The Effects of Galactoflavin on Riboflavin Enzymes and Coenzymes

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This is a record of the effects of feeding galactoflavin on the concentrations of riboflavin-containing coenzymes and enzymes in liver and kidney. Galactoflavin was shown by Emerson, Wurtz, and Johnson (1) to produce a riboflavin deficiency in rats which is reversible by excess dietary riboflavin. Slight inhibitory effects of galactoflavin were later found by Kearney (2) on the enzymatic activities of several flavoproteins purified from yeast, kidney, or snake venom. The enzyme inhibitions in vitro were not sufficiently large to account for the over-all effects of galactoflavin fed to rats.

In order to find out whether greater inhibitions occur in vivo, rats were given galactoflavin until signs of riboflavin deficiency appeared; the tissues were then analyzed for flavin mononucleotide, flavin adenine dinucleotide, D- and L-amino acid oxidase, glycolic acid oxidase, reduced triphosphopyridine nucleotide dehydrogenase, and reduced diphosphopyridine nucleotide dehydrogenases. In addition tissue extracts were examined chromatographically to see whether galactoflavin had replaced riboflavin in either the free or the combined (coenzyme) form. The feeding experiments were supplemented by studies in vitro with the galactoflavin analogue of FMN, galactoflavin monophosphate. It was found that this coenzyme analogue is capable of partially reactivating the apoenzymes of liver and yeast TPNH dehydrogenases.

Riboflavin nutrition has certain special features that are essential for understanding the effects in vivo of galactoflavin. For each tissue there is (a) a limiting upper level for flavin content which is not exceeded, no matter how much riboflavin is fed, and (b) a limiting lower level below which flavin content will not fall on a riboflavin-free diet, no matter how long continued (3). The characteristic upper and lower limiting levels may differ by as little as 35% in some tissues, such as brain, whereas, in liver, at the other extreme, there is a 4-fold difference between “ceiling” and “floor” (4). As a rule a greater proportion of FMN than of FAD is lost in deficiency; free riboflavin is present at very low levels (4). When an animal is placed on a riboflavin-free diet it will grow until tissue flavin has been diluted to the lower concentration limits, after which the body weight will slowly diminish to compensate for inevitable small losses of riboflavin. Traces of riboflavin from intestinal bacterial flora will often compensate for such losses and maintain nearly stationary weights. Studies of a variety of enzymes have shown (5) that the activity of certain flavin-containing enzymes never decreases significantly even in extreme deficiency; enzymes of this type presumably account for the firmly held flavin component. The studies also showed that the activity of other enzymes decreases to a marked degree in deficiency, and enzymes of this type would reasonably account for the variable flavin component.

EXPERIMENTAL PROCEDURE

Animals—Litter mate, 22- and 25-day-old male Sprague-Dawley rats (Holtzman Rat Company, Madison, Wisconsin) were used. Rats from each litter were placed on each of the diets. The basal riboflavin-free diet was previously described (5). Dietary supplements are indicated in the tables.

Preparation of Tissues—The rats were lightly anesthetized with ether, the thorax was opened, and as much blood as possible was withdrawn from the exposed heart. One sample of liver was homogenized at once in a glass tissue grinder for immediate assay of DPNH and TPNH dehydrogenases. The rest of each liver and the kidneys were frozen in liquid nitrogen and stored at -40° or -80° until used.

Coenzyme and Protein Measurements—FAD and FMD determinations were made by a fluorometric method (4, 6) on 10% trichloracetic acid extracts prepared at 0° with 1:200 dilution of tissue. The values obtained in the FMN measurements would include any free riboflavin, but the latter has been shown to be negligible under the conditions of these experiments (4). The same methods were used for the measurement of galactoflavin and its phosphates since these behave fluorometrically like the riboflavin analogues. The protein content of the tissue homogenates or of the purified enzymes was determined colorimetrically (9).

DPNH and TPNH Dehydrogenases—The enzymes in whole homogenates that oxidize DPNH and TPNH were measured spectrophotometrically with either cytochrome c or potassium ferricyanide as electron acceptor (5, 10).

Amino Acid and Glycolic Acid Oxidases—Homogenates were prepared as described (4), insures quantitative extraction of flavin coenzymes except for the small amount of protein-bound flavin of the type found in succinic dehydrogenase (7). This fraction is reported not to exceed 3% of total flavin (8).

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1 This enzyme is frequently called TPNH-cytochrome c reductase.
2 The abbreviations used are: FMN, flavin mononucleotide; TPNH, reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; GFP, galactoflavin monophosphate.
Weanling rats were given a riboflavin-free diet for 4 to 5 weeks supplemented as indicated with galactoflavin (200 mg per kg of diet) or riboflavin (200 mg per kg). The average body and liver weights were 74 and 3.8 g, respectively, for those fed no riboflavin, 49 and 3.2 g for those fed galactoflavin, and 285 and 11 g for those fed riboflavin. The kidney weights for the three groups were 640, 555, and 883 mg, respectively. Each value represents the average of three or four rats with the standard error of the mean. Values without standard errors represent averages of two rats fed the diet plus riboflavin which agreed within 10%.

Table I

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity with dietary supplement*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>Glycolic acid oxidase</td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td>0.07 ± 0.01</td>
</tr>
<tr>
<td>Substrate + FMN</td>
<td>0.12 ± 0.01</td>
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<tr>
<td>L-Amino acid oxidase</td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td>0.030 ± 0.001</td>
</tr>
<tr>
<td>Substrate + FMN</td>
<td>0.031 ± 0.001</td>
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<tr>
<td>D-Amino acid oxidase</td>
<td></td>
</tr>
<tr>
<td>Substrate</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>Substrate + FAD</td>
<td>5.44 ± 1.00</td>
</tr>
<tr>
<td>DPNH dehydrogenase</td>
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<tr>
<td>Urease (m)</td>
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<tr>
<td>Ferrixyanide</td>
<td>29.6 ± 2.1</td>
</tr>
<tr>
<td>FAD</td>
<td>118</td>
</tr>
<tr>
<td>FMN</td>
<td>4.6</td>
</tr>
</tbody>
</table>

* Enzyme activity is expressed as moles of keto acid formed, cytochrome c reduced, or DPNH oxidized, per kg of tissue protein per hour; coenzyme activity, as micromoles per kg of protein.

incubated for 15 or 20 minutes in an atmosphere of oxygen with 0.25 mM d-alanine, 0.2 mM L-leucine, or 0.125 mM glycolic acid. The keto acids formed were determined as 3-hydrazinoquinoline derivatives by the absorption at 305 nm (5, 11, 12). The procedures are at least 100 times more sensitive than assays made in the Warburg apparatus and permitted tissue dilutions of 25- to 1000-fold during incubation.

Purification of GFP—Crude GFP (Merek Sharp and Dohme, Rahway, New Jersey)4 was purified on a column of DEAE-cellulose type 20, as described by Moffatt and Khorana (13) for purification of FMN. A column 26 X 0.8 cm was used at 4° and shielded from light. Crude GFP (1 mg) in water was applied to the column. Fraction I, 167 pg (all fractions are calculated per flavin moiety. Fraction III, 493 pg, contained one phosphate by the method of Frigerio and Harbury (17). TPNH

Preparation of Apoenzymes—Glycolic acid oxidase was partially purified from rat liver according to the method of Kun, Dechary, and Pirot (16) and resolved into the apoenzyme and coenzyme by the method of Frigerio and Harbury (17). TPNH dehydrogenases purified from yeast and calf liver were generously supplied by Dr. George R. Drysdale of the Department of Biological Chemistry. The yeast enzyme had been prepared as described by Haas, Horecker, and Hogness (18) through the alcohol precipitation and lyophilisation steps and then purified by column fractionation with hydroxyapatite followed by DEAE-cellulose. It was resolved by the method of Warburg and Christian (19) as modified by Strittmatter (20). The liver

4 The generous supply of galactoflavin and galactoflavin phosphate by Merek Sharp and Dohme through the courtesy of Dr. Max Tischler is gratefully acknowledged.
enzyme had been prepared by modification of the method of Williams and Kamin (21) and was resolved as described above. The apoenzyme was very unstable unless albumin was added during acid treatment.

RESULTS

Galactoflavin Effects on Flavin Enzymes and Coenzymes—In the first experiment, when galactoflavin was added to a riboflavin-free diet, growth of rats stopped immediately at an average weight of 48 g, with no significant further change. In contrast, rats fed the flavin-free diet grew to weigh 74 g, and the ad libitum controls, to 285 g. The livers of rats fed galactoflavin weighed significantly more in proportion to body weight than those of the flavin-free controls (6.5 versus 5.1%). This is what would be expected from an increase in severity of the riboflavin deficiency and has been interpreted as an attempt to compensate for the metabolic inadequacy (5).

The changes observed in enzyme activities with simple riboflavin deficiency (Table I) illustrate some of the features mentioned in the introduction. Thus in the liver, glycolic acid oxidase and D-amino acid oxidase are reduced to 5% of control values whereas DPNH dehydrogenase is unaffected. Also shown is the greater loss of flavin from liver than from kidney and the greater loss of FMN than FAD in both organs. Two other features of riboflavin deficiency are illustrated: (a) the same enzyme (in this case D-amino acid oxidase) may be more severely affected in one tissue than another, and (b) depressed enzyme activities are not restored to normal by addition of the coenzyme in vitro, suggesting that the apoenzyme has been lost (5,10).

In this experiment galactoflavin exaggerated the deficiency in only two respects. It lowered FMN levels somewhat in kidney, the greater loss of flavin from liver than from kidney and the greater loss of FMN than FAD in both organs. Two other features of riboflavin deficiency are illustrated: (a) the same enzyme (in this case D-amino acid oxidase) may be more severely affected in one tissue than another, and (b) depressed enzyme activities are not restored to normal by addition of the coenzyme in vitro, suggesting that the apoenzyme has been lost (5,10).

In this experiment galactoflavin exaggerated the deficiency in only two respects. It lowered FMN levels somewhat in kidney, and reduced glycolic acid oxidase activity in liver from 5% of control values to 0.5%. Although these concentration changes are relatively minor, the absolute amounts of flavin have probably been reduced by at least one-third, since galactoflavin reduced body weight by this amount.

The effects of galactoflavin were more evident in a second experiment, in which the control rats were given a suboptimal amount of riboflavin rather than none (Table II). FAD, FMN,
glycolic acid oxidase, \(\alpha\)-amino acid oxidase, and TPNH dehydrogenase were all reduced by the antagonist to levels characteristic of severe deficiency. Once again DPNH dehydrogenase was unaffected. The total flavin content per liver was reduced by galactoflavin to 36% of control values. Galactoflavin induced in these animals clinical signs characteristic of extreme riboflavin deficiency. These signs were not seen in the control animals.

As with simple riboflavin deficiency, the decreases in enzyme activity appear to be accompanied by loss of the apoenzymes, since addition of the appropriate coenzyme to the assay systems did not restore more than a small percentage of the normal enzyme activities (Tables I and II).

Search for Galactoflavin Compounds in Tissues—The most complete experiments were made with liver and kidney from rats fed 200 times as much galactoflavin as riboflavin for 8 weeks. In chromatograms of samples equivalent to 0.3 g of fresh tissue, FMN but no GFP was detected in extracts hydrolyzed with hydrochloric acid alone. After acid phosphatase treatment, traces of galactoflavin were found in the samples from liver and kidney of three out of six rats. The amounts did not exceed 0.03 \(\mu\)g of galactoflavin per g of liver or kidney (0.2 \(\mu\)g per g of protein). This is only 0.1 to 0.2% of total flavin present. Clearly, galactoflavin did not replace riboflavin to any significant degree.

Combination of GFP and FMN with Apoenzymes of Flavoproteins—Addition of the natural coenzyme, FMN, to the apoenzyme of purified \(\alpha\)-amino acid oxidase restored the activity to the original level (Table III), but with GFP there was no restoration. Preliminary incubation of the apoenzyme with GFP did not inhibit subsequent reactivation by FMN.

Activity of partially resolved liver TPNH dehydrogenase was increased from 20% of full activity to 80% by the addition of either FMN or FAD at 70 \(\mu\)M. Horecker (22) tested both coenzymes at lower concentrations and found FMN more effective than FAD, although the native enzyme contains FAD. GFP definitely stimulated the activity of this apoenzyme preparation (Table III), but the increase was only one-fourth that obtained with FMN. Incubation of the preparation with GFP prior to the addition of FMN gave the same activity as FMN alone.

The apoenzyme preparation of yeast TPNH dehydrogenase had only 0.5% of the activity of the holoenzyme. Recombination of the apoenzyme with FMN increased the activity 45-fold (Table III). GFP increased the activity only 4-fold, but the effect was reproducible. It has been found that GFP will also partially reactivate the apoenzyme of purified microsomal cytochrome \(b_1\), reductase.

**DISCUSSION**

From the results it is evident that the presence of galactoflavin in the diet can accelerate the onset of riboflavin deficiency in animals on a riboflavin-free diet and can convert a marginal riboflavin diet into a deficient one, but it cannot depress FMN, FAD, or flavin enzyme activities below the rigid lower levels characteristic of simple riboflavin deficiency. This serves to emphasize the extreme tenacity with which tissues hold on to a definite fraction of the normal flavin complement.

The evidence given does not establish the mechanism of the galactoflavin effect, but chromatographic analysis has ruled out replacement of tissue riboflavin by galactoflavin. If galactoflavin is not incorporated into the tissues, there are at least four ways in which it might act: (a) it might inhibit riboflavin absorption from the gut; (b) it might inhibit phosphorylation of riboflavin; (c) it might interfere with uptake of riboflavin by the tissues; or (d) it might accelerate riboflavin excretion. Because galactoflavin accelerates the onset of riboflavin deficiency in rats on a riboflavin-free diet, the first possibility could not alone explain the results. Data of Kearney (2) and McCormick (23) show that galactoflavin does not inhibit phosphorylation of riboflavin or itself undergo phosphorylation by purified yeast or liver flavokinase. In vivo the phosphorylation might be prevented in a more complex manner. Normal plasma riboflavin levels are exceedingly low, only 0.12% of liver flavin levels (24). Therefore, galactoflavin might reasonably interfere with transport of riboflavin into the cells. Attempts to measure the effect of galactoflavin on riboflavin excretion were inconclusive because of the enormous disparity between the amounts of riboflavin and galactoflavin excreted. Nevertheless, it seems very likely that the kidneys might not be able to distinguish completely between riboflavin and galactoflavin.

The studies *in vitro* with GFP do not contribute directly to the question of mechanism of action, but they do show that if the antagonist were phosphorylated, it could replace FMN as a coenzyme at least partially. These latter studies also underline the close similarity in properties of riboflavin and galactoflavin.

**SUMMARY**

Rate fed galactoflavin and no riboflavin for 5 weeks weighed 66% as much as those on a simple riboflavin-free diet, but in the former group there were only minor reductions in the activities of flavin-containing enzymes and in the flavin levels of liver and kidney when compared to the amounts found in riboflavin deficiency per se. In contrast, rats fed galactoflavin plus marginal amounts of riboflavin developed clinical signs and hepatic enzyme and flavin levels characteristic of complete riboflavin deficiency. Riboflavin coenzymes are not replaced in the tissues by galactoflavin analogues.

The galactoflavin monophosphate analogue of flavin mononucleotide, has been shown capable of partially reactivating the *apoenzymes* of yeast and liver reduced triphosphopyridine nucleotide dehydrogenases. The apoenzyme of liver glycolic acid oxidase was not reactivated by the analogue.

**REFERENCES**

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