de Bruin, S. H., Lambert, L. H. M., and van Os, G. A. J. (1973), *Biochem. Biophys. Res. Commun. 55,* 193.
de Bruin, S. H., Rollema, H. S., Janssen, L. H. M., and van Os, G. A. J. (1974), *Biochem. Biophys. Res. Commun. 58,* 204.
Edelstein, S. J. (1974), *Biochemistry 13,* 4998.
Gray, R. D., and Gibson, Q. H. (1971), *J. Biol. Chem. 246,* 7168.
Herzfeld, J., and Stanley, H. E. (1974), *J. Mol. Biol. 82,* 231.
Imai, K. (1973), *Biochemistry 12,* 738.
Imai, K., and Tyuma, I. (1973), *Biochim. Biophys. Acta 293,* 290.
Johnson, M. E., and Ho, C. (1974), *Biochemistry 13,* 3653.
Kilmartin, J. V. (1974), *FEBS Lett. 38,* 147.
Kilmartin, J. V., Fogg, J., Luzzana, M., and Rossi-Bernardi, L. (1973), *J. Biol. Chem. 248,* 7039.
Koshland, D. E., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5,* 2580.
Maeda, T., Imai, K., and Tyuma, I. (1972), *Biochemistry 11,* 3685.
Monod, J., Wyman, J., and Changeux, J. P. (1965), *J. Mol. Biol. 12,* 881.
Ogata, R. T., and McConnell, H. M. (1972a), *Proc. Natl. Acad. Sci. U.S.A. 69,* 335.
Ogata, R. T., and McConnell, H. M. (1972b), *Biochemistry 11,* 4792.
Perrella, M., Kilmartin, J. V., Fogg, J., and Rossi-Bernardi, L. (1975), *Nature (London) 256,* 759.
Perutz, M. F. (1970), *Nature (London), 228,* 726.
Perutz, M. F. (1972), *Nature (London), 237,* 495.
Riggs, A. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2062.
Szabo, A., and Karplus, M. (1972), *J. Mol. Biol. 72,* 163.
Szabo, A., and Karplus, M. (1975), *Biochemistry 14,* 931.
Tyuma, I., Imai, K., and Shimizu, K. (1973), *Biochemistry 12,* 1491.
Tyuma, I., and Shimizu, K. (1970), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 29,* 1112.
Wyman, J. (1964), *Adv. Protein Chem. 19,* 223.
Wyman, J. (1967), *J. Am. Chem. Soc. 89,* 2202.

^{15}N Nuclear Magnetic Resonance of Flavins†

Kunio Yagi,* Nobuko Ohishi, Akiko Takai, Keiichi Kawano, and Yoshimasa Kyogoku

ABSTRACT: Ninety-nine percent ^{15}N-enriched flavins were synthesized and their proton decoupled ^{15}N resonances were observed. The enriched compounds were [1,3-^{15}N]riboflavin, [1,3,5-^{15}N]riboflavin, [1,3-^{15}N]riboflavin 5′-phosphate, [1,3,5-^{15}N]riboflavin 5′-phosphate, [1,3,5-^{15}N]flavin adenine dinucleotide, [1,3,5-^{15}N]lumiflavin, and [1,3,5-^{15}N]lumichrome. By comparison of their spectra and from the nuclear Overhauser effect data each ^{15}N resonance peak could be assigned to each ^{15}N nucleus. The order of the chemical shifts well corresponds to that of the calculated π-electron densities. The N-3 nucleus gives the most intense inverted peak and the N-5 nucleus a small noninverted peak. By changing pH from neutral to alkaline, the chemical shift and the intensity of signal were mostly affected in the N-3 resonance of riboflavin 5′-phosphate. The N-5 signal of flavin adenine dinucleotide showed a fairly large downfield shift with the increase of temperature. These observations can be well interpreted by the chemical structure and the proposed conformation of riboflavin 5′-phosphate and flavin adenine dinucleotide.

Physicochemical properties of flavins such as their electronic spectra on their molecular basis have been amply discussed, but little information is available on the attitude of each atom or group of the isoalloxazine ring of flavins. To reveal the fine mechanism involved in the binding between flavin coenzymes and apoproteins and the oxidoreduction catalyzed by flavoproteins, such information is essential. For this purpose, nuclear magnetic resonance (NMR)[1] studies are quite promising. Accordingly, the structure of flavin coenzymes and their interaction with apoproteins have been studied by the use of proton magnetic resonance (Sarma et al., 1968; Kotowycz et al., 1969; Kainosho and Kyogoku, 1972; Crespi et al., 1972; Raszka and Kaplan, 1974). However, the parts which directly participate in redox reaction and the sites which interact with apoproteins are known to be mainly localized at the 1–5 positions in the isoalloxazine rings. Proton magnetic resonance cannot give us direct information on the positions, since protons are located only at the 6–9 positions. For this reason, carbon and nitrogen magnetic resonances seem to be more useful. ^{13}C resonance of FMN in natural abundance has already been reported (Breitmaier and Voelter, 1972), but some of the assignments were corrected by the ^{13}C spectra of ^{13}C-enriched riboflavin (Yagi et al., 1976). The interaction of riboflavin with egg white flavoprotein was studied using [^{13}C]riboflavin (Yagi et al., 1976). The present paper deals with the preparation of ^{15}N-enriched flavins and their ^{15}N magnetic resonance spectra. This is the first systematic report on the ^{15}N resonance spectra of flavins.

Materials and Methods

To obtain ^{15}N-enriched riboflavin, [^{15}N]urea (99.36 atom %, Prochem., England) and sodium [^{15}N]nitrite (99 atom %, Prochem., England) were used as starting materials. As shown in Figure 1, [1,3-^{15}N]riboflavin and [1,3,5-^{15}N]riboflavin were synthesized through barbituric acid and *N*-ribityl-3,4-dimethyl-6-phenylazoaniline. By phosphorylating ^{15}N-enriched

† From Institute of Biochemistry, Faculty of Medicine, University of Nagoya, Nagoya 466, Japan, and Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan. *Received February 26, 1976.*

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

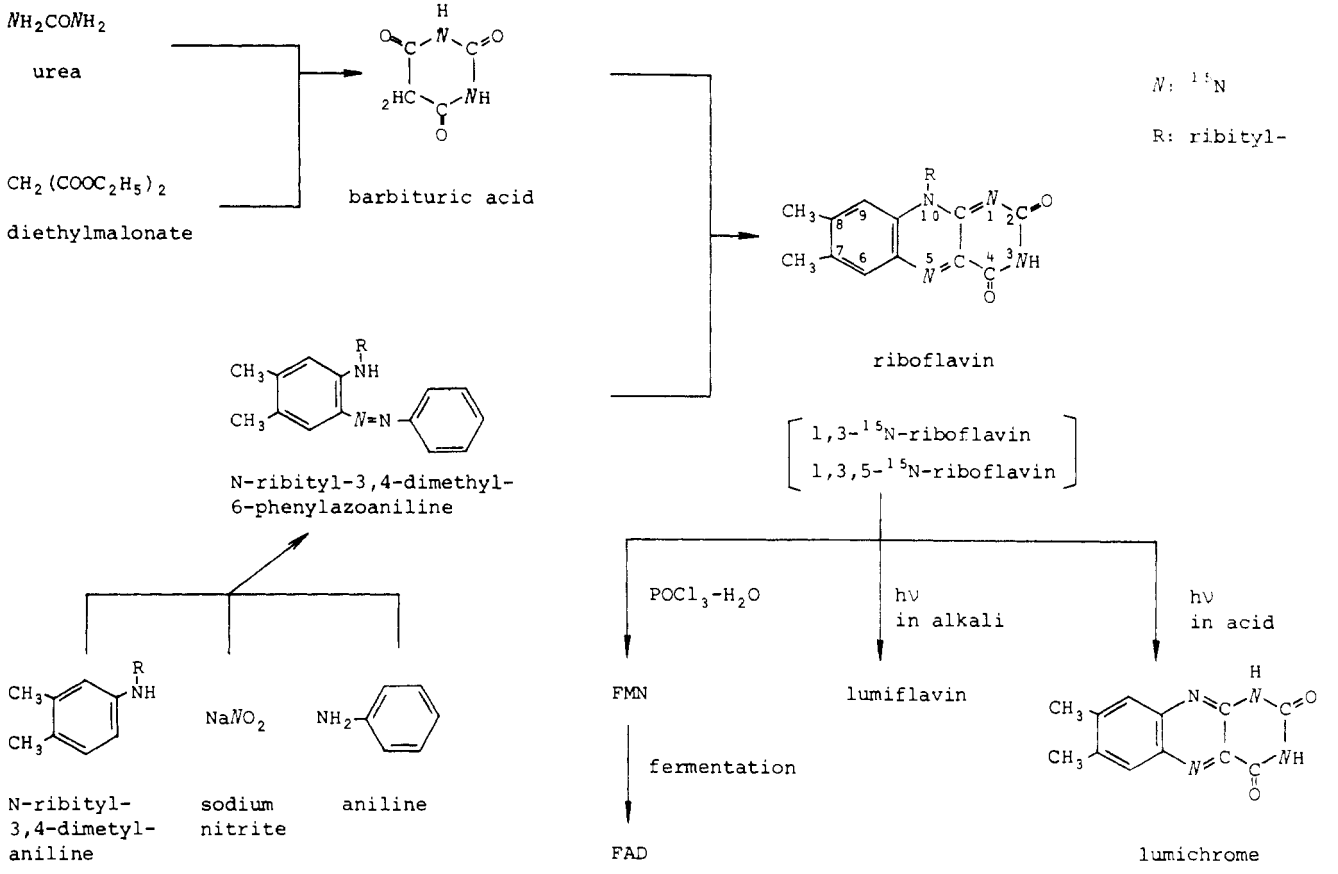

FIGURE 1: Diagram of the synthesis of ^{15}N-enriched flavins.

riboflavin with $POCl_3$–H_2O, [1,3-^{15}N]FMN and [1,3,5-^{15}N]FMN were obtained. [1,3,5-^{15}N]FAD was obtained from the enriched FMN through fermentation procedure. [1,3,5-^{15}N]Lumiflavin and lumichrome were prepared by photolysis in alkali and acid media, respectively. These flavins are chromatographically pure, except for lumiflavin which contained a small amount of lumichrome.

^{15}N spectra were recorded on a JEOL PFT-100 pulse Fourier transform NMR spectrometer operating at 10.14 MHz, with associated proton-decoupling equipment. Pulse-free induction decay curves repeated every 2 s (45° pulse, 18 μs) were Fourier transformed after 100 times accumulations in the time domain, and the accumulations in the frequency domain were continued until a satisfactory spectrum was obtained. Spectra ranged over 8192 points corresponded to 5000 Hz and the resolution was 1.22 Hz, i.e., 0.12 ppm for ^{15}N.

Samples were dissolved in H_2O and spun in 10-mm diameter tubes. Field was locked to the D resonance of D_2O in a 2-mm coaxial tube. Chemical shifts were read relative to the $^{15}NO_3^-$ resonance of 95% enriched $ND_4{}^{15}NO_3$ dissolved in 1 N DCl in the coaxial tube.

TABLE I: ^{15}N Resonances of ^{15}N-Enriched Flavins and Barbituric Acid.

	Chemical Shifts (ppm)[a]		
	5-N	1-N	3-N
[1,3-^{15}N]Riboflavin (in 50% acetic acid)		185.1	216.1_5
[1,3-^{15}N]FMN (pH 7.6)		184.6_5	215.2_5
[1,3,5-^{15}N]Riboflavin (in Me_2SO-d_6)	35.1_5	177.9_5	214.3
[1,3,5-^{15}N]FMN (pH 5.7)	42.1_5	184.4_5	215.8_5
[1,3,5-^{15}N]FAD (pH 5.8)	41.5_5	184.3	215.7_5
[1,3,5-^{15}N]Lumichrome (in Me_2SO-d_6)	39.1	246.5_5	217.9
[1,3,5-^{15}N]Lumiflavin (in Me_2SO-d_6)	—	—	214.3
[1,3-^{15}N]Barbituric acid (pH 2.0)			223.2

[a] Measured from external $ND_4{}^{15}NO_3$.

Results and Discussion

Assignments of ^{15}N Resonances. All of the proton decoupled spectra of 1,3,5-^{15}N-enriched flavins gave two inverted peaks and a noninverted peak (Figure 2). By comparison of these spectra with those of [1,3-^{15}N]riboflavin and [1,3-^{15}N]FMN, a small noninverted peak around 40 ppm was assigned to N-5 (Table I). For the assignment of the remaining two peaks, it is noted that the chemical shift of the N-3 signal should be close to that of barbituric acid because of the resemblance of their chemical structures. Bigger negative NOE enhancement for the signals of the nucleus which is bonded to a proton can be also expected. From these reasons, the signal in higher field is assignable to N-3. Thus, the chemical shifts of ^{15}N resonance are in the order well corresponding to that of the calculated π-electron densities of the nitrogen atoms in the isoalloxazine (Song, 1969). The N-3 nucleus is the most and the N-5 is the least shielded among the three nuclei.

For the signal of the N-1 nucleus, an ordinally noninverted peak is expected, since there is no proton on it. However, it gives

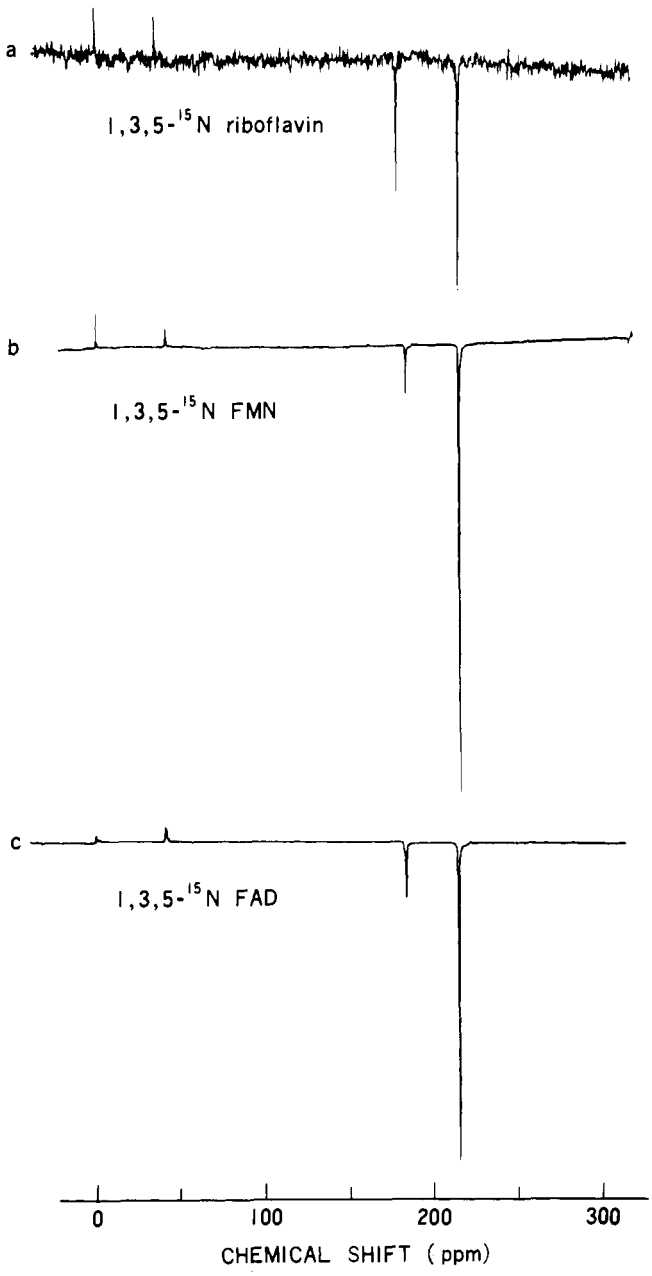

FIGURE 2: Proton decoupled ^{15}N NMR spectra of (a) [1,3,5-^{15}N]riboflavin (15 mg of sample was dissolved in 1.5 ml of Me_2SO-d_6, 32 500 times accumulated at 50 °C), (b) [1,3,5-^{15}N]FMN (20 mg of sample in 1.6 ml of H_2O, pH 4.0, 43 900 times accumulated at room temperature), and (c) [1,3,5-^{15}N]FAD (100 mg of sample in 1.5 ml of H_2O, pH 5.8, 33 600 times accumulated at room temperature).

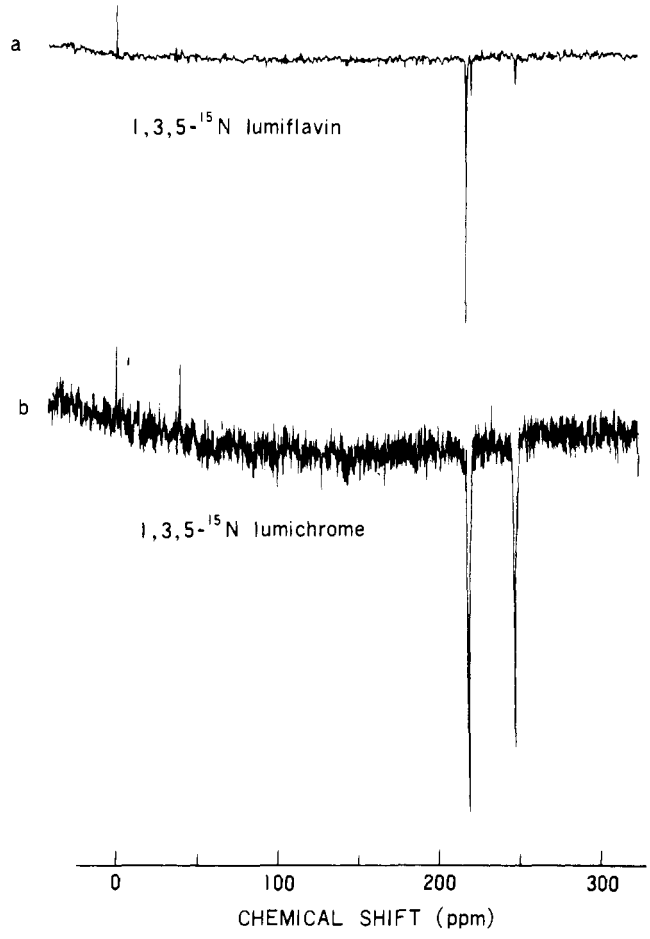

FIGURE 3: Proton decoupled ^{15}N NMR spectra of (a) [1,3,5-^{15}N]-lumiflavin (6.5 mg of sample in 1.5 ml of Me_2SO-d_6, 43 100 times accumulated at room temperature) and (b) [1,3,5-^{15}N]lumichrome (20 mg of sample in 1.5 ml of Me_2SO-d_6, 36 600 times accumulated at 60 °C).

an inverted peak. It may be due to the intramolecular NOE by the protons in the ribityl group (Lichter and Roberts, 1971). The evidence that the resonance of N-1 can hardly be seen around 185 ppm of lumiflavin supports the idea (Figure 3a). It may be considered that the signal becomes weak by the lack of the effect from the ribityl protons. Small inverted peaks in the spectrum are due to lumichrome contaminated as impurities.

If the small negative NOE of the N-1 signal arises from the solvation of water molecules, the intensity of the signal should change when the solvent is replaced by another one. However riboflavin and FMN in Me_2SO-d_6 still give an inverted signal.

In the spectrum of lumichrome, two strong inverted signals are seen besides a weak signal of N-5 at 40 ppm (Figure 3b). Thus, it is evident that protons are bonded to both of the N-1 and N-3 atoms, as shown in Figure 1. The peak at 246.5_5 ppm was tentatively assigned to the N-1 nucleus, since it can be expected that the removal of the ribityl group from N-10 and the protonation at N-1 result in a large change of the electron density around N-1, and induce a large shift for the N-1 signal.

Splits for the N-1 and N-3 signals due to the geminal ^{15}N–^{15}N spin–spin coupling and splitting by ^{15}N-5 for the proton magnetic resonance of ^{1}H-6 have been expected. As such splitting could not be detected in both cases, it has become clear that geminal ^{15}N–^{15}N coupling in the heterocyclic ring is less than 1 Hz. The vicinal ^{1}H–^{15}N coupling at the cis positions is also known to be quite small (Axenrod, 1973).

pH and Temperature Dependence of Chemical Shifts. pH dependency of ^{15}N chemical shifts has been measured for FMN. Sometimes in the spectra of alkaline solutions several lines were observed. Since FMN is known to be unstable in alkaline solutions, some of the lines are considered to be ascribed to the decomposed products of FMN. After the measurement of the spectra, however, FMN was found to be still a main component as checked by chromatography.

In Figure 4 the positions of main peaks are plotted. No shift is seen below pH 8, while above pH 9 there is a trend of a large downfield shift in the plot for the N-3 signals. Small up- and downfield shifts are also seen for N-5 and N-1, respectively. These shifts must be due to the deprotonation at N-3. The pK

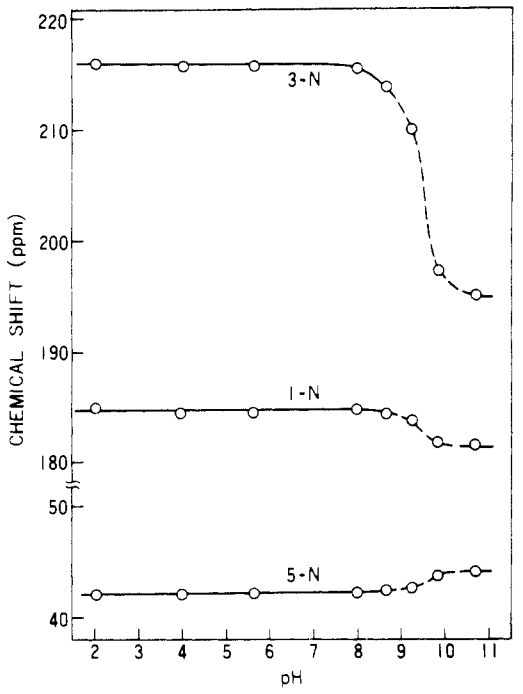

FIGURE 4: pH dependency of the ^{15}N chemical shifts of [1,3,5-^{15}N]-FMN. Twenty milligrams of sample was dissolved in 1.5 ml of H_2O. Each point was obtained after 25 000–35 000 times accumulations.

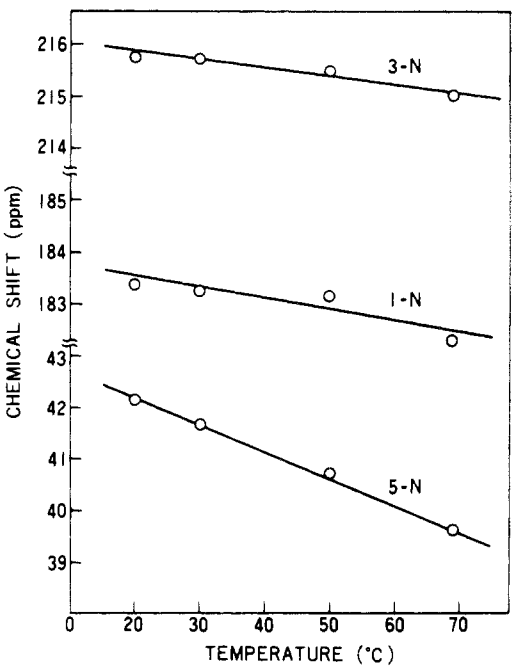

FIGURE 5: Temperature dependency of the ^{15}N chemical shifts of [1,3,5-^{15}N]FAD. Each point was obtained after 10 000–15 000 times accumulations.

value estimated from the curves is consistent with that reported previously (Kavanagh and Goodwin, 1949).

The temperature dependence of the ^{15}N chemical shifts of FAD was also measured (Figure 5). Sometimes satellite peaks accompanied by main signals were observed in the spectra of FAD at higher temperatures. It is suspected that they come from decomposed products after long time accumulations at high temperatures.

All of the three main signals shift to downfield as the temperature is raised. The shift must be ascribed to the break of intra- and intermolecular stacking interaction. The extent of the downfield shift is the largest for the signal of N-5. The result is consistent with the expectation from the previously proposed models for intermolecular interaction of the isoalloxazine ring and also from the most probable conformation of FAD (Kainosho and Kyogoku, 1972). The N-5 is the vertically nearest to the center of associated isoalloxazine and adenine rings.

In the present experiment it has become clear that the chemical shifts of ^{15}N resonances reflect the electron densities of the corresponding nuclei and are also sensitive to the way of interaction. Besides it, the NOE of the signal reveals the site of protonation, as demonstrated with histidine by Kawano and Kyogoku (1975). Thus, the ^{15}N magnetic resonances of flavins are considered to be useful to elucidate the change in charge distribution accompanied by redox reactions and also the mechanism of their interactions to apoproteins. We are now in the process of investigation in this direction.

References

Axenrod, T. (1973), in Nitrogen NMR, Witanowski, M., and Webb, G. A., Ed., London, Plenum Press, p 261.

Breitmaier, E., and Voelter, W. (1972), *Eur. J. Biochem. 31,* 234.

Crespi, H. L., Norris, J. R., and Katz, J. J. (1972), *Nature (London), New Biol. 236,* 178.

Kainosho, M., and Kyogoku, Y. (1972), *Biochemistry 11,* 741.

Kavanagh, F., and Goodwin, R. H. (1949), *Arch. Biochem. 20,* 315.

Kawano, K., and Kyogoku, Y. (1975), *Chem. Lett. (Tokyo),* 1305.

Kotowycz, G., Teng, N., Klein, M. P., and Calvin, M. (1969), *J. Biol. Chem. 244,* 5656.

Lichter, R. L., and Roberts, J. D. (1971), *J. Am. Chem. Soc. 93,* 3200.

Raszka, M., and Kaplan, N. O. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4546.

Sarma, R. H., Dannies, P., and Kaplan, N. O. (1968), *Biochemistry 7,* 4359.

Song, P. S. (1969), *Ann. N.Y. Acad. Sci. 158,* 410.

Yagi, K., Ohishi, N., Takai, A., Kawano, K., and Kyogoku, Y. (1976), in Flavins and Flavoproteins, Singer, T. P., Ed., Amsterdam, Elsevier, p 775.