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Nitrogen-15 and Carbon-13 Nuclear Magnetic Resonance of Reduced Flavins. Comparative Study with Oxidized Flavins†

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ABSTRACT: Nitrogen-15 and carbon-13 nuclear magnetic resonance spectra of the fully reduced form of flavin were studied with riboflavin tetrabutyrate (RBUT), an organic solvent-soluble derivative of riboflavin. For the measurement of ^{15}N resonances, 99% enriched [1,3-^{15}N]RBUT and [1,3,5-^{15}N]RBUT were synthesized. In order to assign the ^{13}C resonances, 90% enriched [2-^{13}C]RBUT, [4a-^{13}C]RBUT, [4,10a-^{13}C]RBUT, and [8-$^{2}H_3$]RBUT were employed. The upfield shift of N(5) resonance upon reduction was remarkable (286 ppm), while the N(1) signal moved only by 79 ppm. The one-bond ^{15}N-H spin-spin coupling constant $^1J[^{15}N(5)-H]$ of the reduced RBUT was smaller than its $^1J[^{15}N(1)-H]$ and $^1J[^{15}N(3)-H]$. These observations indicate that N(5) changed into sp^3 hybridization upon reduction and lost the character of planar nitrogen. Most of the ^{13}C nuclei of the reduced form resonated at higher field than did those of the oxidized form, which is well explained by the increase in π-electron densities. Among the ^{13}C resonances, the upfield shift of C(4a) was remarkable (32 ppm), which explains the reactivity of C(4a) in oxygen flavoprotein complexation. ^{13}C-^{15}N spin-spin coupling constants were obtained from the measurements of ^{13}C magnetic resonance of ^{15}N-enriched RBUT. The values of the one-bond ^{13}C-^{15}N coupling constants increased markedly with protonation at N(1) and N(5) upon reduction.

The elucidation of the electronic and structural properties of the reduced flavin in comparison with those of the oxidized one is essential for understanding the redox reactions of flavoenzymes. Electronic states of the oxidized and reduced flavins have been examined by means of molecular orbital calculations and discussed in connection with their reactivities (Song et al., 1976) and with the electronic spectra (Grabe, 1974; Nishimoto et al., 1978). However, there is no experimental evidence which proves the calculated electronic distribution in a flavin molecule except for the X-ray analysis of flavin derivatives (Kierkegaard et al., 1971).

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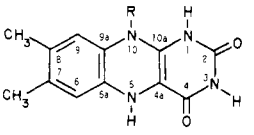
Nuclear magnetic resonance (NMR)[1] is a powerful tool for studying the electronic state of each atom in flavin and its structure. Proton magnetic resonance has been widely used for this purpose (Kainosho and Kyogoku, 1972; Crespi et al., 1972; Raszka and Kaplan, 1974; Grande et al., 1977b). ^{13}C NMR spectra of FMN and FAD with ^{13}C in natural abundance have also been studied (Breitmaier and Voelter, 1972). Yagi et al. (1976a), however, corrected the assignments of carbons, in part by using ^{13}C-enriched FMN and FAD, and studied the interaction of [^{13}C]riboflavin with the apoprotein of egg-white flavoprotein. Recently, Yagi et al. (1976b) reported the ^{15}N magnetic resonance spectra of flavins, which demonstrated that ^{15}N chemical shifts and the NOE are sensitive to the electronic states and to chemical interactions. These studies have dealt only with the oxidized form of free and protein-bound flavin, except for several studies of N-alkylated flavins (Tauscher et al., 1973; Ghisla et al., 1973; Hemmerich and Haas, 1975). This is probably due to the difficulties in practical measurement: line broadening is caused by small amounts of semiquinone radical provoked by a trace of oxygen and by the strong self-association of the reduced flavin. We have found that riboflavin tetrabutyrate (RBUT), one of the organic solvent-soluble derivatives of riboflavin, is a useful tool to overcome these difficulties. Accordingly, the carbons and nitrogens which directly participate in the redox reaction of this compound were selectively enriched with ^{13}C and ^{15}N. The observation of their magnetic resonance was expected to allow characterization of the reduced state of flavin.

Materials and Methods

^{15}N-enriched riboflavins were synthesized from [^{15}N]urea (99.36 atom %, Prochem., England) and sodium [^{15}N]nitrite (99 atom %, Prochem., England) as ^{15}N sources by the method described in the previous paper (Yagi et al., 1976b). ^{13}C-enriched riboflavins were prepared from [^{13}C]urea (91.4 atom %, Prochem., England), [1-^{13}C]malonic acid (92.7 atom %, Prochem. England), and [2-^{13}C]malonic acid (90 atom %, Merck, Canada) as ^{13}C sources (Yagi et al., 1976a). The butyric esters of these flavins were synthesized by the method of Yagi et al. (1961), and the obtained ^{15}N- or ^{13}C-enriched RBUT's were recrystallized according to Yagi et al. (1967).



[8-$^{2}H_3$]RBUT was obtained by a modification of the method of Bullock and Jardetzky (1965). Unlabeled RBUT was heated at 90–95 °C in pyridine/D_2O (1:1) for 3 h, and the deuterium content of the methyl group at C(8) was about 75%, as estimated by ^1H NMR. The purity was checked by cellulose thin-layer chromatography with *n*-butyl alcohol–acetic acid–water (4:1:5, v/v) as the solvent system.

The ^{15}N spectra were obtained at 10.09 MHz on a JEOL PFT-100 pulse Fourier transform NMR spectrometer. The spectra were recorded with or without noise-modulated ^1H decoupling (2.5 kHz). Spectra were spread over a 5-kHz region with 8192 data points; the resolution due to digitalization was 1.22 Hz, i.e., 0.12 ppm for ^{15}N. Throughout the operation, the pulse width was 20 μs (50° flip angle) and the pulse delay was 2 s. Sample tubes used were 10 mm in diameter with a 2-mm coaxial tube containing $^{15}NH_4{}^{15}NO_3$ solution in Me_2SO-d_6, which provides the reference standard and the external lock signal. The chemical shifts were measured upfield in parts per million relative to external $^{15}NO_3^-$ in Me_2SO-d_6. The ^{15}N signal of $^{15}NO_3^-$ in this medium was positioned at 2.05 ppm lower than that in 1 N DCl. The ^{13}C and ^1H NMR spectra were obtained in the Fourier transform mode on a JEOL FX-100 spectrometer operating at 99.60 and 25.05 MHz, respectively. Chemical shifts were read relative to the resonance of internal Me_4Si in both cases. Normal ^{13}C NMR spectra were taken over 5 kHz with 8192 data points, while ^{13}C–^{15}N coupling constants were measured at 2.5 kHz with 16384 points; the digital resolution was 0.3 Hz.

To prepare the reduced RBUT sample, a $CDCl_3$ solution of RBUT in an NMR sample tube was treated with an aqueous solution of sodium dithionite in an amount sufficient to reduce the RBUT. After shaking, the tube was sealed anaerobically. Since a slight amount of paramagnetic flavin radical produces a broadening of the signals, the water layer was held over the $CDCl_3$ solution to keep the fully reduced state as was done by Ghisla et al. (1973) for N-alkylated flavins. Reduced RBUT could be reversibly oxidized by bubbling of oxygen into the solution.

Results and Discussion

^{15}N Resonances of Oxidized and Reduced Flavins. Figure 1 shows the proton-decoupled and coupled ^{15}N resonance spectra of [1,3,5-^{15}N]RBUT in the oxidized state. They are similar to the spectra of 1,3,5-^{15}N-enriched riboflavin, FMN, and FAD, except for slight differences in their chemical shifts (Table I) within the solvent effect (Yagi et al., 1976b). The proton-decoupled spectrum of the reduced [1,3,5-^{15}N]RBUT gave three inverted peaks, as shown in Figure 1. By comparison of the spectrum with that of the reduced [1,3-^{15}N]RBUT, the signal in the highest field (319.0 ppm) was assigned to N(5). For the assignment of the remaining two peaks, it is noted that the chemical shift of the N(3) signal should not be largely changed upon reduction; the peak at 229.15 ppm was assigned to N(3). The chemical-shift difference between N(3) and N(1) of the reduced RBUT has the same value (28.65 ppm) with that of lumichrome (Yagi et al., 1976b), which seems reasonable because of the resemblance of their chemical structures around the two nitrogens.

The upfield shift of the N(5) resonance upon reduction is remarkable (286 ppm) in contrast to that of N(1) (79 ppm). The shift value of N(5) is comparable to the ^{15}N shift difference between pyridine and piperidine (280 ppm) (Witanowski et al., 1977). It indicates that N(5) is changed to sp^3 hybridization, leading the isoalloxazine ring to a bent form (Kierkegaard et al., 1971). The ^{15}N shift differences between the oxidized and the reduced RBUT correspond well to the π-electron densities calculated by Pullman and Pullman (1959), in which N(5) is the most and N(3) is the least affected upon reduction.

It has been postulated in a previous paper (Yagi et al., 1976b) that the unusual negative NOE of the N(1) signal of the oxidized form may be due to the intramolecular NOE by the protons in the ribityl group. This was confirmed by deuterium exchange (Figure 2). On addition of D_2O to the Me_2SO-d_6 solution of [1,3,5-^{15}N]RBUT, the N(3) signal decreased in intensity, while the N(1) signal was not affected. This means that the NOE of the N(1) signal is due to the unexchangeable protons such as those in the ribityl group and is not caused by the protonation by solvent molecules. All of the proton-decoupled ^{15}N signals of the reduced [1,3,5-^{15}N]RBUT gave inverted peaks (Figure 1) and lost their NOE

[1] Abbreviations used are: NMR, nuclear magnetic resonance; FMN, riboflavin 5′-phosphate; FAD, flavin adenine dinucleotide; NOE, nuclear Overhauser effect; RBUT, riboflavin 2′,3′,4′,5′-tetrabutyrate; Me_2SO-d_6, dimethyl-d_6 sulfoxide; Me_4Si, tetramethylsilane.

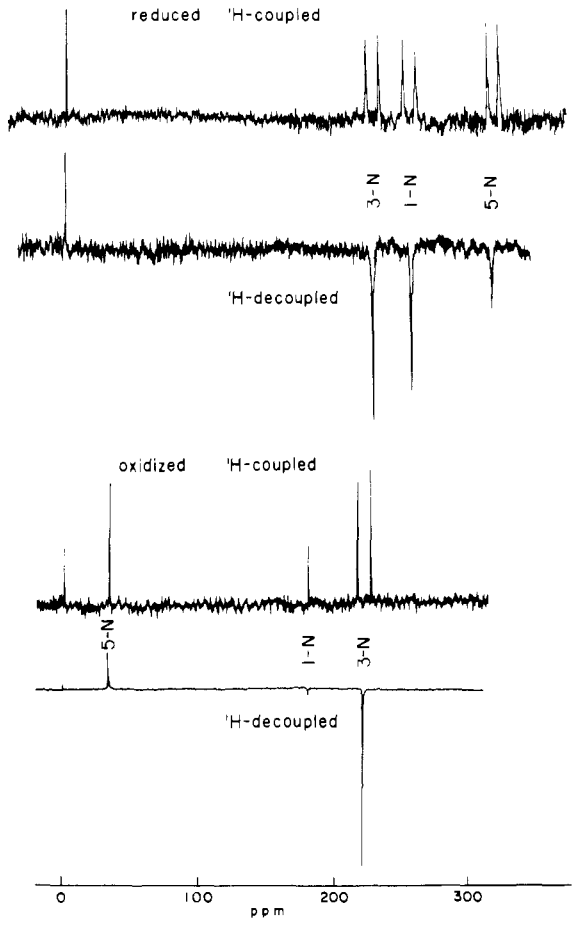


FIGURE 1: Proton-coupled and decoupled ^{15}N NMR spectra of reduced and oxidized [1,3,5-^{15}N]RBUT. About 50 mg of sample was dissolved in 2 mL of $CDCl_3$. Each spectrum was obtained after 5000–20 000 accumulations at room temperature, except for the ^1H-coupled spectrum of the reduced RBUT (−18 °C). Chemical shifts are relative to external $^{15}NO_3^-$ in Me_2SO-d_6.

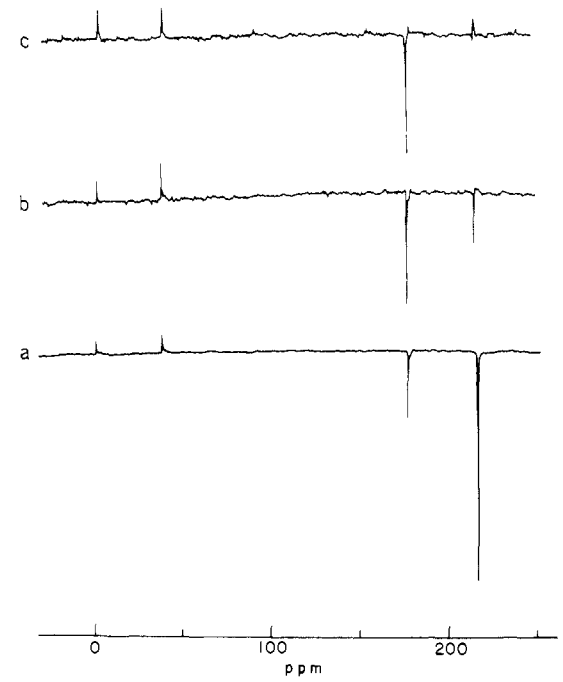


FIGURE 2: Effect of addition of D_2O on the ^{15}N NMR spectrum of [1,3,5-^{15}N]RBUT in Me_2SO-d_6. Thirty milligrams of sample was dissolved in 1.5 mL of Me_2SO-d_6 (a), and then 0.1 mL of D_2O was added (b). To the solution, 0.1 mL of D_2O was further added and stood for several hours (c).

TABLE I: ^{15}N Chemical-Shift Values of ^{15}N-Enriched Riboflavin Tetrabutyrate in Oxidized and Reduced States.[a]

	chemical shifts (ppm)		
	N(1)	N(3)	N(5)
[1,3-^{15}N]RBUT			
ox.	178.5_5	219.1	
red.	257.9_5	229.4	
[1,3,5-^{15}N]RBUT			
ox.	178.7	219.3_5	33.4
red.	257.8	229.1_5	319.0

[a] Measured from external $NH_4{}^{15}NO_3$ in Me_2SO-d_6.

when [1,3,5-^{15}N]RBUT was reduced in D_2O. These observations confirm that the reduced RBUT is 1,5-dihydroflavin.

In the proton-coupled spectrum of the oxidized [1,3,5-^{15}N]RBUT, only the N(3) signal is a doublet (Figure 1). On the other hand, the spectrum of the reduced form gave two doublets [N(3) and N(5)] and a broad peak [N(1)] at room temperature. The broad N(1) signal also became a sharp doublet on lowering the temperature to −18 °C (Figure 1). Since the proton at N(1) is the most acidic in the reduced flavin ($pK \sim 6.5$) (Lowe and Clark, 1956), the temperature dependence of the N(1) signal could be due to the fast exchange of the proton on N(1) at room temperature and to the decreasing rate of exchange upon lowering the temperature. At lower temperature, each N(1), N(3), and N(5) of the reduced RBUT is obviously bound to a proton. Similar discussions on the temperature dependence of ^{15}N–H coupling constant have also been made for porphyrin derivatives (Kawano et al., 1978). The ^{15}N–H spin–spin coupling constants of the oxidized and reduced RBUT are summarized in Table II. The coupling interaction between directly bonded nuclei is generally regarded as being dominated by the Fermi contact term, and the dependence of $^1J(^{15}N\text{–}H)$ on the amount of s character in the bond has been expressed as follows (Binsch et al., 1964):

$$\%\,s = 0.43\ {}^1J(^{15}N\text{–}H) - 6$$

where % s is the percent s character of the nitrogen hybrid orbital. This relation shows that N(3) in the reduced flavin has more sp^2 character than in the oxidized flavin and that N(5) is in the most sp^3-like hybridization among the three nitrogens in the reduced form of the isoalloxazine. The sp^3 character of N(5) is consistent with the aforementioned conclusion from ^{15}N chemical-shift data. Some ^{15}N–^1H coupling constants between the nuclei separated by three bonds were also observed by ^1H NMR, and the values are of the same order as those obtained by Axenrod (1973). Consequently, the three-bond coupling constant, ^3J[N(5)–H(6)], can be used for the assignments of ^1H NMR resonances of C(6)H and C(9)H.

^{13}C Chemical Shifts and Electron Densities. Proton-decoupled ^{13}C resonance spectra of the oxidized and reduced RBUT with ^{13}C at natural abundance level are given with their assignments in Figure 3 and their shift values in Table III.

Signal assignments were performed by the measurements of ^{13}C-enriched materials and [8-$^2H_3$]RBUT, selective proton decoupling, and the use of ^{13}C–^{15}N spin–spin coupling constants. ^{13}C chemical shifts of [2-^{13}C]RBUT, [4a-^{13}C]RBUT, and [4,10a-^{13}C]RBUT in the oxidized and reduced states are summarized in Table IV. These results are in good agreement with those in Table III. Thus, the assignments of 2-, 4-, 4a-,

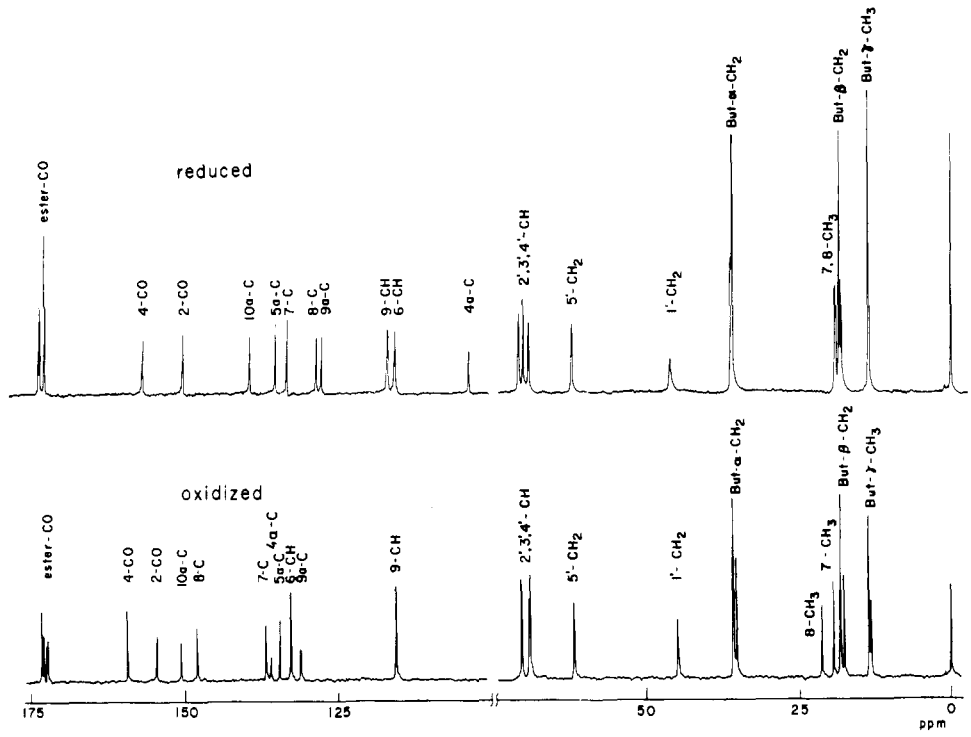


FIGURE 3: Proton-decoupled ^{13}C NMR spectra of reduced and oxidized RBUT with ^{13}C at natural abundance. Reduced RBUT: 60 mg in 2 mL of $CDCl_3$, accumulated 40 000 times; oxidized RBUT: 100 mg in 1.5 mL of $CDCl_3$, accumulated 1600 times.

TABLE II: ^{15}N-H Spin-Spin Coupling Constants of Oxidized and Reduced Riboflavin Tetrabutyrate.

	assignment	$J(^{15}N\text{-}H)$ (Hz) measured by ^{15}N NMR	^{1}H NMR
ox.			
	^{1}J[N(3)-H]	90.3 ± 1.2	90.3 ± 0.15
	^{3}J[N(1)-H(3)]		0.9 ± 0.15
	^{3}J[N(5)-H(6)]		~2[a]
red.			
	^{1}J[N(3)-H]	94.0 ± 1.2	92.5 ± 1
	^{1}J[N(1)-H]	94.0 ± 1.2	~90[a]
	^{1}J[N(5)-H]	86.7 ± 1.2	~84[a]
	^{3}J[N(5)-H(6)]		2.1 ± 0.15

[a] Indefinite values estimated from broad peaks.

TABLE III: ^{13}C Chemical-Shift Values of Riboflavin Tetrabutyrate in Oxidized and Reduced States.[a]

carbon	chemical shifts (ppm) oxidized	reduced
but-γ-CH_3	13.3	13.5_5 (4)
	13.6_5 (3)	
but-β-CH_2	17.7	17.9_5
	18.2_5 (2)	18.2 (3)
	18.4	
7-CH_3	19.4_5	18.8_5
8-CH_3	21.4	19.0
but-α-CH_2	35.5	35.7_5 (2)
	35.8_5	35.9
	36.0 (2)	36.0_5
1'-CH_2	44.9	45.8
5'-CH_2	61.8	61.7_5
2',3',4'-CH	68.9	68.7_5
	69.1	69.6
	70.3	70.3
9-CH	115.7	117.0_5
C(9a)	131.3	127.6_5
6-CH	132.9	115.8
C(5a)	134.6	135.1_5
C(4a)	136.1	103.9_5
C(7)	136.9	133.2_5
C(8)	148.0	128.5
C(10a)	150.7	139.2_5
2-CO	154.5_5	150.0
4-CO	159.3_5	156.4_5
but-CO	172.2_5	172.2_5 (2)
	172.4	173.0
	172.8_5	173.2
	173.1_5	

[a] Measured from internal Me_4Si. The numerals in parentheses indicate the numbers of the overlapped peaks.

and 10a-carbons were established, and eight carbons of the isoalloxazine ring still await assignment. Partial decoupling experiments made it possible to identify the methyl carbons bound to C(7) and C(8) as quartets and the methine carbons at positions 6 and 9 as doublets. The nonprotonated carbons at positions 7 and 8 were distinguished from the other peaks by the loss of NOE from the methyl protons. The remaining two carbons were assigned to the nonprotonated C(5a) and C(9a). From the ^{13}C spectrum of [8-$^{2}H_3$]RBUT, C(8) and 8-CD_3 were distinguished from C(7) and 7-CH_3, respectively, by their intensity. The carbons at positions 6 and 9 were assigned by the selective decoupling with irradiation at the known proton resonance frequency. The carbon at position 5a was distinguished from C(9a) by comparing ^{13}C-^{15}N spin-spin coupling constants (see Table VI): C(5a) was coupled with N(5) but not C(9a). The assignments of all the carbons in the isoalloxazine ring of the oxidized RBUT are compatible with those of 3,7,8,10-tetramethylisoalloxazine reported by Grande et al. (1977a).

By comparing the shift values in Table III with those of FMN in D_2O (Yagi et al., 1976a; Breitmaier and Voelter,

TABLE IV: ^{13}C Chemical-Shift Values of ^{13}C-Enriched Riboflavin Tetrabutyrate in Oxidized and Reduced States.[a]

	position	chemical shifts (ppm) oxidized	reduced
[2-^{13}C]RBUT	2	154.3	149.0
[4a-^{13}C]RBUT	4a	136.0_5	104.0_5
[4,10a-^{13}C]RBUT	4	159.2_5	156.5
	10a	150.7	139.3

[a] Measured from internal Me_4Si.

TABLE V: Comparison of Observed ^{13}C Chemical-Shift Differences between Oxidized and Reduced RBUT with Values Calculated.

position	^{13}C chemical shifts (ppm)[a] obsd	calcd[b]	calcd[c]
4a	$+32.1_5$	+36.8	+44.6
10a	$+11.4_5$	+20.8	+12.5
9a	$+3.6_5$	+8.0	+12.6
5a	-0.5_5	−4.8	−2.7
4	+2.9	+4.8	+5.4
2	$+4.5_5$	+1.6	−2.2
9	-1.3_5	0.0	+6.7
8	+19.5	+9.6	+15.7
7	$+3.6_5$	0.0	+4.6
6	+17.1	+16.0	+18.6
8-CH_3	+2.4	0.0	0.0
7-CH_3	+0.6	0.0	0.0

[a] Positive sign means an increase in ^{13}C shift upon reduction. [b] Calculated from the relation of 160 ppm/π-electron (Spiesecke and Schneider, 1961) by the use of the net charge reported by Grabe (1974). [c] Calculated from the same relation by the use of the net charge reported by Pullman and Pullman (1959).

1972), it was found that C(2), C(4), C(7), C(8), and C(9) of RBUT give signals at a higher field than those of FMN, whereas C(6) gives signal at a lower field. This can be ascribed to the solvent effect: hydrogen bondings between water molecules and the carbonyl groups are eliminated in $CDCl_3$ (Yagi et al., 1976a; Grande et al., 1977a) and the modes of association in different solvents are different.

It is known that variations in local π-electron densities primarily govern the ^{13}C shieldings in aromatic rings (Lauterbur, 1961). The observed ^{13}C chemical shifts of the oxidized and reduced RBUT, however, are not consistent with the calculated π-electron densities of the isoalloxazine ring (Grabe, 1974; Pullman and Pullman, 1959). The differences in the observed ^{13}C chemical shift between the oxidized and reduced RBUT were compared with those obtained by calculation on the assumption that ^{13}C shieldings depend only on π-electron density (Table V). They agree fairly well in their orders and in their signs, though their absolute values do not correspond. This may be ascribed to the accuracy of calculated π-electron densities: differences between the values calculated for two states are more reliable than the absolute values. Also in the observed chemical shift values, the difference between two states would be advantageous in diminishing the factors other than π-electron density that influence the change of ^{13}C chemical shifts such as neighboring-bond anisotropy.

Among the changes in ^{13}C chemical shifts due to reduction, the shift of C(4a) to high field is remarkable (Table V). In addition, the C(4a) signal is located at the highest field among the carbons of the reduced isoalloxazine ring, except for the 7- and 8-methyl carbons (Table III). The C(4a) position has

TABLE VI: ^{13}C–^{15}N Spin–Spin Coupling Constants of Oxidized and Reduced Riboflavin Tetrabutyrate.[a]

carbon	assignment	$J(^{13}C$–$^{15}N)$ (Hz)[b] oxidized	reduced
4	^1J[C(4)–N(3)]	12.2	13.1
	^2J[C(4)–N(5)]	8.5	0
2	^1J[C(2)–N(3)]	11.4[c]	19.5
	^1J[C(2)–N(1)]	7.2[c]	19.5
10a	^1J[C(10a)–N(1)]	7.9	17.6
7	^3J[C(7)–N(5)]	4.0	0
5a	^1J[C(5a)–N(5)]	1.2	11.0
6	^2J[C(6)–N(5)]	8.9	0
4a		*d*	*d*
8,9,9a		0	0

[a] Obtained from the ^{13}C NMR spectra of [1,3-^{15}N]RBUT and [1,3,5-^{15}N]RBUT. [b] Digital resolution is 0.3 Hz. Signals of the reduced RBUT are broad and their actual resolution is about 1 Hz. [c] Interchangeable with each other. [d] Not obtainable owing to the weak and complex peak.

been recognized as the best candidate for the electrophilic addition of oxygen to the reduced flavin model and flavoenzyme (Ghisla et al., 1977; Kemal et al., 1977). The reactivity of C(4a) was supported by the calculation of frontier electron distribution (Song et al., 1976). Accordingly, the higher position of C(4a) may provide an experimental basis for the possibility of O_2 addition to C(4a).

^{13}C–^{15}N Spin–Spin Coupling Constants. The measurements of ^{13}C NMR spectra of [1,3-^{15}N]RBUT and [1,3,5-^{15}N]RBUT made it possible to observe ^{13}C–^{15}N spin–spin coupling constants. They have not been obtained previously because of the low sensitivity of ^{15}N and ^{13}C nuclei and their low natural abundance. The values of $J(^{13}C$–$^{15}N)$ were obtained by the first-order analysis and are summarized in Table VI. The coupling constants of C(4a) with ^{15}N nuclei were not obtained, owing to the weak and complex peak.

It is apparent that the values of ^2J[C(4)–N(5)] and ^2J[C(6)–N(5)] are larger than normal two-bond couplings and are dramatically reduced upon reduction. Large couplings have also been observed for a few other molecules like quinoline and explained by the orientation of nitrogen lone-pair electrons (Wasylishen, 1977). Thus, the large values for ^2J[C(4)–N(5)] and ^2J[C(6)–N(5)] can be interpreted to mean that C(4) and C(6) are under the influence of the nitrogen lone-pair lying cis to the carbons. The values of one-bond ^{13}C–^{15}N coupling constants, ^1J[C(2)–N(3)], ^1J[C(2)–N(1)], ^1J[C(10a)–N(1)], and ^1J[C(5a)–N(5)] increase upon reduction. These results are well explained, except for ^1J[C(2)–N(3)], by the effect that the protonation at a nitrogen atom causes a marked increase in the coupling to the neighboring carbon (Pregosin et al., 1972). The ^{13}C–^{15}N coupling constants of the quinoxaline part in RBUT correspond well to those of quinoline (Pregosin et al., 1972).

In the present experiments it has become clear that the ^{15}N and ^{13}C chemical shifts, $J(^{15}N$–H) and $J(^{13}C$–$^{15}N)$, of flavin are drastically changed upon reduction and, therefore, they may be used as oxidation state markers. The chemical shift of N(5) and its coupling constant with the attached proton suggest that the nitrogen has some sp^3 character and thus the isoalloxazine ring flexes along its N(10)–N(5) axis to form a butterfly-shaped molecule. It is noteworthy that C(4a) is most shielded among the isoalloxazine ring carbons in connection with the capability of C(4a) to react with an electrophile, e.g., O_2. Comparisons of ^{13}C and ^{15}N resonances in free flavin with those of enzyme-bound flavin or with those of flavin in a ter-

nary complex, apoenzyme–coenzyme–substrate (substrate analogue or inhibitor), could afford valuable information on the structure and function of flavoproteins.

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