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, Wurte, 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 sufficient 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-aminolevulinic acid oxidase, glyceraldehyde-3-phosphate dehydrogenase, and reduced diphosphopyridine nucleotide dehydrogenase. 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 anologue 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 FMN determinations were made by a fluorometric method (4, 6) on 10% trichloroacetic 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 in...*
incubated for 15 or 20 minutes in an atmosphere of oxygen with 
1000-fold during incubation.

derivatives by the absorption at 305 mp (5, 11, 12). The pro-
ketoads formed were determined as 3-hydrazinoquinoline

Table I

<table>
<thead>
<tr>
<th>Activity with dietary supplement*</th>
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<tbody>
<tr>
<td>None</td>
</tr>
<tr>
<td>Liver</td>
</tr>
<tr>
<td>Glycolic acid oxidase</td>
</tr>
<tr>
<td>Substrate</td>
</tr>
<tr>
<td>Substrate + FMN</td>
</tr>
<tr>
<td>L-Amino acid oxidase</td>
</tr>
<tr>
<td>Substrate</td>
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<tr>
<td>Substrate + FMN</td>
</tr>
<tr>
<td>D-Amino acid oxidase</td>
</tr>
<tr>
<td>Substrate</td>
</tr>
<tr>
<td>Substrate + FAD</td>
</tr>
<tr>
<td>DPNH dehydrogenase</td>
</tr>
<tr>
<td>Utyochrome c</td>
</tr>
<tr>
<td>Ferriyianide</td>
</tr>
<tr>
<td>FAD</td>
</tr>
<tr>
<td>FMN</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.

centrifuged without further treatment, and the flavins were con-
centrated from the supernatant fluid and chromatographed (see below). Homogenates of other 1-g samples after the acid treat-
ment were brought to pH 5.5 with 3 ml of 4 M potassium acetate
solution and centrifuged. The supernatant fluid was incubated for
18 hours at 38° with 0.2 ml of a preparation of human seminal
acid phosphatase (6) with activity toward p-nitrophenyl phos-
phate of 30

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%.
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 vivo, 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, p-amino acid oxidase, and TPNH dehydroge
enase were all reduced by the antagonist to levels characteristic
of severe deficiency. Once again DPNII 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 apoenzyme
system did not restore more than a small percentage of the normal
enzyme activities (Tables I and II).5

Search for Galactoflavin Compounds in Tissuesthe 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
0.05 µg of galactoflavin per g of liver or kidney (0.2 µg 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 Apenzymes of Flavoproteins—Addition of the natural coenzyme, FMN, to the apoen-
zyme of purified glycolic 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 µM. Horecker (22) tested both coen-
zymes 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. Recombina-
tion 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 b1 reductase.2

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

It seems very unlikely that these results could be explained by
the presence of a galactoflavin inhibitor. Furthermore, during
the assays homogenates were diluted 25- to 1000-fold. No more
than traces of galactoflavin or its derivatives could be found in
the tissues (see below).

P. Strittmatter, personal communication.

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 galacto-
flavin is not incorporated into the tissues, there are at least four
ways in which it might act: (a) it might inhibit riboflavin absorp-
tion from the gut; (b) it might inhibit phosphorylation of ribo-
flavin; (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 pre-
vented 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 trans-
port 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 under-
line the close similarity in properties of riboflavin and galacto-
flavin.

SUMMARY

Rats 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 ac-
tivities of flavin-containing enzymes and in the flavin levels of
liver and kidney when compared to the amounts found in ribo-
flavin 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 mono-
nucleotide, 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.

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