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Riboflavin Status Influences the Biosynthesis of Flavin Peptides and Related Enzyme Activities in Rat Liver Mitochondria

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The effects of riboflavin deficiency on the biosynthesis of flavin peptides and levels of flavoenzymes and catecholamines have been investigated. The percentage of ^{14}C-riboflavin radioactivity formed in mitochondria appeared to increase up to 2 weeks but started to decline at 3 weeks. A significant increase of radioactivity incorporation into mitochondria and into trypsin-digestable plus trypsin-non-digestible flavin peptides was detected in riboflavin-deficient animals. More than 35% of incorporation was observed at the end of the first week and 160% higher incorporation was observed in flavin peptide after the second week. Activities of MAO and succinate dehydrogenase were affected markedly by riboflavin status whereas those of acetylcholinesterase were not affected. Riboflavin deficiency also brought about marked reductions in levels of epinephrine and norepinephrine. It is concluded that the levels of flavin peptides, MAO and succinate dehydrogenase, and catecholamines were affected significantly by the availability of riboflavin and in particular the duration of its depletion.

KEY WORDS: Riboflavin Status, Flavin Peptide, Enzyme Activity, Rat Liver Mitochondria

The biochemical role of riboflavins resides largely in its being the precursor of FMN and FAD, two important coenzymes that are essentially necessary for a wide variety of oxidation-reduction reactions in metabolic pathways (Brady and Hoppel, 1985: Veitch *et al.*, 1989). A number of biologically important flavoenzymes containing covalently bound FMN or FAD have been found in various tissues of animals (Massey, 1994). The fact that monoamine oxidase and succinate dehydrogenase are flavoproteins demonstrates the nutritional requirement of riboflavin for normal enzyme activity (Sourkes and Missala, 1976; Kim and Lambooy, 1978). The formation of flavocoenzymes has been determined to be enzymatic and flavinyl peptides of MAO were synthesized and studied (Falk *et al.*, 1976). Labelled riboflavin as a precursor has been utilized in the study for FAD and FMN biosynthesis in rat liver and kidney. It has been suggested that the radioactivity found in mitochondria fraction was largely bound to macromolecules as FAD (Fazekas and Sandor, 1973). Although the existence of these flavoenzymes has been well documented (Singer and Edmondson, 1980), little information is available on their metabolic roles and biological significances under riboflavin deficiency. Thus the mode of flavoprotein biosynthesis as well as the formation of covalent linkage between flavin coenzymes and apoenzymes in biological systems

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needs to be explored.

In the present study we attempted to address the relationship of riboflavin deficiency to levels of free flavins, flavin peptides, flavoenzymes and catecholamines in rat liver.

Materials and Methods

Materials

FMN, FAD, succinate, 2,4-dichlorophenolindophenol, acetylthiocholine, 5,5′-dithiobis (2-nitrobenzoic acid), bovine serum albumin were purchased from Sigma Chemical Co.(St. Louis, MO, USA). [2-^{14}C] riboflavin was purchased from Amersham Corp. (Arlington Heights, Il, USA). Aquasol-2 and ^{14}C-tryptamine were obtained from Dupont NEN (Boston, MA, USA). The riboflavin deficient diet was purchased from US Biochemical corporation (Clevelavd, OH, USA). All other chemicals need were of analytical grade.

Animal treatment

Male Sprague-Dawley rats weighing 40-45 g were divided into two groups of 20, and they were provided riboflavin deficient and riboflavin supplemented diets for 1, 2 and 3 weeks, respectively (Bates, 1989). The control group was fed the riboflavin deficient diet supplemented with 25 mg riboflavin/kg diet. Throughout the experiment the animals were maintained on a 12 h light and 12 h dark cycle and allowed free access to food and water *ad libitum*. Rats were sacrificed by decapitation and their organs were removed as quickly as possible, and stored frozen at −70°C until analysis.

Determination of free flavins

The levels of free flavins such as riboflavin, FMN and FAD in liver tissue were determined by a reverse-phase HPLC with a fluorometric detection (Park, 1985). The tissue samples were homogenized in a cold 10 % TCA by a Potter-Elvehjem glass homogenizer for 2 min and the homogenate was centrifuged at a low speed. The supernatant was adjusted to pH 7.0 by a dropwise addition of cold 0.2 M K_2HPO_4. A clear supernatant obtained after a low centrifugation was directly injected into HPLC system for the analysis. The column was μ Bondapak C18 (particle size 10 μm, 30 cm × 10 cm, Waters Assoc., Milford, MA) preceded by a guard column (5 cm × 2.2 mm) packed with Corasil/C18 (Waters Assoc.). The detection system was a Schoeffel FS 970 fluorometer equipped with a monochromator at the excitation side, a filter at the emission side and a 5 μl flow cell. The excitation wave length was 360 nm and a 550 nm cutoff filter was used on the emission side. Time-constant was set at 6 sec. The eluant, consisting of 0.01 M KH_2PO_4/K_2HPO_4 buffer (pH 5.0) and 35% (v/v) methanol in aqueous solution, was pumped at a flow rate of 1 ml/min (4,000 psi) at ambient temperature(about 24~26°C).

Preparation of mitochondria

At the end of feeding period, one μCi of riboflavin 2-C^{14} in 1 ml of saline was injected subcutaneously and rats were killed 24 hrs later. A 10% homogenate of the liver was made in 0.25 M ice-cold sucrose with a Potter-Elvehjem homogenizer to prepare the mitochondrial fraction (Johnson and Lardy, 1976). After centrifuging the homogenate at 600 ×g for 15 min the resulting supernatant was centrifuged further at 6,500 ×g for 20 min. The pellet was resuspended in 1/2 volume of 0.25 M sucrose and centrifuged at the same speed. The final pellet resuspended in 0.01 M potassium phosphate buffer (pH 6.8) was used as the mitochondrial suspension.

Preparation of flavin peptides

Mitochondrial suspension was heated at 80°C for 15 min to isolate the free flavin or the heat-dissociable flavin (Cerletti and Giordano, 1971). It was cooled rapidly on ice and centrifuged at 24,000 ×g for 15 min. The majority of the heat-dissociable flavin was contained in the supernatant and the pellet was resuspended in 5 ml of 0.015 M potassium phosphate buffer (pH 6.8) and centrifuged at the same speed. The pellet was repeatedly washed with a sufficient amount of 1% TCA.

The final pellet was resuspended in 3 ml of 50 mM potassium phosphate buffer, pH 7.8, for

proteolytic digestion. To the pellet suspension 20 mg each of trypsin and chymotrypsin was added and incubated at 38°C for 4 hrs. The pH of the reaction mixture was adjusted periodically with 0.1 N KOH. The reaction was terminated with the addition of 0.5 ml of 55% TCA and then heated at 100°C for 3 min. After centrifuging at 4,000 rpm for 20 min, the sepernatant was counted to determine ^{14}C-riboflavin that was associated with trypsin-digestable flavin peptides.

Determination of radioactivity

The liver and mitochondria samples as well as flavin peptides were dissolved in 2 ml of 1 N NaOH and were neutralized with glacial acetic acid prior to the addition of 10 ml of Aquasol-2. The radioactivity was determined with LS 6000 Beckman scintillation spectrophotometer and results were corrected for quenching by external standards.

Determination of enzyme activity

Monoamine oxidase activity was determined according to the radioenzymatic assay of Balsa *et al.*, (1987). The reaction mixture contained 25 mM sodium phosphate buffer, pH 7.4, 100 mM tryptamine-2-^{14}C (2 mCi / mM) and 50 μl of 2% tissue homogenate in a total volume of 500 μl. The enzymatic reaction was performed at 37°C for 20 min after preincubation in the absence of tryptamine and was stopped with the addition of 200 μl of 2 N HCl. After the reaction product was extracted with 4 ml of toluene the radioactivity of the extract was measured by liquid scintillation counting. Enzyme activity was expressed in nanomoles of tryptamine turned over per mg of protein in one hour.

Succinate dehydrogenase activity was determined spectrophotometrically by the method of King (1967). The reaction mixture contained 0.75 ml of 0.2 M sodium phosphate buffer, pH 7.8, 200 μl of 0.6 M succinate, 100 μl of 1.5 mM 2,4-dichlorophenolindophenol, 100 μl of 50 mM potassium cyanide, 300 μl of 1% bovine serum albumin and several volumes of 9 mM phenazine methosulfate with appropriate amount of water to make up to 2.95 ml. The enzymatic reaction was initiated by the addition of 50 μl of crude homogenate and the reduction of 2,4-dichlorophenolindophenol at 600 nm was followed. Changes of absorption on the initial linear slope was employed to calculate succinate dehydrogenase activity by multiplying a factor of 0.0476. The velocity of enzyme reaction was expressed as m moles of succinate oxidized per mg of protein in one minute.

Acetylcholinesterase activity was assayed according to the method of Ellman *et al.*, (1961). The reaction mixture contained 0.1 M potassium phosphate buffer, pH 8.0, 1 mM 5,5′-dithiobis (2-nitrobenzoic acid), 75 mM acetylthiocholine and enzyme solution. The enzymatic reaction was initiated by the addition of enzyme solution to the sample cuvette and the absorbance was monitored at 412 nm for 1 min. The specific activity of acetylcholinesterase was expressed as the amount of enzyme that catalyzed the formation of 1 n mol of product per min milligram of protein at 30°C. The protein content was determined using the method of Bradford (1976) with bovine serum albumin as the standard.

Determination of epinephrine and norepinephrine

A paired ion HPLC method with amperometric detection was employed to measure epinephrine and norepinephrine following purification on alumina (Kawasaki *et al.*, 1989). The brain and liver tissues were homogenized in cold 5% $HClO_4$ for deproteinization for 2 min and the homogenate was centrifuged at 15,000 $\times$g for 25 min. The supernatant was absorbed on acid-washed alumina previously buffered with 0.5 M Tris-HCl, 30 mM EDTA and 3.0 mM sodium metabisulfite, pH 8.6. After washing alumina twice with deionized water, catecholamines were eluted with 0.05 M phosphoric acid and 0.1 M sodium metabisulfite and centrifuged at 16,000 $\times$g for 5 min. The eluate was injected directly into the chromatographic system which consisted of a model 45 solvent delivery system, a model U6K manual injector and a μ Bondapak C18 column (0.39 $\times$ 30 cm, Water Scientific Ltd, Boston, MA, USA). The mobile phase was composed of 8 parts of methanol and 92 parts of 0.1 M NaH_2PO_4, 0.1 mM EDTA and 1 mM sodium

octylsulfate, pH 5.5. The catecholamines were detected by means of carbon paste electrode at a sensitivity of 5 nAV^{-1} and at +7.0V against an Ag/AgCl reference electrode.

Results and Discussion

The degree of riboflavin deficiency was assessed by noting the growth and reduced flavin coenzyme concentrations in the deficient rats. Figure 1 illustrates the growth curves of rats fed riboflavin deficient diet and riboflavin supplemented diet for 3 weeks, respectively. The body weight gain of the riboflavin deficient animals leveling off around 4 days whereas that of the riboflavin supplemented animals shows a linear relationship with the feeding time up to 3 weeks. The reduction in the body weight gain is the first sign of riboflavin deficiency (Dakshinarmuti, 1977).

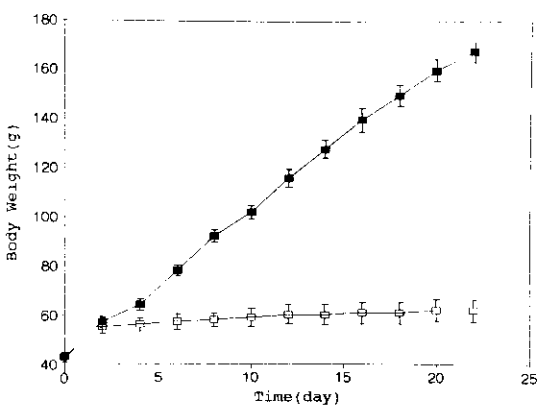

Fig. 1. Growth curves of rats fed with riboflavin deficient diet or riboflavin supplemented diet. Each diet was given to 20 rats for 22 days, respectively. -■-■-, riboflavin supplemented rat; -□-□-, riboflavin deficient rat.

Changes in the levels of FAD, FMN and riboflavin in the liver of riboflavin-deficient rats were presented in Table 1. The levels of FAD in the liver of riboflavin-deficient rats were significantly reduced compared to the control animals after 1, 2, and 3 weeks, respectively. By contrast the levels of FMN and riboflavin were significantly reduced only after 2 weeks but not after 1 and 3 weeks. This suggests that FAD pyrophosphorylase, converting FMN to FAD was more severely affected by riboflavin status and there existed a specific and differential regulation in biosynthesis of flavin coenzymes (Lee and McCormick, 1983).

The ability to incorporate ^{14}C-riboflavin into liver of rats fed on riboflavin-deficient diet and the distribution of ^{14}C-label in mitochondria were tested each week and compared with control (Table 2). The percentage of radioactivity detected in the liver was 21% after 1 week, 14% and 17% after 2 weeks, and 17% after 3 weeks, respectively. The percentage of ^{14}C formed in mitochondria of the liver appeared to increase up to 2 weeks but started to decline at 3 weeks. The incorporation of radioactivity into whole liver and mitochondria showed no differences between the control and riboflavin-deficient groups. The percentage of ^{14}C-labeled free flavins in mitochondria tended to decrease as the duration of dietary treatment progresses but no significant differences were observed between two groups.

Table 1. Changes in liver flavin levels of rats fed on riboflavin-supplemented and -deficient diets

Flavin	1 week		2 week		3 week	
	$+B_2$	$-B_2$	$+B_2$	$-B_2$	$+B_2$	$-B_2$
			μg/g tissue			
FAD	53.40±3.20	41.10±3.10*	61.20±4.10	45.60±3.10**	58.30±3.20	43.70±2.10**
FMN	3.18±0.21	2.90±0.10	3.40±0.02	2.97±0.08**	3.24±0.25	2.94±0.30
B_2	0.05±0.02	0.04±0.01	0.05±0.01	0.03±0.01*	0.05±0.01	0.04±0.01

All values are expressed as mean ± S.E. for five samples. Each sample consisted of tissues pooled from four animals. Each diet was given to rats for 1, 2 and 3 weeks, respectively, at which time the rats were sacrificed for flavin analysis. $+B_2$, riboflavin-supplemented; $-B_2$, riboflavin deficient; B_2, riboflavin. *$P<0.05$; **$P<0.01$, compared to control rats.

Table 2. Distribution of radioactivity in free flavins and flavin peptides of rat liver mitochondria

Treatment	Distribution of ^{14}C radioactivity							
	% recovered in liver	%detected in liver mitochondria	% of free flavins and flavin peptides in mitochondria			CPM per mg protein		
			free flavin	T.D. flavin	T.D. flavin TND. flavin	mitochondria	T.D. flavin	T.D. flavin & TND. flavin
1 week								
$+B_2$	21.0	23.0	100	5.20	7.10	1240±20	64	80±0.1
$-B_2$	20.6	30.0	100	4.15	5.80	1980±80	81	108±7.0*
2 week								
$+B_2$	14.0	48.2	98.0	4.18	5.10	730±15	30	37±0.1
$-B_2$	17.0	49.3	91.0	5.20	6.84	1390±50*	75	95±6.0**
3 week								
$+B_2$	16.5	38.3	90.5	6.80	8.90	654±30	45	56±0.2
$-B_2$	17.0	37.0	90.0	4.38	6.15	1180±60**	43	54±0.3

All values are expressed as mean ± S.E. for five samples. Each sample consisted of tissues pooled from four animals. Rats were fed riboflavin (B_2)-supplemented and deficient diet for 3 weeks' period. One μCi of riboflavin-2-^{14}C in 1 ml saline was administered subcutaneously and subjected to metabolism for 24 hrs before sacrificing rats. The percentage (%) represents the amount of the injected riboflavin-2-^{14}C incorporated into liver and mitochondria. T.D. flavin, trypsin-digestable flavin; TND. flavin, trypsin non-digestable flavin peptide *for $P<0.02$, and **for $P<0.01$.

The percentage of radioactivity detected in trypsin-digestable flavins and trypsin-digestable flavins plus trypsin-non-digestable flavins of riboflavin-deficient group was higher than that of control at 2 weeks only but lower than that of control at 1 and 3 weeks. However, when the amount of proteins in each fraction was taken into account, a significant increase of incorporation into mitochondria and into trypsin-digestable plus trypsin-non-digestable flavin peptides was detected for riboflavin-deficient rats. More than 35% of incorporation was observed at the end of the first week and 160% higher incorporation was observed in flavin peptide fraction after the second week. However, there were no significant differences in incorporation after three weeks. The similarly enhanced incorporation of radioactivity was also found to be associated with covalently bound flavins in flavoprotein fractions in riboflavin-deficient rats (Muttart *et al.*, 1977). This would suggest that apoenzyme synthesis in riboflavin-deficient rats were not significantly affected by riboflavin deficiency.

MAO and succinate dehydrogenase activities in rat livers showed some dependency on the availability of riboflavins (Table 3). However, acetylcholinesterase activity was not affected. The reduced MAO activity paralleled the decrease in levels of both trypsin-digestable flavins and trypsin non-digestable flavins (Table 2) and of FAD (Table 1). Among flavins the largest reduction was accounted for by the loss of FAD. This is of immediate importance to the relationship to MAO and succinate dehydrogenase activities, for FAD is very much likely the intermediate in the formation of covalently bound flavins (Dix and Lambooy, 1981).

Another possible explanations for the decrease of MAO and succinate dehydrogenase activities lie in the fact that some changes in the lipid composition of liver mitochondria by riboflavin deficiency may produce some influences on activities of MAO and succinate dehydrogenase which are embedded in mitochondrial membrane (Veitch *et al.*, 1989).

As shown in Table 4 the riboflavin deficiency brought about marked reductions in concentrations of epinephrine and norepinephrine in rat liver (71% and 42% reduction below the controls, respectively). The pronounced lowering

Table 3. Effect of riboflavin deficiency on activities of monoamine oxidase, succinate dehydrogenase and acetylcholinesterase in rat liver.

	Monoamine oxidase (nm/mg/hr)	Succinate dehydrogenase (mm/mg/min)	Acetylcholinesterase (nm/mg/min)
1week			
$+B_2$	52.8±2.0	28.6±1.2	12.6±1.0
$-B_2$	45.6±1.5*	26.0±1.7	13.0±0.9
2 week			
$+B_2$	54.0±1.6	30.0±1.0	11.7±0.6
$-B_2$	43.0±1.2**	21.3±1.5**	11.0±0.8
3 week			
$+B_2$	55.2±2.0	29.4±1.5	13.0±1.0
$-B_2$	50.8±1.8*	23.8±1.4*	11.8±0.8

All values are expressed as mean ± S.E. for five samples. Each sample consisted of tissues pooled from four animals. Each diet was given to rats for 1, 2 and 3 weeks, respectively, at which time the rats were sacrificed for enzymes analysis. $+B_2$, riboflavin-supplemented; $-B_2$, riboflavin deficient; B_2, riboflavin. *$P<0.05$; **$P<0.01$, compared to control rats.

Table 4. Effect of riboflavin deficiency on levels of epinephrine and norepinephrine in rat liver

Catecholamine	$+B_2$	$-B_2$
	μg/g tissue	
Epinephrine	2.35±0.04	0.68±0.01**
Norepinephrine	6.70±0.10	3.90±0.06**

All values are expessed as mean ± S.E. for five samples. Each sample consisted of tissues pooled from four animals. Rats were fed on each diet for two weeks. $+B_2$, riboflavin-supplemented diet; $-B_2$, riboflavin deficient diet. **$P<0.01$, compared to control rats.

effect on hepatic catecholamines may point to some defects in the metabolic conversion of catecholamine biosynthesis (Sourkes, 1972) in which corresponding enzymes may be involved.

Overall these results suggest thst the biosynthesis of flavin peptides, MAO and succinate dehydrogenase activities, and catecholamine levels were affected significantly by the availability of riboflavin and in particular the duration of its depletion.

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리보플라빈 결핍이 쥐간의 미토콘드리아의 플라빈 펩티드와 관련된 효소 활성에 미치는 영향

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리보플라빈 결핍이 쥐간의 미토콘드리아의 플라빈 펩티드 합성, MAO, 숙신산 탈수소 효소 및 아세틸콜린 에스테라아제 활성 그리고 에피네프린과 노르에피네프린 함량에 미치는 영향을 조사하였다. 미토콘드리아내 탐지된 ^{14}C-리보플라빈의 방사선 함량은 2주 까지 증가하였으나 3주째에는 감소하기 시작하였다. 미토콘드리아내 합성된 방사선 함량과 트립신-가수분해 및 트립신-비가수분해 플라빈 펩티드의 농도의 증가는 리보플라빈 결핍시 현저히 나타났다. 미토콘드리아내 합성율은 첫째주에 35% 이상으로 나타났고, 상기한 트립신 플라빈 펩티드들의 합성율은 2주째에 160% 이상으로 나타났다. MAO와 숙신산 탈수소 효소 활성은 리보플라빈 상태에 따라 현저히 감소하였으나, 아세틸콜린에스테라아제는 영향을 받지 않았다. 에피네프린과 노르에피네프린 함량도 현저히 감소하는 것으로 나타났다. 쥐간의 미토콘드리아내 플라빈 펩티드 합성, MAO, 숙신산 탈수소 효소 활성, 카테콜라민 농도는 리보플라빈 결핍상태와 특히 그 지속기간에 따라 변화하였다.