benzene component of the alloxazine and isoalloxazine systems are nucleophilic centres and can react with electrophilic agents. Beresovskii and coworkers studied the acylation of 8-amino-alloxazines [14]. This showed that the weakly basic amino-group of 8-aminoalloxazines does not react with an excess of acetyl chloride under mild conditions. However, after these compounds had been heated for 20 h with acetic anhydride in pyridine at 60°C, their acetyl derivatives were obtained. 8-Benzoyl- and 8-tosyl-aminoalloxazines were synthesised by acylating 8-aminoalloxazine and 8-amino-7-methylalloxazine with benzoyl chloride and toluene-*p*-sulphonyl chloride [14].

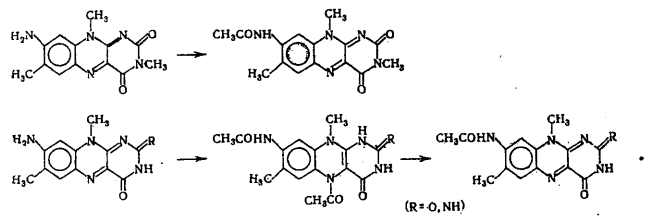
In a study of the acetylation of 7,8-diaminoalloxazine, it was shown that the reaction proceeds in steps owing to the different reactivities of the exocyclic amino-groups [171].

It proved possible to select conditions ensuring the monoacylation of 7,8-diaminoalloxazine with acetic anhydride in dimethyl sulphoxide (DMSO) at 20°C; the structure of 7-acetamido-8-aminoalloxazine was attributed to the monoacyl derivative [171]. Further acetylation yields 7,8-diacetamidoalloxazine:

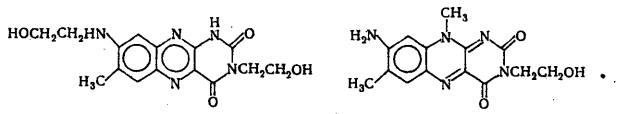


On heating, the acetyl derivatives of 7,8-diamino-alloxazine show a marked tendency to undergo the imidazole cyclisation. The corresponding imidazo-alloxazines are also obtained on refluxing 7,8-diamino-alloxazine with acetic and formic acids: interaction with nitrous acid gives rise to the triazolo[4,5-i]-alloxazine system [171].

On refluxing with a mixture of acetic anhydride and acetic acid, 8-aminoisoalloxazines are acetylated to the 8-acetamido-derivatives [133], which can also be obtained under the conditions of reductive acetylation with subsequent selective hydrolysis of the acetyl group at the 5-N nitrogen atom of the 1,5-dihydro-compound. Hemmerich and coworkers obtained in this way 8-acetamidonorlumiflavin and its 2-imino- and 2-deoxy-analogues [133]:



It has been shown that there is a difference between the reactivities of the amino-groups in the 8-positions of alloxazines and isoalloxazines with respect to hydroxyethylation [164]. Whereas on treatment with ethylene oxide and heating, the 8-amino-group in the sodio-derivatives of alloxazine and 8-norlumichrome is hydroxyethylated together with the cyclic 3-imino-group, in isoalloxazines the amino-group remains inert.



The higher reactivity of the 8-amino-group in the alloxazines ring system compared with isoalloxazine has been confirmed by calculations [17], which established that the electron density (1.830) and the peripheral electron density (0.618) at the amino-group of 8-aminoalloxazine are higher than for 8-iminoisoalloxazine (1.817 and 0.254 respectively).

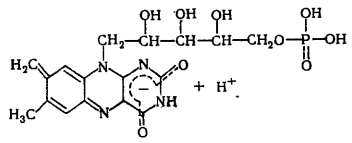
The 9-amino-group in the isoalloxazine ring system is readily diazotised by nitrous acid; the diazonium salt enters into the azo-coupling reaction and is deaminated. Lumiflavin is obtained in this way from 9-aminolumiflavin as a result of diazotisation and decomposition of the diazonium salt [172]. However, the diazo-group in this compound could not be replaced by bromine in the Sandmeyer reaction, possibly owing to steric hindrance [172].

8-Aminoalloxazines and 8-aminoisoalloxazines [133,173] are also diazotised by nitrous acid [133,149-151,174]; the diazonium salts formed are coupled in an alkaline medium to β-naphthol [164,174] and in dilute formic acid to certain aromatic amines (diethylaniline, *NN*-bis-β-chloroethyl-aniline) [175] with formation of azo-compounds.

11. REACTIONS OF THE METHYL GROUP IN THE 8-POSITION

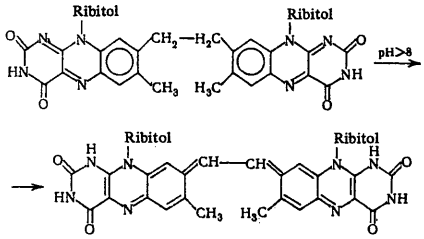
Until recently the biological importance of the two *ortho*-methyl groups in the benzene part of the molecule of riboflavin and its coenzymes (FMN and FAD) was not clear. Although the removal of the methyl groups from the flavin involves relatively small steric and electronic changes in the molecule, it nevertheless entails a sharp decrease or total loss of biocatalytic activity [176]. The replacement of these groups by other substituents (NH_2, NHRibitol, OH, Cl, etc.) yields inhibitors of certain metabolic reactions [177-179].

The 8-methyl group in the isoalloxazine ring system exhibits acidic properties, resembling those of nitrotoluene or methylbenzoquinone [18]. At pH 6.8–6.9 and 90–95°C in D_2O the protons of the 8-methyl group of FMN are exchanged for deuterium [180], which has been explained by the possible formation of an anion with a strongly delocalised charge; the formation of such an anion, which would compensate the loss of aromaticity in the benzene ring, is impossible in the deprotonation of the 7-methyl group, for example:

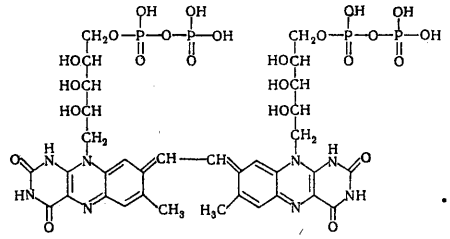


The mobility of the hydrogen atom of the 8-methyl group is shown in flavin dimerisation reactions. Hemmerich et al. established that, under non-aqueous alkaline conditions (BaO in methanol, K_2CO_3 in DMF), flavins are irreversibly dimerised [148] in a manner analogous to the oxidation of nitrotoluenes to stilbenes [18]. The 8-methyl groups in the two molecules are then oxidised and dimerised with the formation of the CH_2-CH_2 group and flavin compounds are

reduced—a diflavin biradical is formed[18] and reacts with a further flavin molecule to form the flavosemiquinone anion and the "diflavin radical"[69]. In the presence of atmospheric oxygen, this radical is oxidised to the stable benzoquinonoid bisriboflavin[148]:

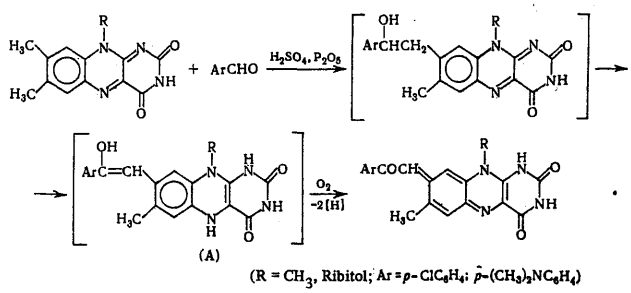


It is noteworthy that, in the phosphorylation of riboflavin under extremely dehydrating conditions (orthophosphoric acid and phosphorus pentoxide), the formation of phosphate esters of flavins with the usual structure is also accompanied to some extent by an analogous oxidative dimerisation involving the 8-methyl group with formation of the benzoquinonoid bisriboflavin 5′-diphosphate[181]:



The ^{1}H NMR spectra of the benzoquinonoid bisriboflavin 5′-diphosphate and the benzoquinonoid bisriboflavin lack the resonance signal due to the protons of the 8-methyl groups, while the signal of the 7-methyl protons is retained[181], which is evidence of a change in molecular structure involving the 8-methyl groups. The benzoquinonoid bisriboflavin 5′-diphosphate and the benzoquinonoid bisriboflavin were found to inhibit oxidative phosphorylation processes in rat liver mitochondria.

The 8-methyl group in the flavin molecule may be regarded as a nucleophilic centre. The enhanced reactivity of the 8-methyl group of flavins compared with the 7-methyl group is reflected in its capacity to interact with electrophilic agents (active carbonyl, nitrosyl, etc. groups). Thus under anhydrous conditions (concentrated sulphuric acid and phosphorus pentoxide) flavins enter into the Knoevenagel reaction with *p*-chlorobenzaldehyde and *p*-dimethylbenzaldehyde, forming the corresponding 8-α-derivatives[148,182]:

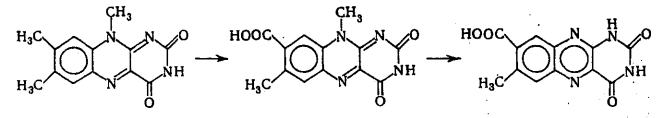


(A)

(R = CH_3, Ribitol; Ar = *p*-ClC_6H_4; *p*-$(CH_3)_2NC_6H_4$)

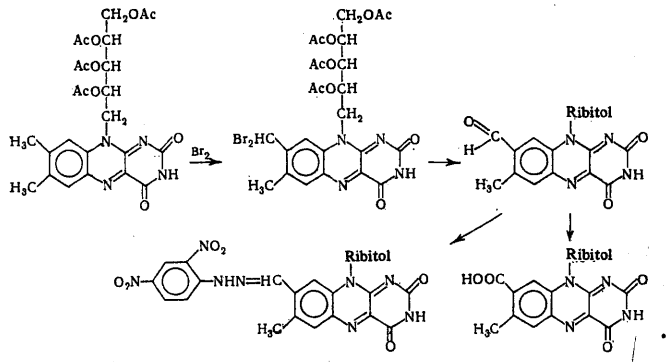
Possibly this reaction proceeds via the intermediate formation of an aldol, which is converted as a result of an intramolecular oxidation-reduction reaction into a compound of type (A) which on oxidation in air gives rise to the corresponding 8-α-derivative of the flavin[182].

The reactivity of the 8-methyl group of flavins is also shown in the reaction of lumiflavin with nitrous acid in boiling acetic acid with formation of 8-carboxynorlumiflavin[148]:



This acid is unstable and is readily converted into 8-carboxy-7-methylalloxazine in a warm alkaline solution.

McCormick achieved the bromination of the 8-methyl group of 2′,3′,4′,5′-tetra-acetylriboflavin with an excess of bromine in pyridine and dioxan; the reaction led to the formation of 2′,3′,4′,5′-tetra-acetyl-8-α-dibromoriboflavin, which was hydrolysed to 8-formylnor-riboflavin on refluxing in 6 *N* hydrochloric acid. This compound is oxidised by dilute aqueous potassium permanganate or periodic acid solution to 8-carboxynor-riboflavin. The condensation of 8-formyl nor-riboflavin with 2,4-dinitrophenylhydrazine led to the formation of the corresponding hydrazone[183]:



Hemmerich and coworkers achieved the monobromination of 2′,3′,4′,5′-tetra-acetylriboflavin at the 8-methyl group by brief treatment with molecular bromine in dioxan in the presence of catalytic amounts of dibenzoyl peroxide and obtained 2′,3′,4′,5′-tetra-acetyl-8-α-monobromoriboflavin[184].

The study of the reactivity of the substituents in the benzene ring of isoalloxazines, in particular the 8-methyl group in riboflavin[148,183], played a decisive role in the determination in 1970 of the structure of a new flavin coenzyme of succinate dehydrogenase (SD) by two groups of investigators: Ghisla et al.[184] in West Germany and Walker and Singer[185] in the USA.

Earlier Kearney, Singer, and Wang established that the flavin present as the prosthetic group in succinate dehyrogenase from ox heart[186] and pig heart[187] is not identical with FMN or FAD[188,189] and, in contrast to these coenzynes, is linked by a covalent bond to one of the protein aminoacids[190].

The flavin of succinate dehydrogenase could not be separated by the usual methods (treatment with strong acids in the cold, boiling in water and 50% ethanol) and only treatment with proteolytic enzymes (trypsin, chymotrypsin) led to the isolation of a flavin peptide derivative, called SD-FAD and containing one mole of 5-adenylic acid, one mole of D-ribose, and two phosphorus atoms joined by the pyrophosphate linkage[190]. The dinucleotide differed from FAD by a hypsochromic shift of the ultraviolet absorption band (from 375 to 350 nm) and certain other properties[190].

SD-FAD was converted into AMP and SD-FMN by brief hydrolysis in 1 *N* hydrochloric acid at 100°C or by treatment with nucleotide pyrophosphatase.

Hydrolysis of SD-FAD (6 *N* HCl, 95°C, 18 h) leads to a riboflavin derivative (SD-riboflavin) and 6–7 aminoacids: alanine, serine, glutamic acid, valine, and threonine with a second serine molecule as the terminal aminoacid residue[190]. The ribityl structure of the substituent in the 10-position was established by periodate oxidation[190].

It was shown that the covalent linkage between the flavin and the aminoacid cannot involve the 1-, 2-, 3-, 4-, and 5-, and 10-positions in the isoalloxazine ring for the following reasons. On alkaline hydrolysis of SD-FAD, urea is formed, which rules out substitution in the 1- and 3-positions[18]. Since the removal of the ribityl side chain by alkaline photolysis[191] does not rupture the bond with the aminoacid in the residual substance, the 10-position was also eliminated[18]. All possible substitutions by alkyl, aryl, acyl, alkoxy-, amino-, and mercapto-groups in the 2-, 4-, and 5-positions are reversible under the conditions of hydrolysis or weak oxidation[5,128].

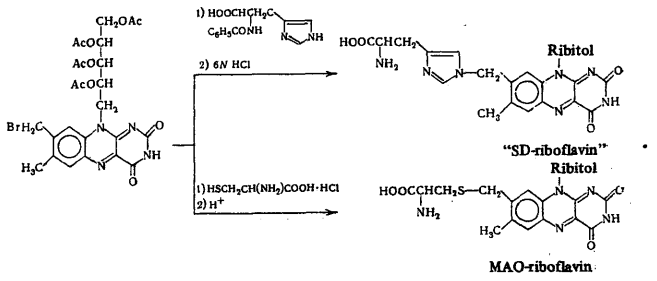
It was also shown that the hyperfine structure of the ESR spectrum of the "SD-flavin" radical differs from that of the riboflavin radical, which made it possible to eliminate the 7- and 9-positions characterised by low spin densities[189]. It was suggested that the addition to aminoacids takes place via the 8-methyl group[191], which is also consistent with the results from double resonance electron spin spectra[192].

On acid hydrolysis of "SD-riboflavin" under severe conditions in an atmosphere of helium, histidine was isolated in 80% yield. The same aminoacid is isolated on neutral photolysis and reductive cleavage of SD-FAD by hydrogen over palladium, i.e. under conditions where 3-benzylhistidine is cleaved[185].

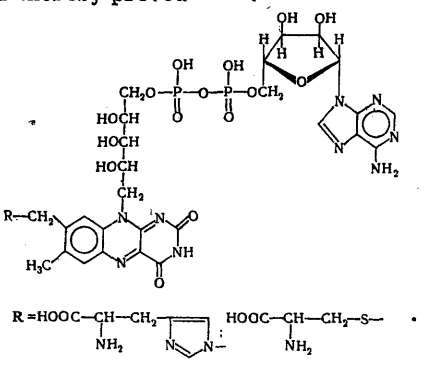
By analogy with the benzylation of histidine at 3-N and also on the basis of steric considerations, it was suggested that the flavin is linked to histidine via the 3-N atom[185].

The results led to the conclusion that "SD-riboflavin" has the structure of 8-α-(1- or 3-L-histidyl)riboflavin[183]. 8-α-(*N*-L-Histidyl)riboflavin, obtained by direct synthesis (heating of 2′,3′,4′,5′-tetra-acetyl-8-α-bromoriboflavin with benzoylhistidine in anhydrous dimethylformamide with subsequent hydrolysis by 6 *N* hydrochloric acid) prove to be spectroscopically and chromatographically identical with "SD-riboflavin"[184].

Next it was shown that the non-dissociating cofactor of monoamine oxidase (MAO) is not FAD but its derivative[193,194]. Stepwise hydrolysis of monoamino-oxidase yielded a mononucleotide and "MAO-flavin"—a riboflavin oligopeptide containing cysteine, serine, tyrosine, and two molecules of glycine. Further hydrolysis of the compound led to the isolation of 8-α-(*S*-cysteinyl)riboflavin[195,196], which Ghisla and Hemmerich also obtained synthetically by the reaction of tetra-acetyl-8-α-bromoriboflavin with cysteine hydrochloride and subsequent deacylation[197]:



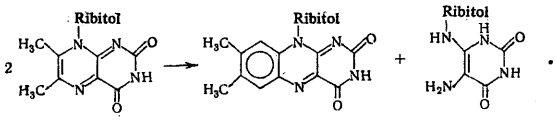
Thus detailed and far-reaching studies of the chemical behaviour of alloxazine and isoalloxazine compounds led to the determination of the unusual structures of new non-dissociating flavin coenzymes of succinate dehydrogenase [8-α-(*N*-L-histidyl)FAD][184,185] and monoamino-oxidase [8-α-(*S*-cysteinyl)FAD],[196,197] the flavin moiety of which is linked covalently to the aminoacid via the 8-methyl group; the earlier suggestion of the possibility of such a linkage was thereby proved[198,199]:



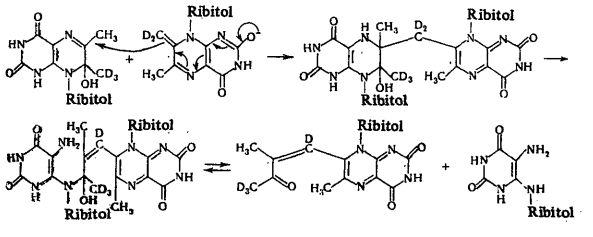
Thus the non-equivalent reactivities of the 7- and 8-methyl groups in the flavin molecule are used in nature as a fine-control factor for the construction with participation of a specific enzyme of the flavin prosthetic groups of succinate dehydrogenase and monoamino-oxidase.

It is interesting to note that the methyl groups of riboflavin are genetically related to the methyl groups of 6,7-dimethyl-8-(1′-D-ribityl)lumazine in the biosynthetic process.

The mobility of the hydrogen atoms of the methyl groups of lumazine, two molecules of which have been shown by Plaut[201,202] to be converted into one riboflavin molecule, is employed in the biosynthesis of riboflavin by the microorganisms *Eremothecium ashbyii* and *Ashbya gossypii*[200]. The same reaction was achieved *in vitro* by Cresswell et al., who showed that in a buffer solution at pH 7.3 6,7-dimethyl-8-(1′-D-ribityl)lumazine condenses into riboflavin in 55% yield on heating under nitrogen in the absence of an enzyme or an external carbon source[203]:



The study of the condensation of deuterated lumazine into riboflavin led to a possible mechanism of this reaction in which there is an initial attack by the carbanion on the 6-position of hydrated pteridine with formation of a carbinolamine in equilibrium with the ketone and finally the formation of the corresponding deuteroriboflavin[204,205]:



Plaut and coworkers propose a different mechanism for the non-enzymatic and enzymatic syntheses of riboflavin from lumazine[206–208].

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12. OTHER REACTIONS

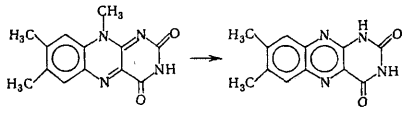
It was shown recently that, on photoexcitation with visible light under anaerobic conditions in an acid medium (pH < 5), lumichrome is hydroxylated in the aromatic ring, possibly in the 9-position, with formation of 9(6)-hydroxylumichrome [142].

An important reaction—the photolysis of riboflavin and other flavins—takes place with partial or complete removal of the side chain in the 10-position depending on the conditions; complete dealkylation leads to the conversion of isoalloxazines into alloxazines.

The studies on the photolysis of riboflavin have been reviewed [1,2,88,209].

Investigations in recent years have been devoted mainly to the identification of the intermediates in the photolysis of flavins and the study of its mechanism [210-217].

It is noteworthy that light is not a specific catalyst of the cleavage of isoalloxazines to alloxazines [4]. This reaction is catalysed also by metal ions such as Fe^{2+}, Sn^{2+}, Co^{2+}, [218,219] and in certain cases by bases [220] and reagents for the carbonyl group [4], quaternary amines, thioethers [87], etc. Thus, when lumiflavin was heated in acetic acid containing hydroxylamine for 120 h, lumichrome was obtained [4]:



The reverse transition, from alloxazines to isoalloxazines, is unknown.

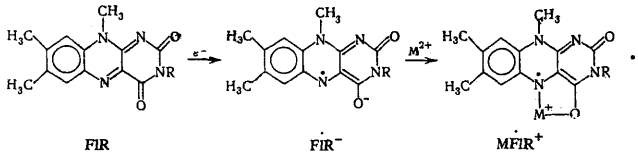
Alkaline or acid degradative cleavage of alloxazines and isoalloxazines with their conversion into quinoxalines has been investigated in fair detail and discussed in earlier papers [1,2,209]. This reaction is widely used to establish the structures of various derivatives of alloxazine and isoalloxazine [118,119,221-223].

Flavins are capable of forming complexes with different organic compounds, as a rule in 1:1 proportions [224]. Such compounds include phenols [225], indoles [226,227], purines [228,229], benzoic acid [230], aminoacids [231,232], etc. There are data showing that the tyrosine aminoacid residue in certain flavoproteins is involved in complex formation with the flavin [233]. Possibly this complex formation between flavin and aromatic aminoacids is in fact the main link between the prosthetic group and the apoenzyme in many flavoproteins [230].

In order to investigate the nature of the bond between flavins and aromatic aminoacids, McCormick and coworkers [234-237] employed as model compounds flavinylpeptides obtained by the interaction of methyl esters of aromatic aminoacids (L-tryptophan, L-tyrosine, and others), with 3- and 10-carboxyalkyl derivatives of flavins.

Riboflavin forms with certain metal salts insoluble intensely coloured chelate complexes, probably via the 5-nitrogen atom and the oxygen of the 4-carbonyl group. The metal ions involved in such complexes are Ag^+, Hg^+, Cu^+, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+}. [238,239] The chelate complexes with univalent metals are more stable than those with bivalent metals. It is noteworthy that the biocatalytic activity of many flavin enzymes is associated with the metal cations they contain, such as those of iron, molybdenum, copper, or manganese [240].

Flavins do not show an appreciable affinity for transition metal cations with a constant valence [238,241], probably owing to the low basicity of the 5-nitrogen atom and the high energy of the enolisation of the 4-carbonyl group [23]. However, in the form of flavosemiquinones, in particular radical-anions, they acquire a specific affinity for metal cations [242]. Under physiological conditions, the half-reduced flavin contains a small amount of radical-anions in consequence of disproportionation. However, in the presence of transition metal cations the equilibrium is displaced towards the formation of radicals owing to complex formation [243]. Under the experimental conditions (in dry DMF) in the presence of a stoichiometric amount of a strong base, for example potassium t-butoxide) 100% of the flavin radical-anions is formed [244]. The formation of the chelate complex can be represented by the following scheme [245]:



FlR FlR⁻ MFlR⁺

Further study of the reactions of alloxazines and isoalloxazines and of their reactivities, the elucidation of the relation between the parameters of the electronic structures in difference positions of the flavin molecule and its interaction with the apoenzyme, the study of the biochemical role of flavin radicals and flavin complexes with aminoacids and metals, as well as investigations of other types will undoubtedly promote a far reaching development of the chemistry of alloxazine and isoalloxazine derivatives and the discovery of the essential features of flavoprotein catalysis.

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