Preparation, Stability, and Compositions of the Crystals of Reduced Riboflavins

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Green crystals were obtained after a reduction of riboflavin (RF) with $Na_2S_2O_4$. These crystals were gradually oxidized to RF when stored in air, even at −10°C. The emission spectra and X-ray powder diffraction analyses of the green crystals clearly showed different patterns from those of RF. The ESR spectra and number of the reduced species determined by the titration with $KMnO_4$ supported the formation of an RF-flavosemiquinone radical (1:1) complex. Reduced riboflavin tetrabutyrate prepared according to the same procedure was too unstable for dioxygen to obtain spectroscopic data.

The biochemical activities of riboflavin (RF), vitamin B2, have long attracted attention, for example, as a precursor of flavin adenine dinucleotide, which has an important role as a coenzyme for the flavoproteins.[1] Although the reduced species of RF were unstable, the presence of flavosemiquinone radicals (RFH) was confirmed with electron spin resonance (ESR) and UV spectroscopy.[2] Further, the in situ reduction of RF with $Na_2S_2O_4$ or $SnCl_2$ in an aqueous solution has resulted in some spectra of mixtures of reduced riboflavins involving RFH, which may be produced according to the equilibrium scheme shown in Chart 1.[2] Unstable RFH, once prepared, was easily oxidized again to RF by exposure to air.[3]

The authors first separated a mixture of RF and RFH as fine green crystals out of a solution of RF reduced with $Na_2S_2O_4$. The stability of the crystals in air was studied quantitatively by reflection spectroscopy. For the separated crystals infrared (IR) and ESR spectra, as well as X-ray powder diffraction patterns, were measured, providing the composition of the green crystals. The same procedures were also applied to riboflavin tetrabutyrate (RTB)[4] to give reduced riboflavin tetrabutyrate (RTBH).

Therefore, the preparation, stability, and composition of RFH and RTBH in the crystals could be described from both chemical and spectroscopic standpoints.

Experimental

Preparation of RFH. RF (5.0 g) was dissolved in boiling water (3 l). Insoluble powdery materials were removed by filtration. To the solution, cooled at 60°C, was added $Na_2S_2O_4$ (0.9 g). Within a few minutes a separated dark solid changed to green. The reaction solution was kept in an ice bath for 3 h. Fine crystals which precipitated were separated and washed twice with ice-cold water. Finally, the crystals were washed with ethanol and dried at 60°C for 24 h under reduced pressure (130 Pa). Dark-green fine needles (ca. 0.1 mm long, yield. 2.89 g) were obtained.

Preparation of RTB. Crude RTB prepared from n-butyric acid, pyridine, $POCl_3$, and RF by heating at 70—75°C for 5 h was recrystallized from 85% MeOH.

Preparation of RTBH. RTB (5.0 g) was dissolved in hot MeOH-H2O (45:55) (2.1 l). To the solution, kept at 60°C, was added $Na_2S_2O_4$ (0.5 g). After the solution was cooled in an ice bath for 3 h, precipitated green crystals were collected by filtration, washed twice with water, and then dried at 60°C for 12 h under reduced pressure. Yield 4.3 g. Mp 148.2°C.

Oxidation of RFH or RTBH with $KMnO_4$. Fine crystals (0.3 g) prepared by the reduction of RF were dispersed in 1% H_2SO_4 (100 ml). The solution was titrated with 2×10^{-3} mol dm^{-3} $KMnO_4$ under magnetic stirring. The end point of titration was determined potentiometrically with an $HgCl_2$-Pt electrode as a standard half cell.

Freshly prepared RTBH (500 mg) was dissolved in acetic acid (50 ml). To the solution was added 10% H_2SO_4 (10 ml) and water (40 ml). Then, RTBH was titrated with 2×10^{-3}
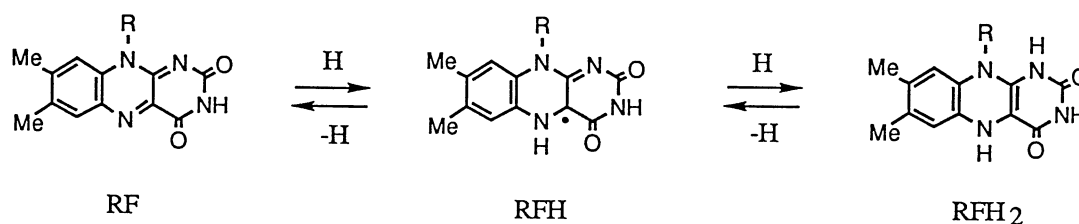
Chart 1.

mol dm^{-3} KMnO$_4$.

Emission Spectra Measurement. Emission spectra were recorded on a Shimadzu RF-502 type spectrophotometer. Powdery samples were packed in a holder and measured under the following conditions: Excitation slit, 15 nm; emission slit 2.2 nm; excitation wavelength, 370 nm.

X-Ray Powder. Diffraction Measurement. An X-ray powder diffraction analysis was carried out using an X-ray diffractometer (Geigerflex D-8C, Rigaku Co.). The conditions were: Target, Cu; filter, Ni; voltage, 30 kV; current, 20 mA; time constant, 1 s; sweep, 2° min^{-1}; divergency slit, 1°; receiving slit, 0.4 mm; detector, Geiger Müller counter.

ESR Measurement. ESR spectra were recorded on a JES-FE3X type spectrometer (X band, 100 kHz modulation). The conditions were: Power, 20 mW; modulation amplitude, 0.63 mT; scan rate, 3.125 mT min^{-1}; time constant, 1 s; gain, 2×1000.

Reflection Spectra Measurement. Reflection spectra were recorded on a Hitachi 228 A type spectrometer equipped with a spherically integrated reflection apparatus (150 mm i.d.). Fine crystals were packed in a dark cell and covered with a glass plate.

IR Spectra Measurement. IR spectra were recorded on a Hitachi 260-50 type spectrometer after preparing KBr tablets by mixing powdery samples (2 mg) with KBr powder (200 mg) and compressing the mixture.

Results and Discussion

The Stability of Reduced RF and Reduced RTB. Fine dark-green needles obtained by the reduction of RF with Na$_2$S$_2$O$_4$ were gradually oxidized by exposure to the air.

The color of the crystals changed from green to yellow according to the progress of oxidation. The time courses in the oxidation of the crystals of reduced RF and RTB in air were traced with the reflection spectra.

As shown in Fig. 1, a crystal of reduced RF stored at room temperature was rapidly oxidized into RF during 10 d, followed by slow oxidation. Reduced RF stored at −10 °C also oxidized into RF, although the rate was slower than that at room temperature. The oxidation rate of reduced RTB was twice as fast as that of reduced RF.

Since an RTBH molecule possesses considerably large substituent groups, the average distance between two isoalloxazine rings in a crystal of RTBH may be greater than that in a crystal of RFH. Then, the overlap of the orbitals of the unpaired electron with those of π-electrons on other isoalloxazine rings for RTBH crystal may be smaller than that for the RFH crystal. Therefore, the instability of RTBH crystal against oxidation may be ascribed to delocalization of unpaired electrons over aromatic rings in the crystal.

The nuclear magnetic resonance and high-performance liquid chromatography analysis of a diluted solution of the fine needles failed, since fast oxidation took place to give RF exclusively. Also, an elemental analysis could not be carried out due to an increase in weight during the weighing operation.

IR Spectra. The IR spectra of the fine needles of reduced RF in KBr tablets were obtained and compared with those of RF. A strong, wide spectral band of RFH observed at 3400 cm^{-1} could be assigned to newly formed NH groups. The spectral bands of amide groups of RF at 1736 and 1650 cm^{-1} were spread and duplicated to make one broad band at 1635 cm^{-1}. On the other hand, the IR spectrum of reduced RTB

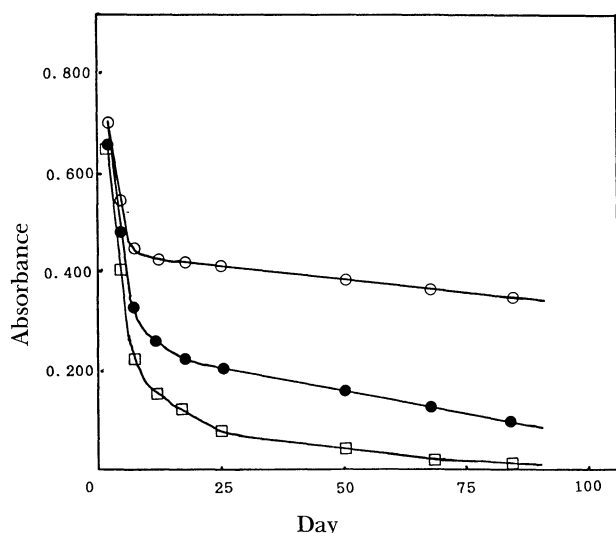

Fig. 1. Decrease in the intensity of reflection spectra of the crystals of reduced RF and reduced RTB in the air.
O: Reduced RF stored at −10 °C,
●: Reduced RF stored at room temperatures,
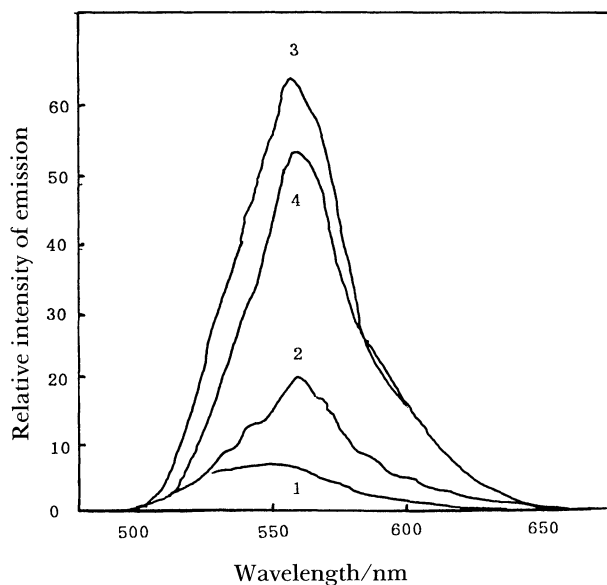□: Reduced RTB stored at room temperatures.

Wavelength/nm

Fig. 2. Emission spectra of the green crystals of reduced RF when oxidized with air or a 2×10^{-3} mol dm^{-3} KMnO$_4$ solution.
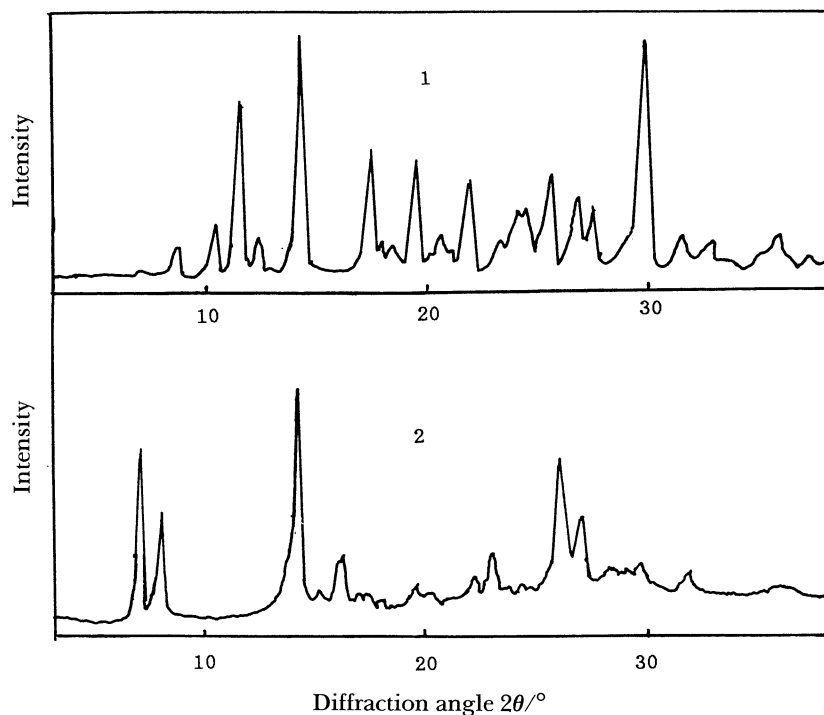1: Reduced RF, 2: Oxidized with air, 3: Oxidized with KMnO$_4$, 4: RF.

Fig. 3. X-Ray diffraction patterns of the green crystals of reduced RF
and orange crystals of RF.
1: RF, 2: Reduced RF.

crystals was the same as that of RTB, suggesting a fast oxidation of the crystal during the preparation of KBr tablets.

Emission Spectra. The emission spectra of crystals of reduced RF and RTB are shown in Fig. 2. The spectral intensity for reduced RF just after preparation is very weak, whereas that for RF is strong. When the crystals of reduced RF dispersed in water came into contact with oxygen by bubbling through air at room temperature, the spectral intensity gradually approached that of RF. Oxidation with a $KMnO_4$ solution (2×10^{-3} mol cm^{-3}) increased the peak intensity for the reduced RF to that of RF. The same tendency was also observed for reduced RTB, except that the oxidation rate with air was faster than reduced RF.

X-Ray Diffraction of Powders. An X-ray powder analysis was carried out for reduced RF. As shown in Fig. 3, the diffraction pattern clearly differed from that of RF. No peaks from RF were observed. The X-ray diffractogram of reduced RF was rather simple and the number of peaks was smaller than those of RF. Sharp peaks were spread from $2\theta=10°$ to $50°$, suggesting the presence of well crystallized solids. The relative intensities of peaks as low as at $7.0°$ and $8.0°$ were very strong in reduced RF. These facts suggest that RF and RFH produced new structures. The crystals of reduced RTB were unstable in air. The patterns of the X-ray spectra taken after 3 d were almost the same as those of yellow RTB crystals.
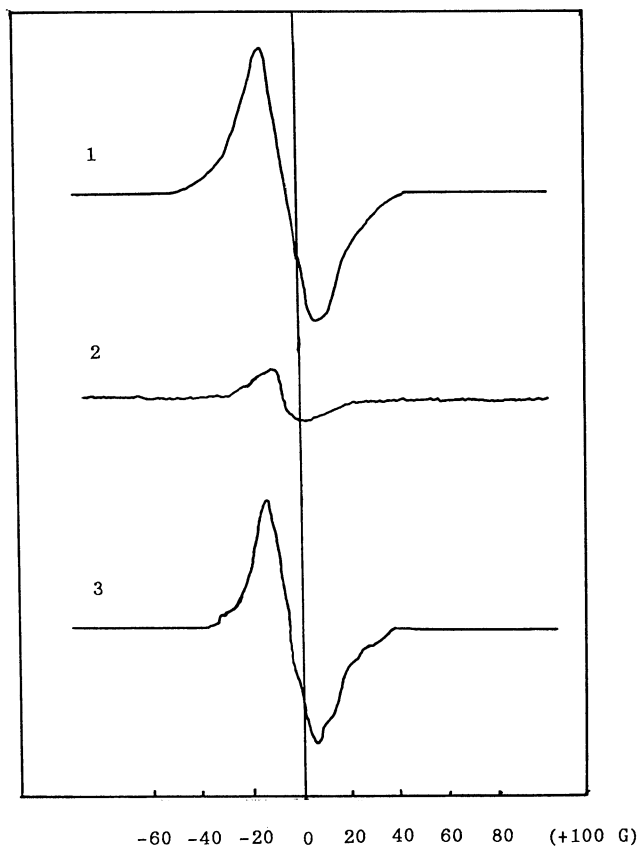

Fig. 4. ESR spectra of the crystals of reduced RF and reduced RTB in the solid state.
1: Reduced RF, 2: RF, 3: Reduced RTB.

ESR Spectra. A solid RF showed weak ESR signals. On the other hand, orange RTB crystals gave no ESR signals.[5] In contrast, the crystals of RFH and RTBH gave strong signals at $g=2.005$ (Fig. 4). The half-width for reduced RF or RTB was 2.278 or 1.964 mT, respectively. Well-resolved hyperfine structures were observed for the latter. The intensities of signals for reduced RF was 5.8-times stronger than that of reduced RTB. These results suggest that the reduced RF crystals contained flavosemiquinone radicals.

Composition of the Crystals of Reduced Riboflavins. The quantitative oxidation of freshly prepared crystals of reduced RF with $KMnO_4$ showed that the molar ratio (RF:RFH) was 47.1:52.9. The purity of RF after oxidation was 99.7%. On the other hand, the molar ratio of RTB:RTBH was 86.9:13.1. To explain these results, two possible cases were considered. In the first case, a mixture or a complex of RF-RFH (1:1) formed a quasi-stable species. The other case involved the formation of a mixture or a complex of RF-RFH$_2$ (3:1). X-Ray powder patterns of RFH contained no peaks from orange RF crystals. The intensity of the emission spectra of the crystals of reduced RF was approximately nil, while those of RF

crystals were strong when excited at 370 nm. Furthermore, the ESR spectra showed the presence of radical species in the crystals of reduced RF. When RF was hydrogenated, the color of crystals changed from orange to dark green, and then faded to pale green.

All of these results suggest that unstable RFH$_2$ formed during the first stage of reduction was quickly oxidized into an RF-RFH (1:1) complex followed by successive oxidation to give RF in an oxidative atmosphere.

References

1) H. Beinert, *J. Am. Chem. Soc.*, **78**, 5323 (1956).

2) F. Muller, *Free Radicals Biol. Med.*, **3**, 215 (1987).

3) "Explanation to Japanese Pharmacopoeia XI," ed by Nihon Kouteisho Kyoukai, Hirokawa, Tokyo (1986), p. C-1659.

4) "Nippon Yakkyokuhogai Iyakuhin Seibun Kikaku," ed by Koseisho Yakumukyoku, Yakugyo Jiho, Tokyo (1986), p. 1122.

5) One of the authors prepared three types of RTB crysals, A (orange), B (brown), and C (yellow) types, respectively. Among those, RTB B, recrystallized from *i*-PrOH, gave a strong ESR signal ($g=2.006$); M. Ebitani, *Yakugaku Zasshi*, **107**, 338 (1987).