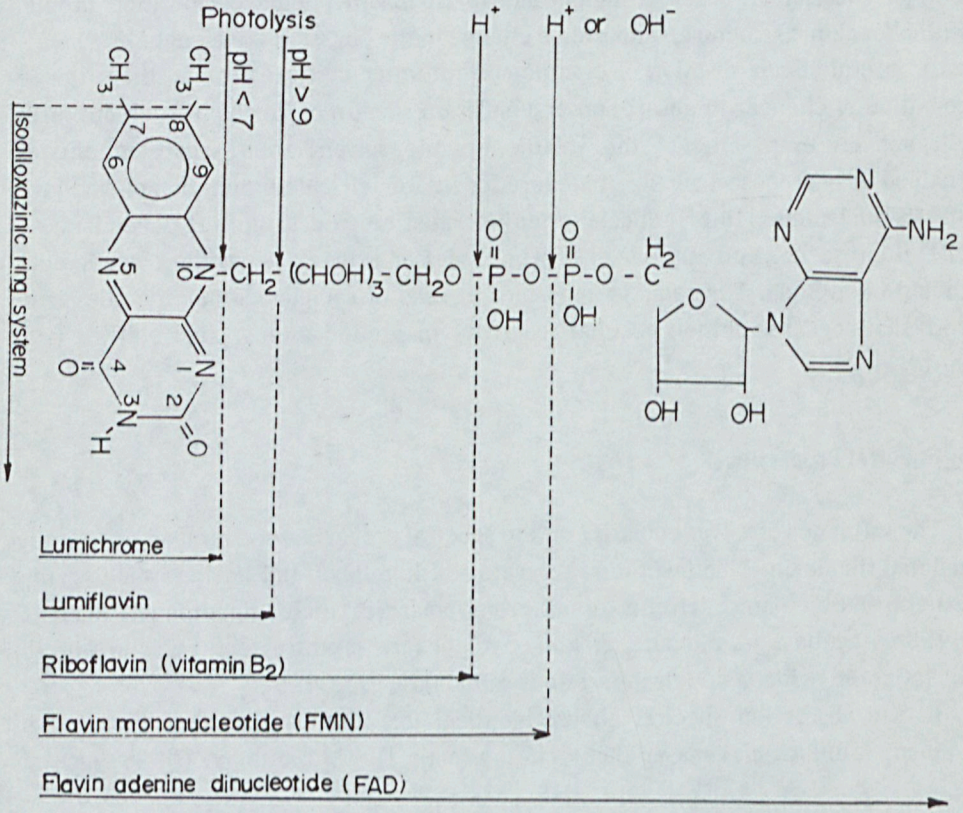
STUDIES ON FLAVINS AND ALLOXAZINES

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1. Introduction

There are many ways in which topics worth to be studied are being selected in science. In this case the idea has been formulated as follows: the real quality of a product depends on its chemical composition and the most labile of the important components should be the best quality index. Vitamins are such suitable labile compounds in food products and have been chosen as study objects. One of the authors, being at that time one of the youngest in the group could not obtain for investigation one of the desirable fat soluble vitamins and had to be content with B_2 (riboflavin). A few years later the second of the authors joined the "vitamin B_2 group" and be-



cause of some non only scientific reasons entered the same field. In such a way the team working on flavins has been established in our Institute.

2. Analytical Problems

In the mid-fifties the laboratory equipment was rather primitive and paper partition chromatography just started to be popular. Looking for suitable analytical methods for qualitative and quantitative determination of flavins in milk, paper and ion exchange chromatography and fluorometry were chosen [1, 2]. It was found that losses of riboflavin due to sunlight irradiation of milk in transparent and colorless glass bottles are rather substantially big [3] and that separation of flavin forms present in milk, liver and yeast can be achieved by means of paper and ion exchange chromatography [1, 4-6]. Further studies allowed to establish that separation of flavins using resorcinol-formaldehyde resins is due to the formation of charge transfer complexes (elution by mixtures of water and organic solvents like acetone, dioxane or concentrated urea solutions) and the method has been rechecked [7].

The use or organic solvents in mixture with leads to some analytical difficulties because of changes in fluorescence intensity of flavins and a strong longwavelength shift of emission in case of lumichrome (a riboflavin photodecomposition product with alloxazinic structure), and even a change in the emission wavelenght [8, 9].

A careful standardisation of organic solvent-water mixtures composition, the recognition of changes in absorption and emission spectra of flavins in such mixtures, alternatively evaporation of the volatile organic solvent from separated eluates, enabled us to propose a simple procedure for fluorometric determination of riboflavin and its nucleotides [10-14]. Special attention must be paid to the pH dependence of FAD fluorescence and any use of organic bases or acids (e.g. pyridine, acetic acid) should be avoided. The same is important in case of samples containing alloxazine derivatives because of their peculiar reactivity in ground and excited states (*vide infra*).

3. Spectral Properties

The influence of solvent polarity on the spectral properties encouraged us to study in detail riboflavin, riboflavin tetrabutyrate and lumiflavin (all having isoalloxazinic structure) in organic solvents of different polarities including ethanol, acetone, pyridine, acetic acid, dioxane, dioxane-cyclohexane mixtures and cyclohexane (in the latter only riboflavin tetrabutyrate is soluble) [8, 9, 15, 16].

In the absorption spectra, almost identical for all flavins studied, showing in aqueous solutions maxima at about 445 nm (max. I), 373 nm (max. II), 268 nm and

225 nm, with decreasing solvent polarity, the following changes were observed: the appearance of fine structures throughout the whole spectrum, a hypsochromic shift and a decrease in absorbancy of max. II. In fluorescence emission spectra the decrease in solvent polarity caused the appearance of a recognizable fine structure, a slight hypsochromic shift of the band and a substantial increase of quantum yield (Table 1) [16].

Table 1. Quantum yields of fluorescence of flavins in different solvents ($\phi \times 10^2$)

Solvent	Lumiflavin	Riboflavin	Riboflavin tetrabutyrate
Water	28.2	25.2	
Acetic acid	23.5	18.4	
Pyridine	52.0	32.6	
Ethanol	42.0	32.0	33.8
Acetone*	56.7	35.0	48.0
Dioxane+	63.6	41.0	52.5
Cyclohexane-dioxane mixture (1:1)	65.2		57.4

*For riboflavin 95 per cent.

+For riboflavin 98 per cent.

Taking into account experimental data and the results of simple semiempirical calculations it was concluded that the inflections in absorption and emission bands are of vibronic character and that max. I and max. II are connected with separate $\pi-\pi^*$ electronic transitions [15].

Apparently similar solvent polarity dependence was observed in absorption spectra of lumichrome [9, 15]. Despite of the different shape of the near UV spectrum in aqueous solution (maximum at about 354 nm and shoulder at about 383 nm) with decreasing solvent polarity the shoulder transforms into max. I and the maximum at about 354 nm shifts hypsochromically down to 326.5 nm thus behaving analogically to max. II in isoalloxazines [15]. The same tendency can be observed in the spectra of alloxazine and the complete set of its mono- and dimethylated in the benzene ring derivatives synthesized in our laboratory [17, 18]. Comparison of the spectral properties of alloxazine derivatives allowed us to state that the electron donating substituent (such as the hyperconjugative methyl group) located at C–7 causes the best separation of max. I and max. II (slightly less separated in alloxazine), located at C–8

causes their mergence and located at C–6 and C–9 gives absorption spectra with bathochromically shifted shoulders instead of max. I. These features appear to be cumulative and 7,8-dimethylalloxazine (lumichrome) exhibits absorption spectrum similar to expected for summarized spectra of 7- and 8-monomethylalloxazines [19].

Alloxazines substituted with electronwithdrawing substituents (cyano group) at C–7 and C–8 show almost opposite features of absorption spectra (well resolved maxima I and II in case of 8-cyano-, merged in case of 7-cyanoalloxazine). The influence of different substituents on absorption spectra of alloxazine derivatives are satisfactorily reproduced by INDO/S and CNDO/S semiempirical calculations [20].

Emission fluorescence spectra of lumichrome and other alloxazine derivatives with decreasing solvent polarity change in a way different than isoalloxazines: a decrease of quantum yield, the appearance of fine structure and a strong hypsochromic shift are observed [15, 18]. Special effects are caused by pyridine, carboxylic acids, phosphoric acids, water etc., (vide infra).

According to experimental data and semiempirical calculations also in case of alloxazine derivatives the main peaks in the near UV regions reflect $\pi-\pi^*$ transitions but at the longest wavelengths most probably $\pi-\pi^*$ transitions overlap one or more $n-\pi^*$ transitions. Shifts of $\pi-\pi^*$ and $n-\pi^*$ transitons in opposite directions with the decrease in solvent polarity were suggested as a possible reason of the observed changes in emission spectra [21].

The shifts of max. II position and quantum yields of fluorescence show a correlation between them and with solvent polarity expressed in Kosower's Z values [15, 16]. Attempts to interprete the observed changes in terms of internal charge transfer complex, nonspecific and specific solute-solvent interactions remained rather unsatisfactory [14, 19].

Recently Yagi et al. [22] using riboflavin tetrabutyrate in carbon tetrachloride in the presence of trifluoro- and trichloro-acetic acids have demonstrated that the characteristic shift of max. II in absorption spectra of isoalloxazines is caused by hydrogen bonding between flavin and acids molecules. Using extended range of hydrogen bonding agents concentrations, additionally also 1,1,1,3,3,3-hexafluoroisopropanol (pKa 9.3) and more polar 1,2-dichloroethane and acetonitrile as bulk solvents, it was possible to demonstrate that in the presence of trifluoroacetic acid in CCl_4 a nonfluorescent species is formed and in more polar solvents protonation of riboflavin tetrabutyrate takes place. The comparison of absorption and excitation spectra indicates that the equilibrium between different forms does not change substantially upon excitation [23]. The use of hexafluoroisopropanol allowed to recognize the limits of spectral changes due to hydrogen bonding (no protonation occurs) which can be responsible for the decrease in fluorescence intensity by no more than 40% [24]. Similar results were obtained in studies on 3-methyllumichrome in the same system. The main differences were: the lack of fluorescence intensity

decrease in hydrogen bonded species and the appearance of phototautomeric emission in presence of carboxylic acids [25].

4. Excited state proton transfer

In the presence of pyridine, acetic acid and to some extent also water, in the fluorescence spectra of lumichrome appears an additional band similar in shape, maximum position and intensity to those of isoalloxazine derivatives, whereas the absorption spectra show solvent polarity dependent changes typical for alloxazines [14, 15]. This phenomenom was preliminarily interpreted as a transformation of excited alloxazine into an excited isoalloxazine species due to abstraction or delocalization of H^+ at N–1 [14]. More detailed studies using pyridine and acetic acid in methanol and dioxane and also semiempirical calculations have lead to the conclusion that this process can be described as excited state proton transfer from N–1 to No–10 position [26]. The driving force of this alloxazine-isoalloxazine phototautomerism appeared to be the electron density distribution changes at N–1 and N–10 upon excitation. The formation of the isoalloxazinic species was confirmed by the fluorescence lifetime and time resolved spectra measurements [27, 28]. In a detailed study on the spectral properties of alloxazine derivatives singly and doubly methylated in the benzene ring in media of different polarity and composition it was established that alloxazine-isoalloxazine phototautomerism can indeed be observed only in case of N–1 unsubstituted alloxazines, that the phototautomeric form is different from anionic species and that the formation of a ground state complex of alloxazine with the catalysing molecule is a prerequisite condition for this excited state proton transfer [18, 29]. It was also established that many molecules having both proton donor and acceptor functions (like carboxylic, phosphoric, sulphuric acids and water) can serve as catalysts [30]. Phototautomerism, but less efficient, takes also place in case of 5-deaza- alloxazine [31].

There are, however, some unanswered questions and puzzling properties of this system. In contradistinction to practically all other known systems undergoing phototautomerism in case of alloxazines a huge excess of catalysing molecules has to be used which has led to the proposal that alloxazines belong to a special strong--catalysis proton transfer class of molecules [32]. Systematic studies on the 3MLc phototautomerism in methanol-acetic acid mixtures using fluorescence lifetime measurements have lead to the conclusion that a complicated system of differently hydrogen bonded structures must be considered [33].

An additional analysis of the properties of methanol-acetic acid mixtures revealed that both components react and their NIR and IR spectra change in time. While checking a series of organic solvents it was found that the efficiency of alloxazine-

-isoalloxazine phototautomerism in the presence of acetic acid shows dramatic diversity (Table 2).

Table 2. Efficiency of alloxazine-isoalloxazine phototautomerism of 3-methyllumichrome catalysed by 0.64 M acetic acid in different organic solvents

Solvent	Dielectric constant	ET value	Efficiency F_{isoAll}/F_{All}	Efficiency Δ^*
Cyclohexane	2.023	31.2	7.81	7.64
Carbon tetrachloride	2.238	32.5	7.70	7.51
Dioxane	2.209	36.0	0.78	0.61
Chlorobenzene	5.621	37.5	8.07	7.81
1,2-dichloroethane	10.36	41.9	6.70	6.43
Acetone	20.70	42.2	2.86	2.66
Acetonitrile	37.50	46.0	6.52	6.24
Methanol	32.7	55.5	0.77	0.37
Ethylene glycol	37.7	56.6	0.77	0.14

$$\Delta^* = {}^{3MLc}\left(\frac{F_{515}}{F_{435}}\right) - {}^{1,3MLc}\left(\frac{F_{515}}{F_{435}}\right)$$

where F_{435} – fluorescence emitted at 435 nm, F_{515} – emitted at 515 nm, 1,3MLc (1,3-dimethyllumichrome) unable to phototautomerize.

Apparently the bulk solvent used should not react with carboxylic acid (methanol does) and should break its dimers (dioxane does not). Under such conditions alloxazine-isoalloxazine phototautomerism takes place in the presence of carboxylic acids concentrations similar to that observed in case of molecules triggering excited proton transfer in other systems [32].

Studies on 3MLc phototautomerism performed in 1,2-dichloroethane using acetic acid as the catalysing molecule have revealed that: the formation of 3MLc-acetic acid cyclic structure via hydrogen bonds at N–10 and N–1 shows a bathochromically shifted excitation spectrum and originates phototautomeric emission with maximum at 495 nm. Increasing acetic acid concentrations cause shifts of this maximum to about 540 nm most probably due to polarity and solvation changes within the solvent cage, a complex equilibrium between 3MLc molecules hydrogen bonded to 1,2-dichloroethane and to acetic acid molecules at different sites (partly blocking the possibility of phototautomerism) is responsible for the existence of an alloxazinic

form (emission at about 435 nm) even in the presence of the highest acetic acid concentrations applied. These observations were confirmed by the results obtained from fluorescence lifetime measurements (2 distinctly different lifetimes: alloxazinic about 1 ns, isoalloxazinic about 5 ns), a succesful analysis of the fluorescence emission spectra into 3 lognormal curves and a reconstruction of absorption spectra by adding 2 types of excitation spectra [35].

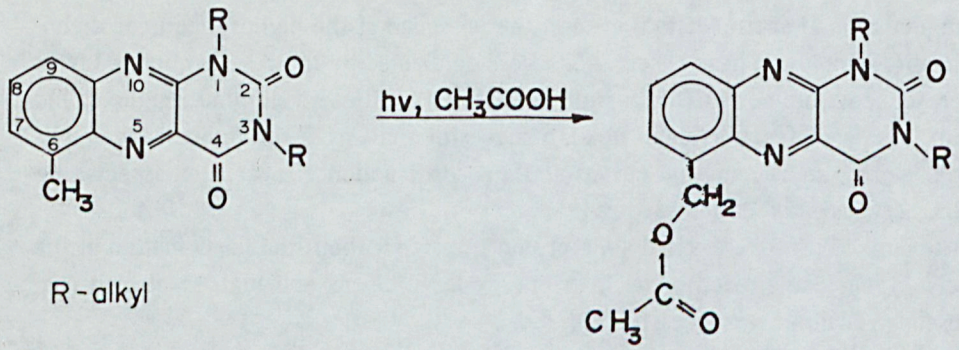
Attempts to provoke excited state proton transfer without the participation of the catalysing molecule, even using high energy laser beams and high resolution spectroscopy remained unsuccessful [36].

5. Photoreactivity

The photolability of riboflavin in milk [3] and in standard solutions exposed to light under laboratory conditions enforced the use of special arrangements and focused attention of the research team. Already during the first experiments with organic solvents the enhanced photolability of riboflavin in low polar media was observed [8, 37]. Surprisingly, among riboflavin derivatives [38] also the very light stable lumiflavin was found to be slightly photolabile, especially in the presence of acetic acid [13].

Alloxazines, were for a long time considered to be photoresistant molecules and indeed this is true in aqueous solutions. However, in low polar media alloxazines undergo photoreactions, which according to resulting spectral changes and thin layer chromatographic analysis lead to different products. The photoreactions of alloxazines in acetic acid appeared to be the most interesting [39]. These quite efficient reactions studied using alloxazine and its monomethyl derivatives according to changes in absorption spectra seem to represent two different types, one building up a new spectrum with maxima at about 320 nm and 370 nm (6- and 9-monomethyl alloxazines), the second forming a new broad maximum at about 350 nm, a shoulder at about 295 nm and a tail at the long wavelength end (alloxazine, 7- and 8-monomethylalloxazines). A thorough analysis of the isolated photoproducts using TLC, UV-VIS, ^{1}H, ^{13}C-NMR, mass spectroscopy and elemental analysis allowed to state that in case of 6- and 9-monomethylalloxazines photoaddition of acetic acid at methyl groups in the benzene ring of alloxazine molecule takes place (Scheme) [40].

In the course of studies on spectral properties in low temperature and polarization of fluorescence of isoalloxazines and alloxazines it was accidentally noticed that solutions in glycerol left on the lab table in a dim light were bleached. A detailed study has revealed that isoalloxazines and alloxazines in glycerol undergo efficient photoreduction and remain photoreduced when the dissolved oxygen is exhausted. The reaction is fully reversible upon the admission of oxygen and the electron donor



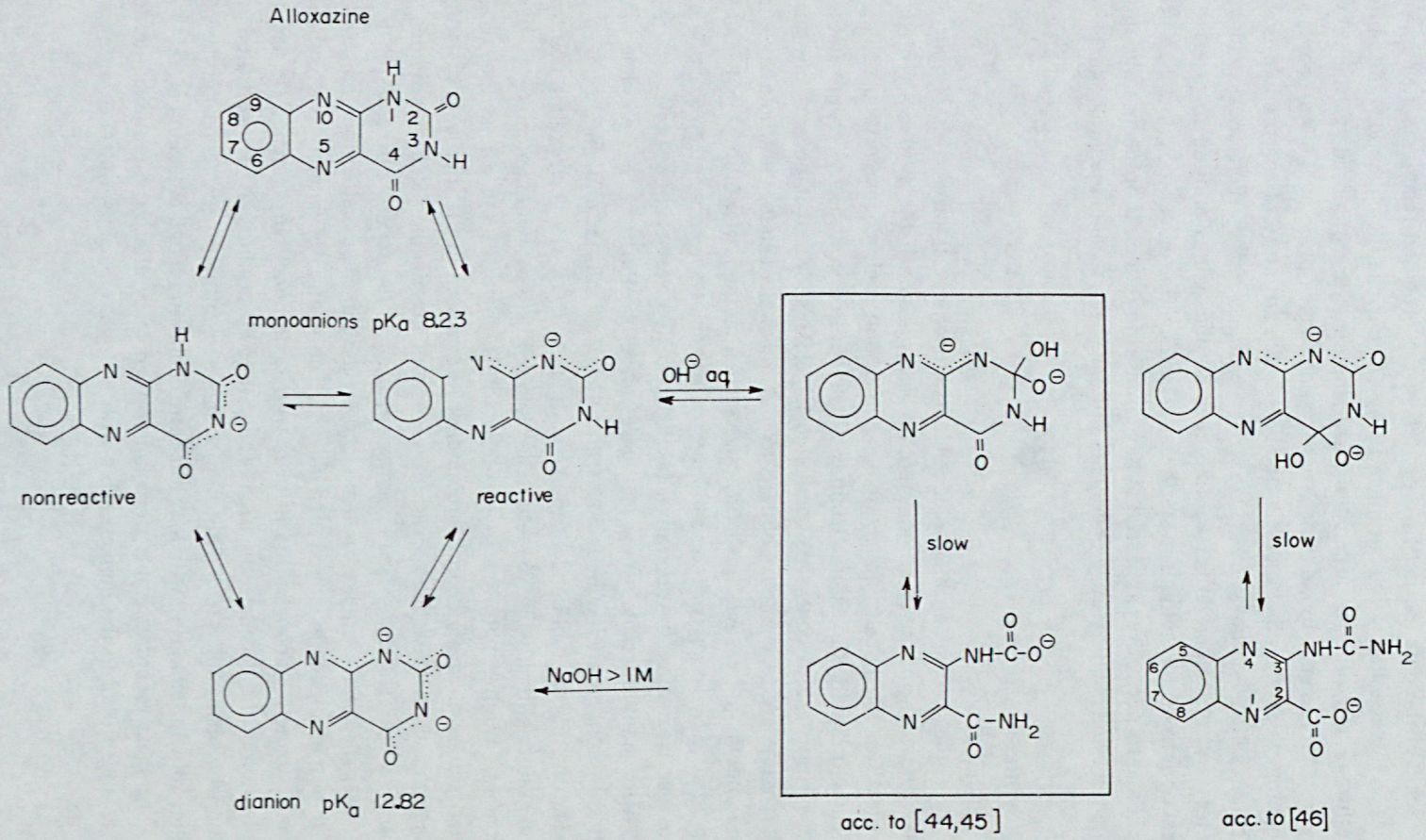
most probably is the nonhydroxylic hydrogen at C–2 of glycerol [41]. In 1,3-propanediol the reaction is about 10 times and in 1,2-propanediol about 20 times slower, it does not proceed at all in ethylene glycol or in glycerol containing more than 50% of water or methanol. This photoreduction of flavins and alloxazines in glycerol has explained the lack of reproducibility of fluorescence polarization data (during measurements photoreduction can considerably lower the fluorescence intensity) obtained in this laboratory and reported in the literature.

Almost in the same way i.e. accidentally it was recently noticed that solutions of 7-cyano-10-hydroxyethylisoalloxazine got bleached when left on the lab table.

It was immediately checked and rechecked and confirmed that there proceeds an efficient photoelimination of the hydroxyethyl substituent at N–10 position [42]. This reaction appears to be the most fortuituous finding allowing to finish succesfully the long lasting attempts to synthesize 7-cyanoalloxazine.

6. Covalent hydration

Studies on spectral properties of alloxazines, particularly on phototautomerism, included also spectral properties of protonated and de-protonated forms. Already first attempts to estimate the pKa values of deprotonation at N–1 and N–3 have lead to a statement that in dilute aqueous alkaline solutions alloxazines unsubstituted at N–1 undergo a reversible reaction resulting in a stable product of higher polarity and changed spectral properties [43]. More detailed studies revealed that in this reaction a molecule of water (OH^- attack and then protonation) is attached to alloxazine and hence the reaction was named "covalent hydration". From the observed reaction course (first rapid and then slow) it was inferred that a gem-diol is formed at one of the carbonyls in the pyrimidine ring and then ring opening at one of the neighbouring bonds takes place [44]. The final structure of the product was not quite clear but taking into account the results of spectral (UV, VIS, fluorescence, IR also at low temperatures, ^1H NMR, mass spectroscopy) data and analogy to the well known product


Alloxazine
monoanions pKa 8.23
nonreactive
reactive
OH⊖ aq
slow
slow
NaOH > 1M
dianion pKa 12.82
acc. to [44,45]
acc. to [46]

of alkaline hydrolysis of 1,3-dimethyllumichrome ring the opening between C–2 and N–3 and a respective structure were postulated [45].

Some of the data did not fit very well in the proposed reaction scheme and studies on this reaction were continued. Using ^{13}C enriched compounds and different pathways to synthesize a compund with the structure proposed for the "covalent hydrate" it has been found that: the OH^- attack takes place at C–4 carbonyl, a ring opening takes place between C–4 and N–3, the structure of the product is 3-ureidoquinoxaline-2-carboxylic acid [46]. The easiness with which such acids, their esters and other derivatives cyclize back to alloxazines allowed to propose such reactions as useful methods in syntheses [47]. The 3-ureidoquinoxaline-2-carboxylic acid and its derivatives appeared to have interesting spectral properties due to intra- and intermolecular hydrogen bondings and have been studied in more detail [47-50].

7. Syntheses

In studies on spectral, photochemical and chemical properties of flavins and alloxazines one of the most important problems has been the availability of suitable derivatives. Biologically active isoalloxazines and their decomposition products or metabolites with alloxazinic structure all substituted with methyl groups in the benzene ring were of course of immediate importance. When it became obvious that alloxazines are very reactive both in ground and excited state, the whole set of mono- and dimethylalloxazines was synthesized using methods of Kühling [51] and Goldner et al. [52]. To improve the solubility in less polar solvents and to exclude one of the reactive centers the derivatives alkylated at N–3 were of special interest.

After many attempts a new method of direct synthesis of N–3 alkylated alloxazines by condensation of o-diamine with suitably substituted alloxane has been found [53]. A modification of condensation conditions allowed also for reaction course control resulting in only one (6-methylalloxazine) of two possible monomethylalloxazine isomers. Another pathway in synthesis of N–3 substituted alloxazines is N–10 dealkylation of isoalloxazines. Aboundant information in the literature claiming the discovery of such reaction conditions were found erroneous. After an extended study the N–3 dealkylation of isoalloxazines was found to proceed by refluxing in most of organic solvents with boiling temp. above 100°C, in some melted inorganic salts and with best efficiency in melted urea [46, 54]. The heating of suitably N–3 substituted isoalloxazine for about 30 hrs in urea with some dimethylformamide (to avoid solidification of the decomposing urea) at about 130°C produces N–3 substituted alloxazine with about 30% yield. This method is now routinely used to synthesize alloxazines substituted at N–3 with long aliphatic chains (undecyl, octadecyl etc.).

The most recent attempts were undertaken to synthesize the alloxazine derivatives substituted in the benzene ring with a single electronwithdrawing group. The

condensation of suitable cyanophenylene-o-diamine with alloxan leads always almost exclusively to 8-monocyanoalloxazine. After many unsuccessful approaches a new pathway was chosen and starting from 3-chloro-4-nitro-benzonitrile 7-cyano--10-hydroxyethyloisoalloxazine was obtained. Photochemical N–10 dealkylation of this derivative resulted in a high yield of 7-monocyanoalloxazine (vide supra, [42]).

8. Semiempirical calculations

The experimental results of the studies performed were in many cases difficult to explain and from the very beginning some theoretical background was necessary. A succesful application of the results of semiempirical calculations of Song [55] has encouraged us to perform calculations using the same PPP-MO-SCF method and parametrization of Song for all possible mono- and di-methyl alloxazine derivatives [56]. The results of these calculations have been used successfully to interprete the experimental results [17, 19, 29]. These calculations, however, because of the complete lack of crystallographic data, were based on bond lengths and angles of isoalloxazine. After many efforts suitable crystals were obtained, analyzed and the first exact data for alloxazines, namely for 9-methyl- and 1,3,8,9-tetramethylalloxazine were calculated [57]. On the basis of these data further calculations were immediately performed using INDO/S and CNDO/S methods for methyl derivatives of alloxazines [58, 59]. Although the results do not fit exactly in the experimental data some very important properties of alloxazines are well reproduced (e.g. the ability to photo-tautomerize, basicity, reactivity of particular sites).

The same approach was applied also to cyano-derivatives of alloxazines which differ from methyl derivatives in many respects and that is again very well reproduced by calculations [60].

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STUDIA NAD FLAWINAMI I ALLOKSAZYNAMI

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Streszczenie

Autorzy artykułu przedstawili historię powstania i poszczególne etapy pracy zespołu zajmującego się problematyką flawin. W tematyce tej od pewnego czasu zaczęły dominować badania nad alloksazynami. Szczegółowo omówione zostały wyniki prac zespołu dotyczące oznaczania ryboflawiny i jej pochodnych, spektralnych własności flawin i alloksazyn, tzw. kowalentnej hydratacji alloksazyn, syntezy pochodnych flawin i alloksazyn, a także obliczeń semiempirycznych.

ИЗУЧЕНИЕ ФЛАВИНОВ И АЛЛОКСАЗИНОВ

Яцек Козиол, Анна Козиол
Отдельение Инструментального Анализа

Резюме

Авторы статьи представили историю возникновения и очередные этапы работы коллектива, занимающегося проблематикой флавинов. В последнее время в этой проблематике стали доминировать исслелования аллоксазинов. Подробно обсуждены результаты работы коллектива, касающиеся определения рыбофлавина и его производных, спектральных свойств флавинов и аллоксазинов, так называемой ковалентной гидратации аллоксазинов, синтеза производных флавинов и аллоксазинов, а также квантовомеханических вычислений.

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