Analysis of Flavins in Ocular Tissues of the Rabbit

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Riboflavin is the precursor of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), coenzymes required for the activity of flavoenzymes involved in the transfer of electrons in oxidation-reduction reactions. Flavins are light sensitive and rapidly degrade when exposed to light in the near ultraviolet and visible wavelengths. Some of the byproducts of flavin photodegradation are toxic. A quantitative survey of flavins in rabbit ocular tissues is reported. Adult male Dutch-Belt Rabbits were fed purified diets containing 3, 30, or 300 mg riboflavin/kg for 1 month. A method of aqueous extraction and high-performance liquid chromatography with fluorescence detection was used to measure riboflavin, FMN, and FAD in cornea, lens cortex, lens nucleus, retina, and blood. The retina contained the highest flavin concentration. In all tissues, the primary flavin was FAD followed by FMN and riboflavin. The highest concentration of riboflavin occurred in the cornea followed by the retina, lens cortex, and lens nucleus. A trend toward increasing concentrations of riboflavin occurred in the retina and blood in response to excess dietary riboflavin, but the concentration changes were not statistically significant. The highest concentration of FAD and FMN occurred in the retina followed by the cornea and the lens cortex and nucleus. The relative contribution of riboflavin, FMN, and FAD to the total flavin pool was markedly different in the various tissues of the eye. The proportion of tissue flavins present as riboflavin decreased from anterior to posterior. It was highest in the cornea followed by lens and retina. The pattern of distribution for FMN was: cornea > retina > lens cortex and nucleus. The proportion of flavins present as FAD was opposite that of FMN and followed the pattern: lens nucleus and cortex > retina > cornea. These differences in the proportion of flavins may reflect differences in the rate of flavin turnover between the ocular tissues. Invest Ophthalmol Vis Sci 32:1981-1985, 1991

The essential nutrient riboflavin is required as a coenzyme in many metabolic reactions. Its biologic necessity is based on its function as a coenzyme in flavoprotein-catalyzed oxidation-reduction reactions. Symptoms of a riboflavin deficiency include retarded growth, cheilosis, angular stomatitis, seborrheic dermatitis, corneal vascularization, and photophobia.1 These symptoms can be reversed by reintroduction of riboflavin into the diet. Isolated riboflavin deficiency in humans is rare; deficiencies usually occur in populations with multiple deficiencies of essential nutrients.

The biologic role of the coenzyme forms of riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), is to mediate the transfer of electrons in oxidation-reduction reactions.2,3 In these reactions electrons are transferred between the isoalloxazine nucleus of riboflavin and the other reactants. The versatility of the flavins in this role can be seen in their ability to transfer one or two electrons at a time as required by a specific reaction.4

Flavins are light sensitive and rapidly photodegraded to inactivated forms of the vitamin, some of which are toxic to biologic tissues.3,6 Flavin coenzymes bound to the active site of the holo form of flavoenzymes are much less sensitive to photodegradation. However, when unbound flavins are exposed to light they can generate toxic photodegradative by-products and activated oxygen species such as superoxide anion, singlet oxygen, hydrogen peroxide, and peroxy- and oxyradicals. When illuminated with visible light, riboflavin and tryptophan can combine to form adducts.7,10 These adducts cause cellular structural damage and increase the rate of mutagenicity in cells in culture. They also have been linked to the pathogenesis of hepatic dysfunction in patients receiving light-exposed parenteral nutrition solutions.11-13

The eye and the skin are the two parts of the body which are exposed to ambient light. They are also the sites of the first clinical symptoms observed in a progressing riboflavin deficiency. The popular notion that the overall health of an individual will increase with an increased intake of vitamins has been questioned in regard to riboflavin since the consequences of this practice on ocular tissues is not...
known. The differentiated cells in the tissues of the eye, which do not undergo cellular renewal, could be particularly vulnerable to photoactivated riboflavin or toxic photodegradative products.

Increased fragility of the photoreceptor outer segments was reported in normal Royal College of Surgeons rats on a 12 mg riboflavin/kg diet, a concentration double the recommended level. In addition to evidence of tearing from light microscopy, biophysical measurements indicated that the retinas from rats fed excess riboflavin had a lower specific gravity owing to a loss of their dense rod outer segments. The basis for these riboflavin-associated changes is not known.

There is little information available on the concentrations of flavins in the eye. Our study therefore was undertaken to obtain normal data on the concentration of the major flavins in ocular tissue. In addition, the experiment was designed to determine if excessive dietary riboflavin levels resulted in an accumulation of flavins in ocular tissues. The Dutch-Belt rabbit was chosen as a model because its eyes were large enough to provide ample tissue for flavin analysis and because of its similarity to humans in both pigmentation and diurnal behavior.

**Materials and Methods**

**Materials**

Standards (FAD, FMN, and riboflavin) were purchased from Sigma (St. Louis, MO); the FMN and FAD were purified further on a column of DEAE-Sephadex, A-25 (Pharmacia) using an elution buffer of 2% (NH₄)₂SO₄. High-performance liquid chromatography (HPLC)-grade solvents methanol and acetonitrile were purchased from Fisher. The HPLC column was an Analytichem Separyte CH column (4.6 x 150 mm, 5-mm particle size). The HPLC apparatus consisted of a Varian series 5500 equipped with a rhodiney injector linked to a Shimadzu RF-530 fluorescence detector. Detector wavelengths were set at 447 nm for excitation and 530 nm for emission. Integration and data analysis were done on a Varian 654 work station. Protein analysis was done using the BCA system from Pierce. Samples were homogenized with a Potter-Elvehjem or Vertishear mechanical homogenizer from Virtis Co.

**Animals**

Care and treatment of animals adhered to the ARVO Resolution on the Use of Animals in Research. Twenty-seven adult male Dutch-Belt rabbits were divided into three groups and maintained on purified diets for 30 days. At this time their eyes were enucleated and retinas analyzed as groups of three rabbits/day. The diets were identical except for the riboflavin content which was varied for the different diets to give a riboflavin concentration of 3, 30, and 300 mg riboflavin/kg diet. A description of the purified diet has been provided previously. The diets contained double the recommended level of vitamin E and were prepared in dim light and stored in the dark at 4°C. All animals were maintained in the same room which was adjusted to a 12-hr light (0600)/12-hr dark (1800) cycle. The room was equipped with overhead fluorescent lights. The light intensity at the wire mesh front of the otherwise enclosed stainless steel cages was <10 lux.

**Sample Preparation**

All procedures were done under dim red light to prevent the photodegradation of flavins in the samples. On each day of analysis, one rabbit from each of the three diet groups was killed by CO₂, and its tissues analyzed. Blood was drawn from the vena cava, and the animal was perfused for 10 min with cold saline solution. The eyes were enucleated. The eyes were dissected in ice-cold phosphate buffered saline (pH 7.4). A circumferential incision was made at the limbus, and the cornea was removed and minced. The lens was separated into two fractions: the cortex (with the anterior capsule included) and the nucleus. The nuclear fraction was composed of the center portion of the lens measuring approximately 5 mm in diameter. The retina was gently teased away from the eyecup and fully separated by a cut at the optic nerve. The respective tissues from both eyes of a rabbit were combined and treated as a single sample.

The retinas were incubated in a water bath at 80°C for 5 min and then homogenized with 15 strokes in a Potter-Elvehjem homogenizer equipped with a Teflon pestle. Lens and cornea samples were heated similarly, but they were homogenized with a Polytron homogenizer. The homogenates were incubated again at 80°C for 15 min, cooled on ice, and particulate debris removed by centrifugation at 8000 g for 15 min.

**Table 1. Analysis of flavins in the rabbit retina**

<table>
<thead>
<tr>
<th>mg Riboflavin/kg diet</th>
<th>FAD</th>
<th>FMN</th>
<th>Riboflavin</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>39.9 ± 3.0</td>
<td>11.2 ± 1.6</td>
<td>0.77 ± 0.07</td>
<td>51.9 ± 4.3</td>
</tr>
<tr>
<td>30</td>
<td>47.6 ± 7.3</td>
<td>14.9 ± 2.4</td>
<td>1.00 ± 0.30</td>
<td>63.5 ± 9.8</td>
</tr>
<tr>
<td>300</td>
<td>43.7 ± 6.8</td>
<td>13.2 ± 1.9</td>
<td>1.09 ± 0.26</td>
<td>58.0 ± 8.8</td>
</tr>
</tbody>
</table>

Values are expressed as pmol flavin/mg protein ± SEM. The values are based on repetitions of the analysis of pooled retina from both eyes of one animal. The number of repetitions for each diet group was, eight for the 3 mg/kg diet, and seven for both the 30 and 300 diet groups.
pellet from the sample in a Beckman microcentrifuge for 5 min at 4°C. The supernatant was removed, deproteinized in a final concentration of 4% trichloroacetic acid (TCA), and centrifuged for 10 min at 4°C. An aliquot of 50 ml of cold 4 M KH₂PO₄ was added to 800 ml of the supernatant, giving a solution of pH 5.5. The extract was filtered through a 0.2-μm filter and used directly for HPLC analysis. Standard solutions were processed in tandem with samples. Treatment at 80°C was used as a precautionary measure to inactivate tissue phosphatases and pyrophosphatases that could cleave the flavocoenzymes. A comparison of heat-treated tissues with tissues maintained on ice gave similar results, and this was not a major potential source of error. In the analyses the percentage recovery, as measured by internal standards for each of the three flavins, was greater than 98%.

Whole blood was prepared for analysis by dilution with an equal volume of ice-cold H₂O and setting this mixture on ice for 5 min. A volume of the diluted blood was added to an equal volume of 5% NH₄Cl, 10 mM NaH₂PO₄, pH 5.5. This mixture was then processed through heat extraction, TCA precipitation, neutralization, and filtration as described.

HPLC Analysis

Details of this procedure were described previously. Sample volumes of 100 ml were injected for analysis. The mobile phase used was 90:10 (NH₄)₂HPO₄:acetonitrile (pH 5.5). The gradient was isocratic at a flow rate of 2.0 ml/min.

Table 2. Analysis of flavins in the rabbit cornea

<table>
<thead>
<tr>
<th>mg Riboflavin/kg diet</th>
<th>FAD</th>
<th>FMN</th>
<th>Riboflavin</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>16.9 ± 2.40</td>
<td>5.63 ± 0.71</td>
<td>2.86 ± 1.18</td>
<td>25.4 ± 3.6</td>
</tr>
<tr>
<td>30</td>
<td>16.5 ± 3.11</td>
<td>4.32 ± 1.35</td>
<td>2.24 ± 1.36</td>
<td>23.1 ± 4.0</td>
</tr>
<tr>
<td>300</td>
<td>14.8 ± 0.73</td>
<td>4.76 ± 0.85</td>
<td>1.26 ± 0.54</td>
<td>20.8 ± 1.4</td>
</tr>
</tbody>
</table>

Values are expressed as pmole flavin/mg protein ± SEM. The values are based on repetitions of the analysis of pooled cornea from both eyes of one animal. The number of repetitions for each diet group was, seven for the 3 mg/kg diet, and six for both the 30 and 300 diet groups.

Statistical Evaluation

Results were tested for statistical significance using a two-tailed student t-test.

Results

The concentrations of riboflavin, FMN, and FAD in the retina are shown in Table 1. A trend was seen toward an increase in riboflavin when the dietary content of riboflavin was increased above the physiologic requirement of 3 mg/kg diet. However this trend was not statistically significant (P < 0.1). Flavin concentrations in the cornea, lens cortex, and lens nucleus are shown in Tables 2–4. The amount of FAD and FMN in these tissues was substantially lower than the retina. The concentration of these two flavins in the cornea (pmol flavin/mg protein) was approximately one half that of the retina, and values for the lens cortex and nucleus were at least an order of magnitude less than the retina. The concentrations of riboflavin were greatest in the cornea followed by retina, lens cortex, and lens nucleus.

The relative proportion of the individual flavins to total flavin content was markedly different in the various tissues of the eye (Table 5). The proportion of flavins present as FAD followed the pattern: lens nucleus and cortex > retina > cornea. The pattern of distribution for FMN was the opposite: cornea > retina > lens cortex and nucleus. The riboflavin concentration decreased from anterior to the posterior. It was highest in the cornea followed by lens and retina.

The flavin concentrations in the blood are given in Table 6. There was a trend toward increased flavins with an increase in dietary riboflavin; however it was not statistically significant (P < 0.1).

Discussion

The distribution of the major flavins is known to vary between organs and is shifted when riboflavin is deficient. The relative ratios of FAD:FMN:riboflavin have been reported to be approximately 30:10:1 in rat liver, kidney, and intestine, but 300:32:1 in heart and 15:3:1 in brain. Under conditions of limited riboflavin, the ratio shifts toward FAD. In rats provided a

Table 3. Analysis of flavins in the rabbit lens cortex

<table>
<thead>
<tr>
<th>mg Riboflavin/kg diet</th>
<th>FAD</th>
<th>FMN</th>
<th>Riboflavin</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2.43 ± 0.23</td>
<td>0.154 ± 0.028</td>
<td>0.067 ± 0.038</td>
<td>2.65 ± 0.29</td>
</tr>
<tr>
<td>30</td>
<td>2.01 ± 0.18</td>
<td>0.131 ± 0.025</td>
<td>0.020 ± 0.009</td>
<td>2.16 ± 0.22</td>
</tr>
<tr>
<td>300</td>
<td>2.03 ± 0.17</td>
<td>0.157 ± 0.030</td>
<td>0.026 ± 0.009</td>
<td>2.20 ± 0.19</td>
</tr>
</tbody>
</table>

Values are expressed as pmole flavin/mg protein ± SEM. The values are based on repetitions of the analysis of pooled cortex from both lenses of one animal. The number of repetitions for each diet groups was eight.
Table 4. Analysis of flavins in the rabbit lens nucleus

<table>
<thead>
<tr>
<th>mg Riboflavin/ kg diet</th>
<th>FAD (%)</th>
<th>FMN (%)</th>
<th>Riboflavin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>1.23 ± 0.16</td>
<td>0.044 ± 0.010</td>
<td>0.016 ± 0.004</td>
</tr>
<tr>
<td>30</td>
<td>1.12 ± 0.12</td>
<td>0.039 ± 0.012</td>
<td>0.024 ± 0.008</td>
</tr>
<tr>
<td>300</td>
<td>1.12 ± 0.12</td>
<td>0.039 ± 0.008</td>
<td>0.014 ± 0.004</td>
</tr>
</tbody>
</table>

Values are expressed as pmole flavin/mg protein ± SEM. The values are based on repetitions of the analysis of pooled nuclei from both lenses of one animal. The number of repetitions for all diet groups was eight.

Table 5. Distribution of flavins in ocular tissues of the rabbit

<table>
<thead>
<tr>
<th></th>
<th>FAD (%)</th>
<th>FMN (%)</th>
<th>Riboflavin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cornea</td>
<td>66.7 ± 4.0</td>
<td>22.7 ± 1.7</td>
<td>10.5 ± 4.1</td>
</tr>
<tr>
<td>Lens cortex</td>
<td>92.2 ± 1.5</td>
<td>5.6 ± 0.5</td>
<td>2.2 ± 1.1</td>
</tr>
<tr>
<td>Lens nucleus</td>
<td>94.8 ± 1.1</td>
<td>3.8 ± 1.0</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Retina</td>
<td>77.9 ± 2.6</td>
<td>20.6 ± 2.6</td>
<td>1.6 ± 0.2</td>
</tr>
</tbody>
</table>

Values are determined from animals receiving 3 mg riboflavin/kg diet and are expressed as relative percent of total flavins ± SEM in each tissue.

The intestinal absorption of riboflavin occurs though an active transport mechanism which imposes a limit on the amount of the vitamin that can be absorbed under normal conditions.19 Active transport of the vitamin is Na⁺ dependent, inhibited by ouabain, and saturable with Kₘ = 3 mM.20 At concentrations greater than 100 mM, intestinal absorption occurs by passive diffusion.21 Once in the blood most of the circulating riboflavin is bound to albumin with smaller amounts bound to immunoglobulins.22 Transport into cells occurs by facilitated diffusion, followed by rapid trapping through phosphorylation by flavokinase to form FMN.23 Studies with isolated hepatocytes have shown that the Kₘ for cellular uptake is approximately the same as for flavokinase. Renal clearance increases to a maximum at flavin plasma levels of 7 mg/ml, and it is inhibited by probenecid, a compound which inhibits renal transport processes.24

The amount of dietary riboflavin required to maintain the activity of the flavoenzyme erythrocyte glutathione reductase is influenced by the wavelength and duration of light exposure in newborn infants. Grosmich et al25 demonstrated that the degree of saturation of erythrocyte glutathione reductase with FAD was reduced in infants treated for hyperbilirubinemia by phototherapy. The longer the phototherapy, the greater the reduction in the level of the saturation.26 The breakdown of bilirubin has been shown to be accelerated by riboflavin in vitro.27 Supplementing neonates with 0.3 mg riboflavin/day during phototherapy prevented riboflavin depletion and resulted in a more rapid destruction of serum bilirubin.

The effects of pharmacologic doses of riboflavin depend on whether the vitamin is given orally, subcutaneously, or intraperitoneally.28,29 Intraperitoneal injections of the vitamin are toxic when the vitamin is given in large amounts. Blood levels of riboflavin exceeding 20 mg/ml riboflavin in the rat result in precipitation of riboflavin in the kidney. The lethal dose for 50% of rats is 560 mg riboflavin/kg body weight, with death resulting from anuria and azotemia due to concretion of riboflavin crystals in the kidney. Injections of a dose of 125 or 500 mg of the sodium salt of riboflavin intraperitoneally have also been reported to induce cytologic changes in the heart, pancreas, and pituitary gland.
Harmful effects of excess riboflavin on the retina have been reported in rats provided excess dietary riboflavin. Our results demonstrate that the retina contains the highest concentration of flavins of the tissues studied, but although a trend toward increasing retinal concentrations occurs with increasing dietary concentrations of the vitamin, the changes in concentration are small. It is possible that significant localized increases in FMN and riboflavin occurred which were lost in pooling the entire retinal tissue. Future studies using radiolabeled riboflavin will be needed to determine the cellular distribution of flavins in the retina to evaluate this possibility.

Key words: flavins, riboflavin, retina, lens, cornea, diet, rabbit

Acknowledgments

The authors thank Professor Joseph Horwitz for his helpful discussions and for teaching them the procedure for separating lens cortex and nucleus properly.

References


Table 6. Analysis of flavins in rabbit whole blood

<table>
<thead>
<tr>
<th>mg Riboflavin/kg diet</th>
<th>FAD</th>
<th>FMN</th>
<th>Riboflavin</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>2.42 ± 0.28</td>
<td>0.074 ± 0.009</td>
<td>0.053 ± 0.013</td>
<td>2.55 ± 0.32</td>
</tr>
<tr>
<td>30</td>
<td>3.00 ± 0.25</td>
<td>0.086 ± 0.013</td>
<td>0.108 ± 0.025</td>
<td>3.19 ± 0.28</td>
</tr>
<tr>
<td>300</td>
<td>3.18 ± 0.26</td>
<td>0.121 ± 0.016</td>
<td>0.137 ± 0.019</td>
<td>3.44 ± 0.57</td>
</tr>
</tbody>
</table>

Values are expressed as pmole flavin/mg protein ± SEM. The values are based on repetitions of the analysis of blood from one animal. The number of repetitions for all diet groups was eight.