231. A Comparative ^{13}C-NMR. Study on Various Reduced Flavins[1])

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Summary

Various two-electron reduced flavin derivatives have been investigated by natural abundance ^{13}C-NMR. spectroscopy. Some selectively ^{13}C-enriched compounds were synthesized to ensure the assignment of some of the quaternary C-atoms of the flavin molecule. Addition of two electrons to oxidized flavin leads to upfield shifts of all resonances except for those due to C(5a), C(9) and C(10'α). The largest upfield shift is observed for C(4a). Also some direct and two-bond coupling constants are reported. Theoretical calculations by INDO show that a rather good correlation exists between the calculated π-electron densities and the observed chemical shifts of the two-electron reduced molecule. For the oxidized molecule, the correlation is less satisfactory. Most substitution effects are additive, but some deviations in some compounds are observed indicating structural differences between the compounds in question.

The chemical shifts are also discussed in terms of the chemical reactivity of the oxidized and reduced flavin molecule.

1. Introduction. – Flavins[3]), *i.e.* isoalloxazines, are involved as coenzymes in many biological reactions (see [1]). Although many of these reactions have been studied by kinetic methods the diversity of biological reactions catalyzed by flavoproteins is not well understood. It has been suggested that the particular reaction catalyzed by a given flavoprotein is mainly determined by the specific interaction of the apoflavoprotein with its coenzyme [2] [3]. If this attractive hypothesis is correct then the electron distribution within the flavin molecule should be altered by the interaction with the apoflavoprotein. A method suitable to detect subtle differences of the electron distribution in a molecule is the NMR. technique. Therefore, we have start to investigate free and protein-bound flavins by the NMR. method with

[1]) Part of this work was presented at the International Symposia on Flavins and Flavoproteins held in Cracow, Poland (September 28–29, 1976) and in Kobe, Japan (March 13–17, 1978).

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[3]) Flavin = 7,8-dimethyl-isoalloxazine; isoalloxazine = 10-substituted 2,3,4,10-tetrahydrobenzo[g]pteridine-2,4-dione; lumiflavin = 7,8,10-trimethyl-isoalloxazine.

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the aim to contribute to a better understanding of the underlying physical principles responsible for the diversity of reactions catalyzed by flavoproteins.

In the past oxidized and reduced flavins have mainly been characterized by light absorption [4] and fluorescence techniques [5] [6] yielding only a limited contribution on the desired information. More recently we have reported a detailed account on the ^{1}H- and ^{13}C-NMR. properties of oxidized flavin derivatives [7] [8]. Recently the latter results have been fully confirmed by *Kawano et al.* [9].

In contrast to the oxidized molecule the 1,5-dihydrostate of flavin has not received te same detailed attention although the two-electron reduced state of flavin is biologically of great importance. The main reason for the lack of detailed physical data on the reduced molecule is due to its easy oxidation by molecular oxygen.

In the present paper we describe results on the ^{13}C-NMR. properties of reduced flavin. The main aim of this investigation was to complete the previous study [8] by the assignments of the resonance lines in the spectra of various 1,5-dihydroflavin derivatives. It is expected that these results will provide a basis for a more profound understanding of the chemical properties of reduced flavins. In addition these studies are needed as a comparative basis for an investigation of flavoproteins.

Tentative [10] and preliminary [11] results on this objective have been published. While this paper was in preparation the assignment of the chemical shifts of the spectrum of one particular 1,5-dihydroflavin derivative has been reported by *Kawano et al.* [9] and by *Ghisla et al.* [12].

2. Experimental Part. – ^{13}C-NMR. spectra were recorded on a *Varian* XL-100 spectrometer operating at 25.2 MHz and equipped with a 16 K *Varian* 620-L computer. All spectra were acquired in the *Fourier* transform mode using 12 mm taperlock tubes purchased from *Wilmad.* For the accumulation of the spectra 8 K data points were used in the time domain and converted to 4 K data points in the frequency domain. The spectral width was 5120 Hz for recording full spectra and 2560 Hz for determining the coupling constants. Frequency and proton noise decoupled spectra [13] were recorded mixing the single frequency from a *Schomandl* NO-60 M synthesizer with the gated, noise modulated frequency from the spectrometer. The power ratio was 200:1. The total signal could be amplified by an ENI-3010 amplifier up to a maximum of 15 W. Although the synthesizer was not coupled to the *Master* oscillator of the spectrometer the largest measured variation in the frequency between the two sources was less than 4 Hz. The other instrumental conditions used were: pulse width 10 μs (30° pulse), repetition rate 2 s. The peak positions were determined from the computer generated printout, using TMS as internal standard. The accuracy of the chemical shifts given is better than 4 Hz. The sample temperature was 26°. Internal deuterium ($CDCl_3$) served as a lock signal. To obtain good natural abundance spectra about 25,000 transients per spectrum were accumulated. The compounds were dissolved in $CDCl_3$ (99.8 atom %, *Merck*, Germany). Saturated solutions were prepared resulting in concentrations varying from 10 to 50 mM. Reduction of solutions of the oxidized flavin to the 1,5-dihydro state was effected directly in the NMR. tube by vigorous shaking of a two-phase solution consisting of $CDCl_3$ and an aqueous solution of 0.5M sodium phosphate (pH 8, saturated with KCl) containing a 10–20 fold excess of sodium dithionite with respect to flavin. The clear 1,5-dihydroflavin solutions thus optained exhibit a reddish orange colour in contrast to usually lighter colour of the oxidized solutions. Also the reduced flavin derivatives which can be isolated in crystalline state (e.g. **9**, *cf. Scheme 2*) were kept under the reducing solution to prevent oxidation (radical formation).

The synthesis of the selectively ^{13}C-enriched flavin derivatives was described earlier [8]. The following compounds *(cf. Scheme 2)* were prepared according to published procedures: 1,3-dimethyl-1,5-dihydrolumiflavin (**9**) and its *N*(5)-acetyl derivative (**8**) [14]; 3-methyl-*N*(5)-benzyl-1,5-dihydrolumiflavin (**12**) and 3-methyl-4a-benzyl-4a,5-dihydrolumiflavin (**13**) [15]; tetraacetylriboflavin (**1**) was prepared from riboflavin [16]; *N*(5)-methyl-(**10**) and *N*(5)-ethyl-1,5-dihydrolumiflavin (**11**) [17]; 2*a*,3-dimethyl-1,5-dihydrolumiflavin (**14**) and its *N*(5)-acetyl derivative (**17**), 3,7-dimethyl-(**15**) and 3,7,8-

trimethyl-*N*(1,10)-ethano-*N*(5)-acetyl-1,5-dihydroisoalloxazine (**16**), 2*a*,4*a*-dimethyl-*N*(5)-acetyl-1,5-dihydrolumiflavin (**18**) [7]; 3,7,8-trimethyl-*N*(10) (ε-acetopentyl)-*N*(5)-acetyl-1,5-dihydroisoalloxazine (**7**) [18]; 3,7,8-trimethyl-*N*(10)-undecyl-*N*(5)acetyl-1,5-dihydroisoalloxazine (**6**) [19], **4** and **5** were prepared from their oxidized molecules by known procedures [14]. Theoretical data were obtained using the INDO program described by *Pople & Beveridge* [20]. We used the original program [21] and modified it for use on the DEC-10 Agricultural University Computer.

3. Results. – 3.1. *Assignments.* To facilitate the assignments of the quaternary C-atoms in the flavin molecule a few selectively ^{13}C-enriched derivatives were synthesized. Also derivatives containing more than one ^{13}C-label in one molecule were prepared in order to obtain from the coupling constants some further information on the submolecular structure of reduced flavin. Spectra of such derivatives in the oxidized and reduced state are given in *Figure 1* (see also *Scheme 1*). The chemical shift most affected upon reduction of oxidized flavin is that due to C(4a) followed by that due to C(10a) *(Fig. 1 A, B, C).* The chemical shifts due to C(2) and C(4) are affected much less upon reduction.

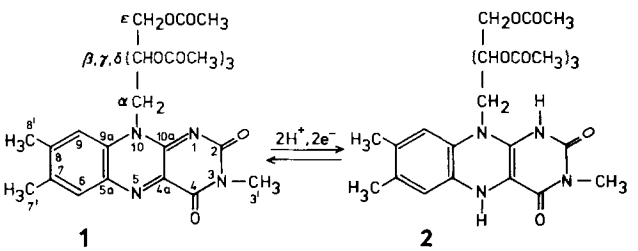
The through-bond coupling constant between C(4) and C(10a), the direct coupling constant between C(4) and C(4a), and that between C(4a) and C(10a)[4]) are considerably influenced upon addition of two electrons to oxidized *flavin (Table 1).*

The assignments of the residual C-atoms in the spectrum of reduced flavin are based on experiments as illustrated in *Figure 2* for 1,3,7,8,10-pentamethyl-1,5-dihydro-isoalloxazine (**9**). The assignments were aided by selective hetero-decoupling techniques and especially by the frequency- and noise-decoupling method [13]. The latter method has the advantage that the NOE[5]) for all resonances is preserved in the spectrum in contrast to the usual, selective single frequency hetero decoupling technique. The assignments are further based on the known fact [22] that two-bond CH-coupling constants are usually small as compared to one- and threebond CH coupling constants. In addition the assignment of some resonances is supported further by the use of various derivatives, *e.g.* **3** *(Table 2).*

The chemical shifts of all compounds investigated are collected in *Table 2,* and the chemical structures of the compounds are presented in *Scheme 2.*

Some of the resonances due to the N(10) side chain of various models (**1** to **7**) could not be assigned to individual C-atoms due to lack of resolution. Nevertheless

Scheme 1



[4]) The coupling constants were verified by homonuclear decoupling experiments.

[5]) NOE = Nuclear Overhauser Effect.

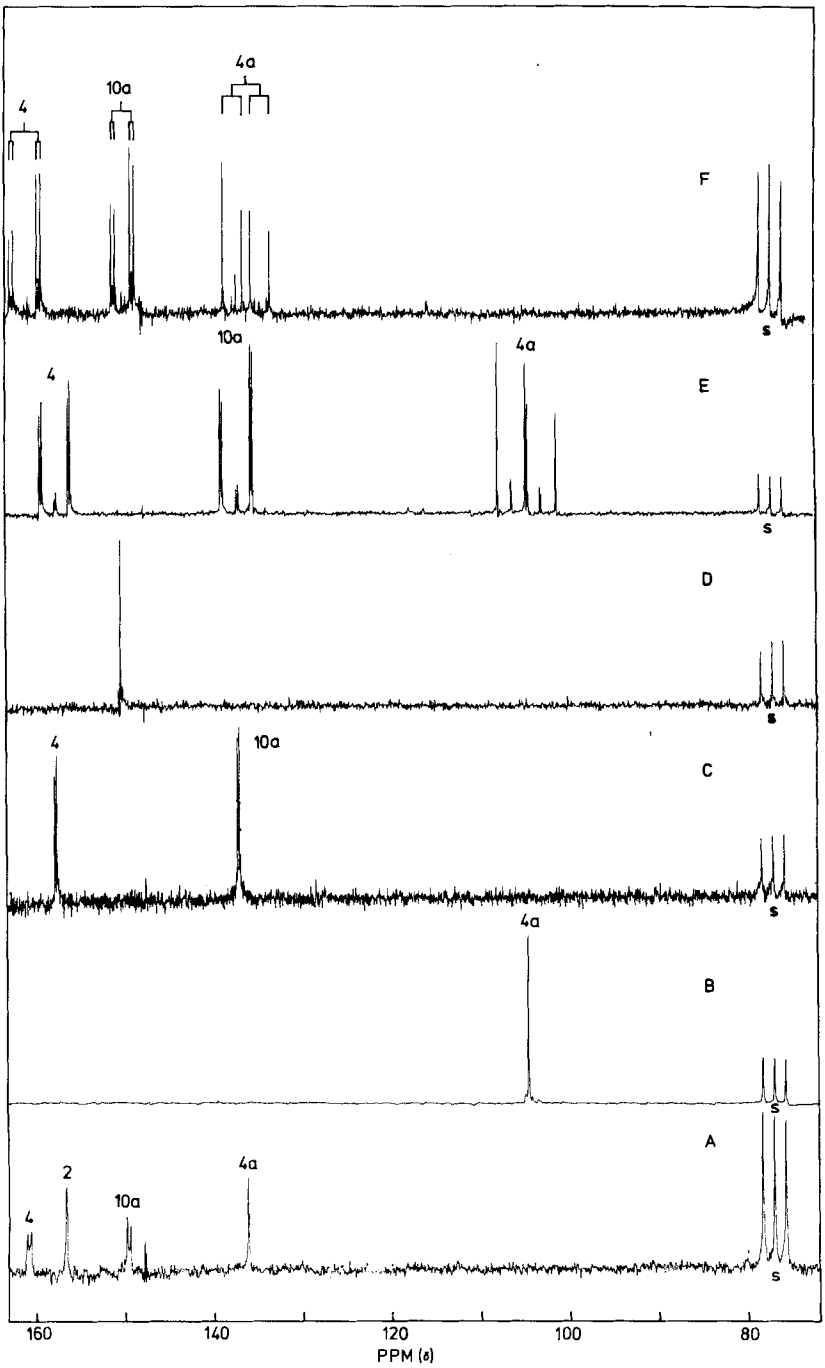


Fig. 1. *Proton noise decoupled ^{13}C-NMR. spectra of various selectively ^{13}C-enriched tetraacetylriboflavin* (TARF) *derivatives in the oxidized and reduced state in $CDCl_3$ solutions*

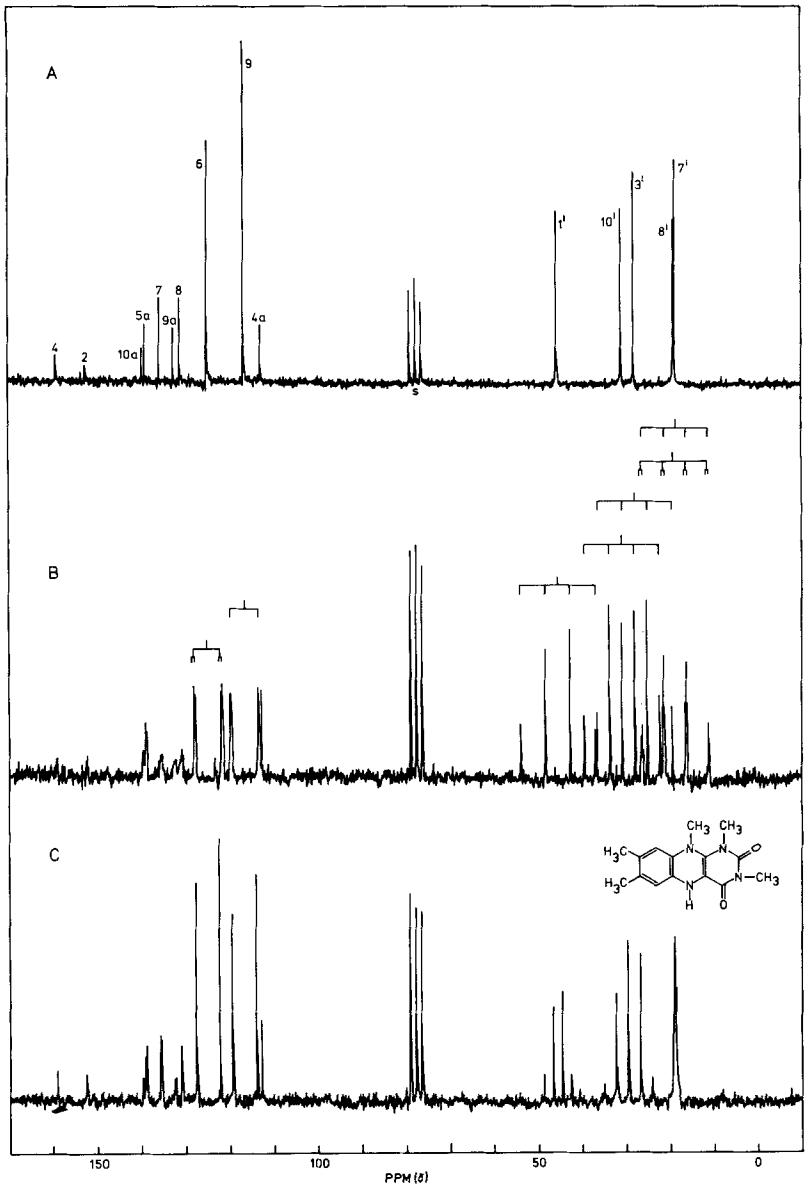


Fig. 2. *Natural abundance ^{13}C-NMR. spectra of 1,3,7,8,10-pentamethyl-1,5-dihydroisoalloxazine* (**9**) *in $CDCl_3$*

A: Proton noise decoupled; B: gated decoupled; C: off resonance spectrum, irradiation frequency at 2 ppm from TMS in the ^1H-NMR. spectrum; s = as in Fig. 1.

← A: A mixture of equal concentration of [2-^{13}C]-, [4a-^{13}C]- and [4,10a-$^{13}C_2$]-TARF in the oxidized state; B: [4a-^{13}C]-TARF in the reduced state; C: [4,10a-$^{13}C_2$]-TARF in the reduced state; D: [2-^{13}C]-TARF in the reduced state; and [4,4a,10a-$^{13}C_3$]-TARF in the oxidized (F) and reduced (E) state; s = resonance due to the solvent.

Table 1. *Experimental*[a]) *and Calculated Coupling Constants* (in Hz) *in [4,4a,10a-$^{13}C_3$]- and [4,10a-$^{13}C_2$]-3-methyltetraacetylriboflavin in the Oxidized (1) and Reduced State (2)*

Redox State	Coupling Constant					
	^1J(C(4),C(4a))		^1J(C(4a),C(10a))		^2J(C(4),C(10a))	
	Exp.	Calc.	Exp.	Calc.	Exp.	Calc.
Oxidized	76.5	100.4	53.3	80.1	10.4	−9.3
Reduced	79.2	98.3	84.5	103.1	5.5	−10.1

[a]) The accuracy of the experimental coupling constants is ±0.2 Hz.

it should be noted that the resonances due to the methyl groups of the acetyl groups become magnetically more equivalent upon reduction of the isoalloxazine ring system. This suggests a small (conformational) perturbation of the side chain upon reduction of the molecule.

3.2. *Comparison of the chemical shifts of various derivatives.* In order to separate possible effects of the side chain acetyl groups on the chemical shifts in the spectrum of **4** compound **5** has been investigated[6]) *(cf. Scheme 2)*. Compound **5** possesses only the terminal acetyl group. In the spectrum of **5** the resonance lines due to C(9a), C(5a) and C(10a) are shifted downfield by 5.0, 1.0 and 0.6 ppm, respectively, and that due to C(4a) is shifted upfield by 1.5 ppm, as compared to those of **4**. It should be noted that the chemical shifts of **5** and **6** are identical as far as the C-atoms of the isoalloxazine skeleton are concerned. Therefore, the difference in shifts between **4** and **5** are most probably caused by the dipole of the acetyl group at C(10'β) *(Fig. 3)*.

Substitution of H–N(5) in **2** by an acetyl group, giving **4**, leads to large upfield shifts of the resonances due to C(5a) (7.8 ppm), C(4a) (5.3 ppm) and C(9) (2.2 ppm) and to large downfield shifts of the resonances due to C(6) (11.7 ppm), C(10a) (10.0 ppm), C(8) (6.1 ppm) and C(9a), C(2), C(4) (about 2 ppm). Similar shifts, but different in magnitude, are observed by comparing the spectra of **9** and **8**, and those of **14** and **17** except that the resonance due to C(9) in **9** is shifted upfield as compared to the corresponding atom in **8** (*Table 2* and *Fig. 3*). In addition C(9a) in **8** and **17** undergoes a larger downfield shift than that in **4**.

Methylation at N(3) (**7** *vs.* **4**) does not influence the chemical shifts except that the chemical shift of C(10a) in the spectrum of **4** is shifted upfield by 2.5 ppm.

Replacing the methyl groups at N(1,10) by an ethano group (**8** *vs.* **16**) leads to upfield shifts of the resonances due to C(4a), C(5a), C(8), C(9), C(9a), C(10a) and C(2) *(Table 2)*.

Methylation of C(2α) instead of N(1) (**14** *vs.* **9**, *Scheme 2*), causes an upfield shift of the resonance lines of C(4), C(4a), C(5a), C(6), C(8), C(9) and C(9a) whereas downfield shifts are observed for C(10a) (8.5 ppm) and C(10') (24.1 ppm). On the other hand, placing the methyl group of N(1) onto N(5) (**9** *vs.* **10**) drastically influences the resonance positions of the peaks of C(4a), C(9a) and C(10a) and, to a lesser degree, those of C(5a), C(6), C(7), C(8) and C(9).

[6]) It was not possible to compare **2** with the corresponding compound of **5** or **6** because of the low solubility of the later compounds in $CDCl_3$.

Replacement of the N(5) methyl by an ethyl (**11**) or benzyl group (**12**) has some influence on the resonance lines of C(4a), C(6), C(9a) and C(10a) *(Table 2)*.

The effect of substitution of a particular atom of the flavin molecule on the chemical shift by various substituents is summarized in *Figure 3* for a few compounds. From *Figure 3* it can be concluded that only the chemical shifts of C(2), C(4) and C(7) are relatively insensitive with respect to both the kind of substituent introduced and the place of substitution. The chemical shifts most affected by substitution are those due to C(4a), C(5a), C(9a) and C(10a).

Substitution of C(4a) (**13**) results in a chemically completely different compound which thus cannot be directly compared with **12** or the other flavin derivatives. However, **13** serves as a model compound for postulated, biologically important intermediates [23][7]). For this reason this compound has been included in this study. Compared to the spectrum of **12**, that of **13** exhibits large downfield shifts for C(2), C(4), and C(10a), and a small downfield shift for C(9). The resonances of all other C-atom are shifted upfield. The largest upfield shift is observed for C(4a) in accord with a change of hybridization ($sp^2 \rightarrow sp^3$) of this atom. It is interesting to note that in the spectrum of **13** the following pairs of atoms become magnetically equivalent: C(7) and C(8), C(7′) and C(8′), and C(6) and C(9). Moreover, the absolute difference between the chemical shifts of these pairs is much smaller than that of the corresponding pairs in the spectrum of **12**, indicating a more symmetrical charge distribution in the benzene subnucleus of **13** than in that of **12**.

4. Discussion. – It is well documented [25] that ^{13}C-chemical shifts are mainly dependent on the charge and the π-electron density at the C-atom under consideration. This fact should, therefore, be helpful in explaining the chemical reactivity of reduced flavin towards various reactants and to predict which atom most likely could be expected to be the reactive center for a nucleophile or an electrophile. To correlate these properties of the molecule with the chemical shifts observed some theoretical calculations were performed. Using published, theoretical data [26–28] and the established relationship [29]:

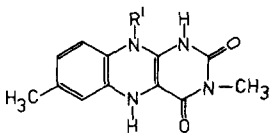
$$\delta_C = -159.5\,\rho + 288.5 \qquad (1)$$

where ρ is the π-electron density, only a rough correlation was found between experimental and calculated chemical shifts. The disagreement is probably caused by the fact that the geometry of the molecules, especially that of reduced flavin, had to be assumed in the calculations due to lack of crystallographic data. In the meantime such data have become available for both the oxidized and reduced flavin molecule, *e.g.* [30]. In our calculations by INDO [20] we used the crystallographic data of *Wang & Fritchie* [31] for the oxidized (coplanar structure) and those of *Norrestam & Glehn* [32][8]) for the reduced (folded structure) molecule. For economic

[7]) Studies on model compounds support the idea that interaction of 1,5-dihydroflavin with molecular oxygen leads to the formation of a flavinhydroperoxide intermediate [24]. It was shown recently that this interaction occurs at C(4a) of reduced flavin [12].

[8]) The authors mentioned in the text that the proton at N(5) is in axial position which is in contradiction with their actual data. The theoretical data obtained with the structure where the proton is in equatorial position gave also better agreement with the experimental data than using the other stereoisomer (*cf.* also [33]).

Scheme 2



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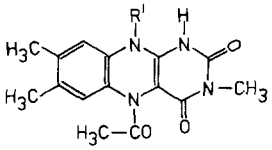

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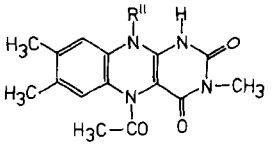

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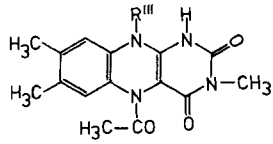

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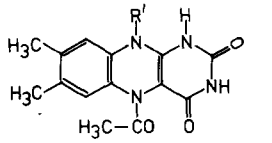

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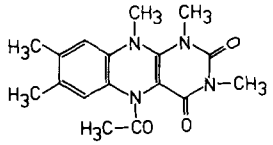

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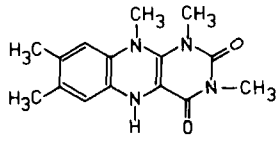

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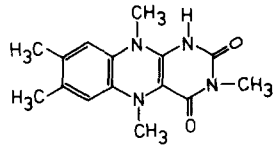

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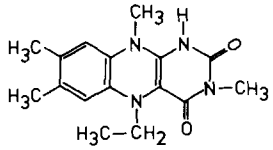

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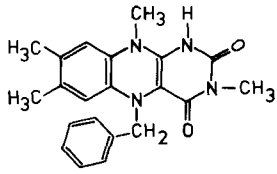

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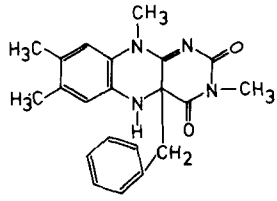

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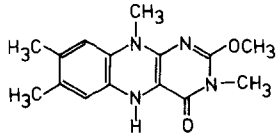

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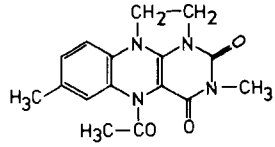

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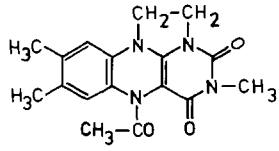

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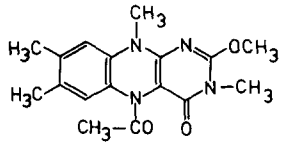

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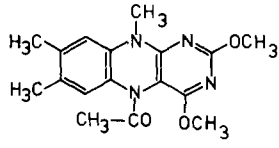

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$R^{I} = CH_2(CHOCOCH_3)_3\,CH_2OCOCH_3$

$R^{II} = (CH_2)_4CH_2OCOCH_3$

$R^{III} = (CH_2)_{10}\,CH_3$

Table 2. *^{13}C-Chemical Shifts* (in ppm) *of Various 1,5-Dihydroflavin Derivatives*[a])

No.[b])	Atom																		
	C(2)	C(4)	C(4a)	C(5a)	C(6)	C(7)	C(8)	C(9)	C(9a)	C(10a)	C(1′)	C(3′)	C(7′)	C(8′)	C(10′)	C(10′α)	C(10′ε)	C(5′α)	C(5′β)
1	155.9	161.4	136.0	134.9	133.1	136.8	147.8	115.6	131.9	149.2	–	28.7	19.4	21.4	–	45.3	62.0[c])	–	–
2	150.6	157.0	105.2	136.0	116.1	133.6	129.0	118.0	128.2	137.1	–	27.3	18.9	18.9	–	47.4	62.0[d])	–	–
3	150.4	156.9	104.5	137.0	115.1	135.7	121.6	116.0	128.6	138.1	–	27.3	20.7	–	–	47.7	62.1[d])	–	–
4	152.3	158.3	99.9	128.2	127.8	132.9	135.1	115.8	129.9	147.1	–	27.7	19.1	19.5	–	44.1	62.0[d])	171.4	21.8
5	152.3	158.1	98.4	129.2	127.7	132.7	135.2	115.8	134.9	147.7	–	28.3	19.3	20.0	–	44.3	64.0[e])	171.5	21.6
6	152.4	158.2	98.1	129.0	127.6	132.5	135.1	115.8	134.9	147.9	–	27.7	19.3	20.0	–	44.4	14.1[f])	171.6	21.5
7	152.0	158.7	99.4	129.6	127.7	132.7	135.4	116.1	129.7	149.6	–	–	19.3	19.8	–	44.2	62.1[d])	171.0	21.4
8	152.2	158.3	105.8	133.0	126.7	133.2	135.3	119.3	138.6	150.8	40.3	28.8	19.5	19.7	33.8	–	–	171.0	22.0
9	151.5	157.7	111.8	137.7	123.7	134.3	129.8	115.6	131.2	138.4	45.5	28.2	19.0	19.3	31.1	–	–	–	–
10	152.2	158.5	103.8	135.2	122.8	132.7	131.3	114.5	138.2	145.6	–	27.3	19.0	19.4	32.0	–	–	44.5	–
11	152.0	158.8	101.3	135.9	124.4	132.5	131.7	114.4	137.1	147.6	–	27.3	19.0	19.4	31.8	–	–	50.5	11.3
12	151.9	159.0	101.2	n.o.[g])	124.8	132.3	137.5	114.8	n.o.[g])	147.8	–	27.3	19.1	19.5	31.8	–	–	60.6	–
13	161.7	169.1	59.0	n.o.[g])	117.3	110.4	110.0	117.5	n.o.[g])	155.6	–	27.6	19.4	19.4	31.1	–	–	–	[h])
14	151.3	154.3	106.3	133.8	114.3	133.8	127.9	114.5	127.7	146.9	–	28.9	18.7	19.0	27.7	–	–	–	[i])
15	150.5	158.4	93.2	127.8	127.3	134.2	128.1	112.3	134.4	148.5	47.0	28.2	20.9	–	44.0	–	–	172.7	22.0
16	150.4	158.4	93.3	124.9	128.3	132.4	134.4	113.8	134.3	148.5	47.0	28.2	19.4	19.7	44.1	–	–	172.8	21.8
17	154.6	157.4	99.9	126.6	127.1	130.8	134.8	114.4	136.8	156.0	–	28.2	19.2	19.9	30.6	–	–	171.2	21.3[k])
18	161.0	161.9	100.1	126.6	127.1	130.8	135.2	114.9	136.8	163.2	–	–	19.2	19.9	30.6	–	–	171.2	21.3[l])

[a]) Spectra were obtained in $CDCl_3$.

[b]) For the structure of the compounds, see *Scheme 1* and *2*.

[c]) Chemical shifts of the other side chain C-atoms: 69.2, 69.5, 70.6 (C(10′β, γ, δ)); 21.0, 20.8, 20.7, 20.3 (CH_3CO(10′β,γ,δ,ε)); 170.8, 170.5, 170.1, 169.9 (CH_3CO(10′β,γ,δ,ε)).

[d]) Chemical shifts of the other side chain carbon atoms: 69.7, 70.0, 70.1 (C(10′β,γ,δ)); 20.6 (CH_3CO(10′β,γ,δ,ε)); 170.8, 170.1, 170.0, 169.8 (CH_3CO(10′β,γ,δ,ε)).

[e]) Chemical shifts of the other side chain C-atoms: 27.4, 27.8, 28.2 (C(10′β,γ,δ)); 171.5 (CH_3CO(10′ε)); 20.9 (CH_3CO(10′ε)).

[f]) Chemical shifts of the other side chain C-atoms: 31.9, 29.6, 29.3, 29.1, 26.6, 22.7, 21.4.

[g]) n.o. = not observed.

[h]) C(4a′a): 43.5.

[i]) C(2′β): 55.2.

[k]) C(2′β): 55.5.

[l]) C(2′β): 54.9; C(4′β): 54.2.

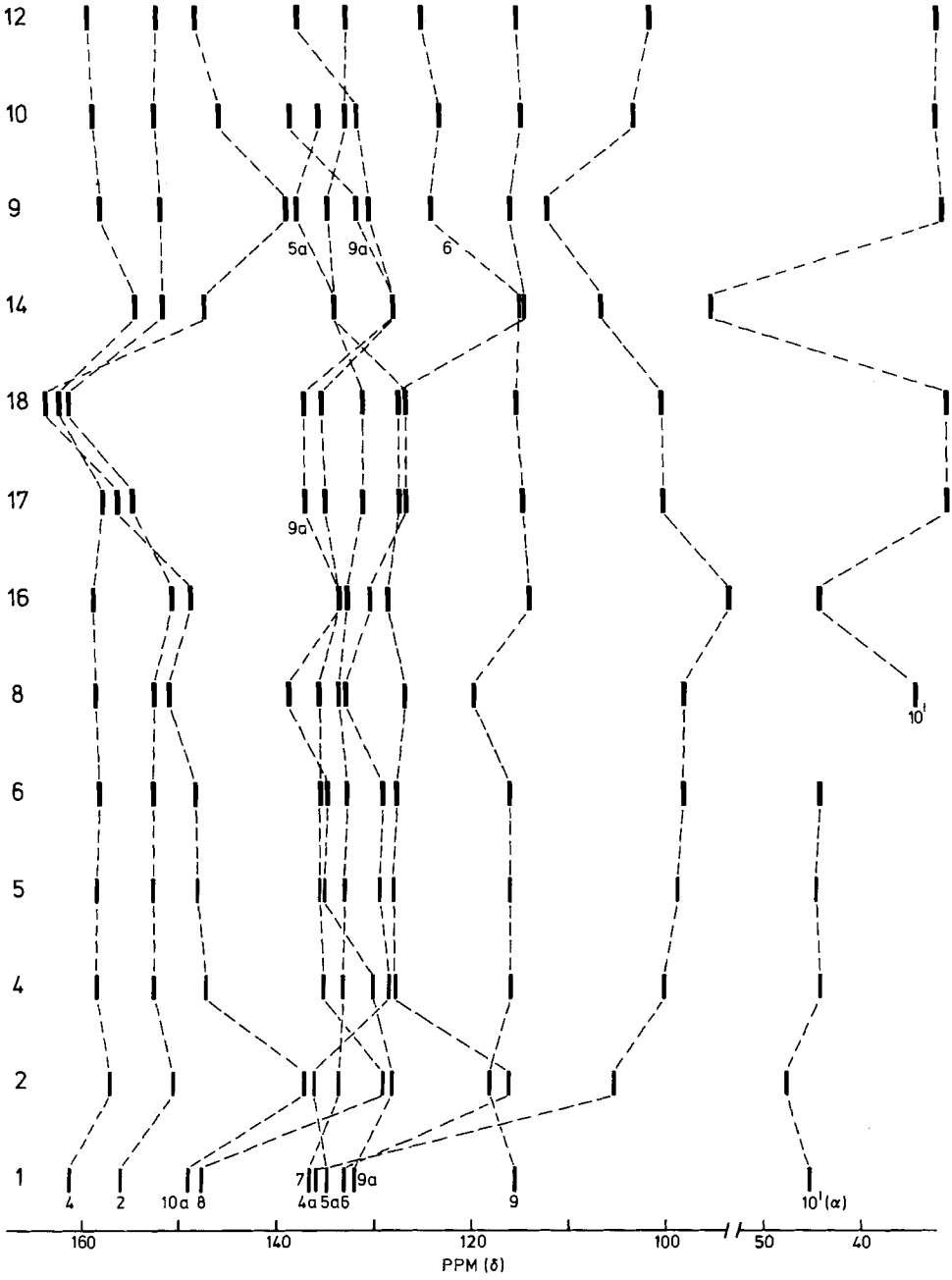

Fig. 3. *^{13}C-chemical shift correlation diagram for various flavin derivatives.* The numbers on the horizontal axis refer to the C-atoms of the flavin molecule *(cf. Scheme 1)*, the numbers on the vertical axis are related to the structure of the individual derivatives *(cf. Scheme 2)*.

reasons in our calculations the methyl groups in the structures mentioned were replaced by protons. The calculated electron densities and chemical shifts are given in *Table 3*. The results show that for the oxidized molecule a fair and for the reduced molecule a rather good correlation exists between experiment and theory. Plotting the calculated chemical shifts against the experimental ones gives a correlation coefficient (r^2) of $\geqq 0.96$ for the reduced and of $\geqq 0.85$ for the oxidized molecule. The slope of a plot of calculated π-electron densities against the experimental chemical shifts gives a value of 147 ppm/π-electron, in fair agreement with the theory [29]. For the reduced molecule only the chemical shift for C(2) and C(9) are predicted less accurately. The downfield shift of the resonances of C(5a), C(9) and C(10') (*Table 2* and *Fig. 3*) and the upfield shift of the resonances of all other C-atoms in the flavin molecule observed upon reduction can thus be explained by a decrease and an increase, repectively, of π-electron density at the atom under consideration. The only exception to this statement is observed for C(7) where the theory predicts a downfield shift but an upfield shift is found experimentally. The fact the experimental chemical shifts of C(2) and C(4) of the oxidized and reduced molecule are inversed as compared to the theoretical calculations might indicate that neighbouring bond anisotropy influences the chemical shifts considerably. This suggestion is supported by findings on uracil derivatives where the corresponding C-atoms show an identical behaviour as those in the flavin molecule [34].

From the relationship 160 ppm/π-electron [29] it is estimated that only about 20% of an electron is added to the benzene ring of flavin upon reduction of the molecule. Furthermore, the theoretical data also show a remarkable change in the density matrix of the p_z-orbitals of C(4a) and C(10a). In spite of an increase of the π-electron density at C(4a) and C(10a), the cross terms between the p_z-orbitals of C(4a) and C(10a) are increased as compared to those of C(5a), C(9a), C(6), C(7), C(8) and C(9). This indicates that a fairly localized double bond exists between C(4a) and C(10a), which is in agreement with crystallographic studies [30]. This fact and the π-electron density increase at C(4a) and C(10a) as well as the rather drastic increase of π-electron density at N(5) and N(1) explain reasonably the observed upfield shift of the resonances due to C(4a) and C(10a).

It should be noted that the theory *(Table 3)* predicts a π-electron density decrease at N(10) upon reduction of the flavin molecule. Preliminary ^{15}N-NMR. experiments [35] showed that N(10) indeed undergoes a downfield shift upon reduction whereas the other three N-atoms of flavin shift upfield as expected. This decrease in π-electron density at N(10) can thus explain the downfield shift of C(10'α) and C(9).

The higher magnetic equivalence of the pairs of C(6) and C(9), and C(7) and C(8) in **2** as compared to those in **1** indicates that the conjugation *via* C(8)–C(6)–N(5)–C(10a)–C(2) is decreased in **2** as compared to that in **1** [8].

The finite pertubation theory [36] makes it possible to estimate ^{13}C, ^{13}C-coupling constants. The computed constants are in reasonable agreement with the experimental values *(Table 1)*. The latter values are in the range expected for sp^2, sp^2-interactions [37]. Available data suggest that direct ^{13}C-coupling constants are approximately correlated to the *s* character of the orbitals making up the bond [38]. Neglecting possible influences of the neighbouring heteroatoms on the direct

Table 3. *Calculated Total and π-Electron Densities of Oxidized and Reduced Flavin and their Comparison with Calculated and Observed Chemical Shifts*

Atom	Oxidized Electron density				Reduced Electron density			
	Total	π	δC_{calc}	δC_{obs}[a])	Total	π	δC_{calc}	δC_{obs}[b])
C(2)	3.49	0.80	160.5	155.9	3.48	0.82	157.3	150.6
C(4)	3.60	0.82	157.3	161.4	3.59	0.82	157.3	157.0
C(4a)	3.98	1.03	123.7	136.0	4.09	1.16	102.9	105.2
C(5a)	3.94	1.05	120.5	134.9	4.10	0.96	134.9	136.0
C(6)	3.98	0.97	133.3	133.1	4.04	1.05	120.5	116.1
C(7)	3.99	1.02	125.3	136.8	4.03	0.99	130.1	133.6
C(8)	3.96	0.96	134.9	147.7	4.00	1.02	125.3	129.0
C(9)	4.03	1.05	120.5	115.6	4.00	1.01	126.9	118.0
C(9a)	3.89	0.96	134.9	131.9	3.92	1.02	125.3	128.2
C(10a)	3.72	0.85	152.5	149.2	3.75	0.95	136.5	137.1
N(1)	5.41	1.35			5.24	1.70		
N(3)	5.24	1.70			5.24	1.67		
N(5)	5.09	0.94			5.17	1.73		
N(10)	5.06	1.59			5.19	1.35		

[a]) Chemical shifts of compound **1**.
[b]) Chemical shifts of compound **2**.

coupling constants the results in *Table 1* indicate that in the oxidized molecule the C(4)–C(4a) bond possesses a higher *s* character than the C(4a)–C(10a) bond. In the reduced molecule the *s* character of both bonds is more similar. Keeping in mind the above mentioned restrictions it can be expected that the experimentally determined coupling constants show a correlation to the bond lengths determined crystallographically [30]. The following bond lengths were published [30]. For the oxidized and reduced molecule, respectively: 0.148 and 0.142 nm for the C(4),C(4a)-bond, and 0.145 and 0.136 nm for the C(4a),C(10a)-bond. Comparing the corresponding values (coupling constant *vs.* bond lengths) of the oxidized with those of the reduced molecule a fair relationship is observed but the values within the oxidized molecule correlate less. This suggests that the effect of the neighbouring polar atoms on the direct coupling constants is probably appreciable.

The experimental two-bond coupling constant is larger in the oxidized molecule *(Table 1)*. Since two-bond coupling constants are also dependent on the bond angles [22] the results are in agreement with crystallographic data, *i.e.* coplanar *vs.* folded structure of oxidized and reduced flavin, respectively.

The assignment presented in this paper for **1** and **2** are in full agreement with the results of *Kawano et al.* [9] who used riboflavintetrabutyrate in their study. The more recent published data of *Ghisla et al.* [12] agree also with our data except for C(4a) and C(7) in **1**, where the order must be reversed. In addition these authors could not assign unequivocally the resonance lines due to C(6), C(7), C(8) and C(9) in **2** and **13**.

In this work and the published work of others [9] [12] tetraacetylriboflavin (**1**) was used as a model compound because of the high solubility of its oxidized and reduced form in $CHCl_3$. It has not been realized, however, that the carbonyl groups of the ester functions of the ribityl side chain might influence some of the chemical

shifts of the isoalloxazine ring. The influence of these ester functions is negligible in **1**, because the chemical shifts of **1** are identical with those of lumiflavin [8] and the oxidized form of **6** (this work, not shown) as far as the C-atoms of the isoalloxazine ring are concerned. The situation is different for **2** (= the reduced form of **1**). A comparison of the chemical shifts of **4** to **6** reveals that the shifts for **5** and **6** are very similar, if not identical, and that the most significant difference between **4** and the compounds **5** and **6** is observed for C(9a) which is shifted upfield by 5.0 ppm in **4** as compared to that in **5** and **6**. These results indicate that the upfield shift of C(9a) in **2** to **4** is induced by the electric field effect of one of the carbonyl groups of the side chain ester functions. The small comformational change of the side chain observed upon reduction of **1** must, therefore, be related to the upfield shift of C(9a) in **2** to **4** as compared to that in **5** and **6**. Hence, one must be careful in relating the chemical shifts of the esterified compounds with those of *e.g.* reduced riboflavin monophosphate.

The effects of substitution at different atoms in reduced flavin are partly rather complex. But this fact is not surprising considering the complex structural behaviour of reduced flavin. From crystallographic data, describing only static structures, it is known that the conformation of reduced flavin is influenced by substitution at N(1) and/or N(5) [39–41], *i.e.* the angle between the normals of the benzene and the pyrimidine subnucleus of the flavin molecule is increased upon substitution. In addition the relative orientation of the N(5)-substituent with respect to the pyrazine subnucleus is also altered [40] [41]. Preliminary studies on the temperature dependence of the ^{13}C-chemical shifts of *e.g.* **4** show that already at $-18°$ at least two different molecular forms are present in solution. These findings are in contradiction with published work of others [42] who showed that the ^{1}H-NMR. spectrum of a similar compound was not influenced at all by a decrease of the temperature down to $-107°$. Since the ^{13}C-chemical shifts are obviously more sensitive to structural changes than the ^{1}H-chemical shifts we have decided to devote a separate study to the observed temperature dependence of the ^{13}C-chemical shifts in order to elucidate the structural features of reduced flavin in more detail. These results will be published in a forthcoming paper. Nevertheless, at this moment we wish to discuss shortly some of the substitution effects. Methylation of C(2*a*) of the flavin molecule does not affect the chemical shifts of the C-atoms of the benzene subnucleus whereas the chemical shifts of the C-atoms of the pyrimidine subnucleus are additive (**17**, **18**). Acetylation of N(5)H (*cf. e.g.* **4** *vs.* **2**) leads to large upfield shifts of C(4a) (5.3 ppm) and C(5a) (7.8 ppm) and large downfield shifts of C(6) (11.7 ppm), C(8) (6.1 ppm) and C(10a) (10.0 ppm). Similar shifts are observed when **17** and **14** are compared with each other. On the other hand comparing **8** and **9** it is learned that the upfield shift of the C-atoms in question and the downfield shift of C(8) and C(10a) is still about the same as mentioned above but the downfield shift of C(6) is decreased by about 9 ppm. Since in **4**, **8** and **17** the N(5) substituent is the same and N(1) methylation the chemical shift of C(6) not affects (*cf.* **8** *vs.* **6**) the observed effect must be related to the relative stereochemical position of the acetyl group with respect to the C(6)-atom[9]). From a comparison of **11** *vs.* **2** it

[9]) It is a reasonable assumption that the acetyl group excerts the same electronic influence *via* the bonds on the shielding of the aromatic C-atoms in all three compounds.

can be concluded that the methyl group of the N(5) ethyl substituent causes a downfield shift of C(6) (δ-effect). Hence the acetyl group in **4** and **17** must be closer to the C(6)-atom than in **8**. This interpretation is in agreement with crystallographic data [41] [42] and results obtained with stereoisomers of a partially related system, *i.e.* *N*-acetyl-tetrahydrochinolin [43].

Finally the relationship observed between the calculated π-electron densites and the experimental ^{13}C-chemical shifts suggests that a similar correlation can be expected with the chemically active atoms of the flavin molecule. Oxidized flavin in the ground state adds nucleophiles preferentially at the centers N(5) (SO_3^{2-}, $P(C_6H_5)_3$) [44] [3] and C(10a) (CH_3O^-) [45]. The centers C(2) and C(8) are chemically less reactive but undergo nucleophilic substitution reactions [46]. An electrophilic substitution reaction is known to occur at C(9) [47]. The reduced flavin molecule, on the other hand, reacts with electrophiles preferentially at the centers N(5) and C(4a) [17, 12] and probably also at C(10a) [24, 48]. These observations demonstrate that indeed a good correlation also exists between the calculated π-electron densites and the observed chemical shifts, and the experimentally observed centers of chemical reactivity of the flavin molecule. This relationship should, therefore, be very helpful for the chemical characterization of new flavin derivatives and flavoenzymes.

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