Communication

Effects of Riboflavin Deficiency on the Synthesis of
Ester Forms of Riboflavin in the Rat Lens

Shigeru ONO, Hiroko HIRANO, Susumu HAMAJIMA,
and Saburo HORIUCHI¹

Department of Biochemistry, School of Medicine,
Iwate Medical University, Morioka 020, Japan

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A few reports were found on the riboflavin (B_2) in the eye tissues in various species (1-3).

Flavin compound in the eye of *Rana nigromaculata* concentrates in the choroid, iris, ciliary body and is not present in other parts. The amount and distribution of flavin compound in the eye varies considerably in different species of frogs (4).

The presence of B_2 in the lens of haddock (*Gadus aeglefinus*), rabbit and other species could not be demonstrated (5).

The synthesis of ester forms of B_2 in the lenses of various species has not yet been determined, however, it should be noted that flavin-containing enzymes such as glutathione reductase exist in the lens (6, 7). Furthermore, we have reported that ester forms of B_2 are synthesized from free riboflavin in the rat lens *in vitro* as well as in other tissues, and that synthesis of ester forms of B_2 is carried out fairly actively (8).

In the present study, the changes in formation of ester forms of B_2 in the lenses of B_2 -deficient rats were investigated.

Wistar strain male rats weighing about 50-70 g were maintained on a B_2 -deficient diet as reported previously (9). Experiments were carried out at 4 and 8 weeks, respectively, after being fed a B_2 -deficient diet (Oriental yeast Co., Japan). NADPH-cytochrome *c* reductase activity (10), glutathione reductase activity (11) and B_2 content (12) in the liver, and glutathione reductase activity in the lens (7) were determined as a marker of B_2 -deficient status.

Lenses from excised eyes were carefully removed by the posterior approach and homogenized in 0.1 M phosphate buffer (pH 7.5) containing ATP 1 mM, $MgCl_2$ 1 mM and $ZnSO_4$ 0.1 mM to give a final concentration of 60 mg wet weight per ml. Two ml of this homogenate was incubated with 0.02 ml of 1.5 $\mu Ci/ml$ D-[2- ^{14}C]-riboflavin solution (specific activity 31.0 mCi/mmol) for 60 min at 37°C after which it was

¹ 小野 繁, 平野浩子, 浜島 進, 堀内三郎

cooled to 4°C and centrifuged at $10,000 \times g$ for 60 min to remove insoluble protein and particulate matter. Unless otherwise stated, all experimental procedures were carried out in a laboratory illuminated with dimmed or red light. Quantitative determination of radioactivity of flavin fractions was carried out using a Sephadex G-15 column (1.5×20 cm). An aliquot of supernatant of incubation medium was eluted with 0.025 M phosphate buffer (pH 7.0), fractions of 2 ml were collected at room temperature. One ml aliquots of each tube were added to 10 ml of dioxane containing 7 g of 2,5-diphenyloxazole, 300 mg of 1,4-bis[2-(4-methyl-5-phenyl-

Fig. 1.

oxazoly]]benzene and 100 g naphthalene per liter and the radioactivities of the samples were counted in a liquid scintillation counter (Aloka Liquid Scintillation System, LSC-900). The radioactivity was eluted in three peaks when the supernatant of incubation medium was chromatographed using a Sephadex G-15. The first, minor peak containing a lesser amount of radioactivity also contained lens proteins as measured by UV absorption at 280 nm showing the presence of riboflavin binding protein in the lens as reported previously (8). The second, large peak of radioactivity represents the fraction of ester forms of B₂ synthesized from ¹⁴C-riboflavin and the third, larger peak was ¹⁴C-riboflavin used as a substrate (Fig. 1).

Identification of the two major peaks was carried out as follows. Fractions of the second and third peaks were pooled, respectively, and flavin compounds were concentrated according to the method of Crammer (13), then separation of flavin compounds was carried out using a DEAE Sephadex A-25 ion exchange column according to the method of Fazekas (14), and paper partition chromatography. When labelled riboflavin alone was filtered through a Sephadex G-15 column, all radioactivity emerged in the position of the third peak (Fig. 1). Furthermore, an authentic standard of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and riboflavin, respectively, was eluted on a Sephadex G-15 and each fraction of FAD, FMN and riboflavin was monitored by measurement of the absorbance at 450 nm (Fig. 1). The fraction of ester forms of B₂ formed by lens homogenate from ¹⁴C-riboflavin was a mixture of FAD and FMN and the amount of ester forms was expressed as nmol/g wet weight of lens for 1 hr at 37°C. ¹⁴C-Riboflavin was purchased from Radiochemical Centre, England. NADPH-cytochrome *c* reductase, glutathione reductase and B₂ content in the respective tissues of B₂-deficient rats showed a significantly low level of B₂ status as shown in Table 1.

Decreases in synthesis of ester forms of B₂ were observed in the lenses of rats which had been fed a B₂-deficient diet for 8 weeks but not for 4 weeks as shown in

Fig. 1. Elution profiles of radioactive flavins in the incubation mixture of lens homogenate of normal and B₂-deficient rats as compared with authentic standards of three forms of B₂ on a Sephadex G-15 column. Lens homogenate containing ATP and some chemicals was incubated with ¹⁴C-riboflavin for 1 hr at 37°C. An aliquot of supernatant of incubation medium was eluted with 0.025 M phosphate buffer (pH 7.0), fractions of 2 ml were collected at room temperature. One aliquots of each tube were added to 10 ml of dioxane scintillator and the radioactivity of the samples were counted in a liquid scintillation counter. Protein concentration in each fraction was monitored by measurement of the absorbance at 280 nm. Authentic standard of FAD, FMN and riboflavin, respectively, was eluted on a Sephadex G-15 and each fraction of FAD, FMN and riboflavin was monitored by measurement of the absorbance at 450 nm, furthermore, ¹⁴C-riboflavin alone was filtered through a Sephadex G-15 column and its radioactivity was counted. Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; FR, riboflavin.

Table 1. Effects of B₂-deficiency on the B₂ content and some enzyme activities in the liver and lens.

	NADPH-cytochrome <i>c</i> reductase (μ mol/min/mg microsomal protein)		Glutathione reductase (units/mg 9,000 $\times$ <i>g</i> sup. protein)		B ₂ content (μ g/g)	
	4 weeks	8 weeks	4 weeks	8 weeks	4 weeks	8 weeks
Liver						
Normal	0.112 $\pm$ 0.011*	0.120 $\pm$ 0.013	48.1 $\pm$ 1.8	44.0 $\pm$ 5.5	37.0 $\pm$ 1.9	35.5 $\pm$ 2.1
B ₂ deficiency	0.069 $\pm$ 0.010**	0.044 $\pm$ 0.005**	28.2 $\pm$ 2.3**	19.9 $\pm$ 3.0**	21.0 $\pm$ 2.1**	13.0 $\pm$ 2.3**

	Glutathione reductase (munits/mg 10,000 $\times$ <i>g</i> sup. protein)	
	4 weeks	8 weeks
Lens		
Normal	0.339 $\pm$ 0.042	0.367 $\pm$ 0.050
B ₂ deficiency	0.232 $\pm$ 0.048**	0.183 $\pm$ 0.022**

*Mean $\pm$ SD, obtained from 5 experiments. ** p < 0.01.

Table 2. Formation of ester forms of riboflavin from ¹⁴C-riboflavin by lens homogenate of B₂-deficient rats.

Wistar strain male rats weighing about 50 g were maintained on a B₂-deficient diet. Determination of formation of ester forms of riboflavin in the rat lens was carried out at 4 and 8 weeks, respectively, after being fed a B₂-deficient diet. See text for details. The amount of ester forms of B₂ formed by lens homogenate from ¹⁴C-riboflavin was expressed as nmol/g wet weight of lens for 1 hr at 37°C.

	Ester forms of riboflavin (nmol/g lens wet weight/hr)	
	4 weeks	8 weeks
Normal	1.474 ± 0.152*	1.310 ± 0.108
B ₂ deficiency	1.518 ± 0.146	1.089 ± 0.027
	N.S.	p < 0.05

*Mean ± SD, obtained from 4 experiments. N.S., not significant; p, t-test.

Table 2.

We have reported that the amount of high molecular phosphate compounds was dropped in the lens of B₂-deficient rat (15), furthermore, we have demonstrated that the activity of glucose-6-phosphate dehydrogenase (G-6-PD) which is the enzyme of the main ATP-generating system in the lens was also reduced, then suggested that lowered G-6-PD activity may lead to a phosphorylation insufficiency in the lens of B₂-deficient rats (9).

These results are consistent with those found in this B₂-deficient experiment, i.e., phosphorylation of B₂ in the lens was reduced in B₂-deficient rats.

It is suggested that B₂ deficiency would bring about metabolic disorder in the lens which requires flavin ester as a coenzyme of the oxidoreduction system to maintain transparency.

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