THE EFFECT OF RIBOFLAVIN ANALOGUES UPON THE UTILIZATION OF RIBOFLAVIN AND FLAVIN ADENINE DINUCLEOTIDE BY LACTOBACILLUS CASEI*

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Since new analogues of riboflavin have been synthesized which inhibit the growth of rats and of microorganisms which require riboflavin (1–3), it was decided to test the effect of some of these compounds upon the utilization of riboflavin and flavin adenine dinucleotide (FAD) for growth of Lactobacillus casei. Other compounds structurally related to riboflavin were also studied.

It has been shown that isoriboflavin, 5,6-dimethyl-9-(d-1’-ribityl)-isoalloxazine, which inhibits the growth of rats on diets free of or low in riboflavin (3), has less than 0.5 per cent of the activity of riboflavin for Lactobacillus casei and has no effect upon acid production by this organism in the presence of maximal amounts of riboflavin (4). The present experiments show that isoriboflavin markedly stimulates acid production by Lactobacillus casei in the presence of suboptimal levels of riboflavin or FAD. Snell and Strong (5) observed similar effects of other riboflavin analogues on the growth of lactic acid bacteria.

The ribitylamino compound, 1-ribityl-2-amino-4,5-dimethylbenzene, also has slight riboflavin-like activity and an augmenting effect upon the utilization of riboflavin. However, the addition of alloxan (which can be condensed synthetically with the ribitylamino compound to form riboflavin (6)) permits better utilization of the ribitylamino compound and suggests some synthesis of riboflavin by the bacteria.

The diaminophenazine compound (2,4-diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine), synthesized by Woolley, competitively inhibits the utilization of riboflavin for growth of Lactobacillus casei, as was reported (2) and has the same effect upon the use of FAD.

Lumiflavin, 6,7,9-trimethylisoalloxazine, has both an inhibitory and a stimulatory action upon the growth of Lactobacillus casei. In the presence of relatively large amounts of lumiflavin the growth with riboflavin is

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inhibited. As the amount of riboflavin is increased, a zone is reached in which the acid production is increased in the presence of lumiflavin. However, lumiflavin inhibits the utilization of FAD for growth of *Lactobacillus casei* at much lower concentrations than are required for inhibition of riboflavin activity. In this respect the data presented on inhibition by lumiflavin are similar to those reported for thiamine and diphosphothiamine inhibition by pyrithiamine (7).

**EXPERIMENTAL**

*Methods*

The experiments were carried out with *Lactobacillus casei*, and either the alkali-treated peptone medium of Snell and Strong (8) or the hydrolyzed casein medium of Landy and Dicken (9). The biotin content of the latter medium was decreased to one-fifth of that of the original, since it was found to be present in large excess. Growth was determined by *pH* measurements after 24 hours of incubation at 37° or by titration of the acid produced in 72 hours. For experiments involving titration of acid produced, the glucose concentration of the medium was doubled and the acetate content tripled to provide better growth curves (10). However, for best results with *pH* values the original concentrations of glucose and acetate were used.

Graded amounts of riboflavin or FAD and other substances to be tested (at *pH* 6.8) were measured into tubes, diluted to 5 ml., and 5 ml. of medium added. (Some of the compounds studied were sterilized by filtration and added to the cooled tubes after autoclaving, but prior to inoculation.) The tubes were covered with glass caps, autoclaved, and each inoculated with 1 drop of a suspension of *Lactobacillus casei*, prepared by diluting 1 ml. of an actively growing 20 to 24 hour culture with 15 ml. of sterile saline. Unnecessary exposure of all solutions to light was avoided.

*Results*

*Isoriboflavin (5,6-Dimethyl-9-(d-1'-ribityl)-isoalloxazine)—*The utilization of isoriboflavin¹ and its effect upon growth with suboptimal amounts of riboflavin and FAD were studied with the peptone and casein media. Table I shows the riboflavin-like activity found on both media with 0 to 0.1 γ of added riboflavin in the presence of 1 to 300 γ of isoriboflavin. The response obtained with isoriboflavin alone is not proportional to the amount present. It is not known whether any part of this activity is due to traces

¹ A sample of isoriboflavin was generously supplied through the courtesy of Dr. Gladys A. Emerson and her associates at the Merck Institute for Therapeutic Research.
of riboflavin in the isoriboflavin. With the peptone medium the per cent activity of isoriboflavin (compared to riboflavin) decreases from 0.4 to 0.01 as the amount of isoriboflavin is increased from 1 to 300 \( \gamma \). Conversely, the per cent activity of isoriboflavin added to the casein medium increases from 0.01 to 0.05 as the level of isoriboflavin is increased to 300 \( \gamma \). The peptone medium supports more growth in the blank tubes than does the casein medium (Fig. 1). This is presumably due to the presence of more riboflavin in the basal peptone medium than in the casein medium.

**Table I**

*Stimulation by Isoriboflavin of Utilization of Riboflavin for Growth of Lactobacillus casei*

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>Isoriboflavin (( \gamma ))</th>
<th>Riboflavin present</th>
<th>Riboflavin-like activity found*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0 ( \gamma )</td>
<td>0.025 ( \gamma )</td>
<td>0.05 ( \gamma )</td>
</tr>
<tr>
<td>Peptone†</td>
<td>1</td>
<td>0.004</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0.025</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.06</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.07</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.11</td>
<td>0.22</td>
</tr>
<tr>
<td>Casein†</td>
<td>10</td>
<td>0.001</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.01</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0.06</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0.14</td>
<td>0.27</td>
</tr>
</tbody>
</table>

* Measured by the acid produced in 72 hours. All values are given in terms of microgram per tube (10 ml.).
† See the text.

Since the apparent activity of isoriboflavin is increased in the presence of small amounts of riboflavin, this may partially account for the greater response to the smaller amounts of isoriboflavin on the peptone medium.

In the presence of added riboflavin, however, isoriboflavin stimulates acid production more with the casein medium than with the peptone medium and shows a greater riboflavin-like activity. This may be seen in Table I and Fig. 1. Fig. 1 shows the acid production of *Lactobacillus casei* in the presence of graded amounts of riboflavin on both media, and with 10 \( \gamma \) of isoriboflavin per tube. The same effects of isoriboflavin are obtained when FAD is substituted for riboflavin. The difference in stimulatory action of isoriboflavin on the two media may be due to differences in the protein degradation products in the two media, as is suggested by the following experiments on riboflavin assay of foods and tissues. Isoriboflavin was added to the basal medium (10 \( \gamma \) per tube) in an attempt to increase
the sensitivity of *Lactobacillus casei* to riboflavin (Fig. 1) and to permit more accurate assay of low potency foodstuffs and tissues. The stimulation of isoriboflavin decreased as increasing amounts of food extracts (enzymatic digests or acid hydrolysates) were added, resulting in a "downdrift" of riboflavin values at higher levels of assay. This "downdrift" was greater when the casein medium was employed and was especially marked when foods of high protein content were analyzed.

*Ribitylamino Compound (1-Ribitylamino-2-amino-4,5-dimethylbenzene) and Alloxan*—The ribitylamino compound\(^2\) has about 0.003 per cent of the activity of riboflavin when incorporated into the medium at levels of 200 and 400 \(\gamma\) per tube (Table II). In the presence of graded suboptimal amounts of riboflavin, its riboflavin-like activity increases. Alloxan, which can be added to the ribitylamino compound to produce riboflavin synthetically (6), cannot replace riboflavin for growth of *Lactobacillus casei* and has no effect upon its utilization of riboflavin. However, when both the ribitylamino compound and alloxan are added, activities as high as 0.35 per cent of that of riboflavin are obtained (based upon the ribitylamino compound added). Table II shows the effect of the addition of mixtures of these two compounds either before or after autoclaving. The growth obtained with no added riboflavin and the stimulation in the presence of riboflavin are decreased by autoclaving the ribitylamino compound

\(^2\) The ribitylamino compound was made available by Dr. H. M. Wuest of Hoffman-La Roche, Inc. This compound was also used in the synthesis of the diaminophenazine analogue of riboflavin.
and alloxan. In the presence of 10 $\gamma$ of the ribitylamino compound, the molecular equivalent of alloxan (5 $\gamma$ of the monohydrate) produces the same effect as does a large excess of alloxan (100 $\gamma$). The effect of these compounds is the same in the presence of FAD or of riboflavin. Foster (4) has shown that this ribitylamino compound is oxidized by Pseudomonas riboflavina 63 per cent as rapidly as is riboflavin. This compound competes with riboflavin for the active centers on the riboflavin-oxidizing enzyme of this organism (4).

Diaminophenazine Compound (2,4-Diamino-7,8-dimethyl-10-ribityl-5,10-dihydrophenazine)—The diaminophenazine compound was synthesized by the method of Woolley (2), who has shown that this compound competitively inhibits the utilization of small amounts of riboflavin for growth of Lactobacillus casei. The present experiments (Table III) show that riboflavin and FAD are inhibited to the same extent by the diaminophenazine. The inhibition is competitive and is overcome by excess riboflavin or FAD. The inhibition by the diaminophenazine differs from that of lumiflavin (shown below) in that the effects on riboflavin and FAD are the same.

It is interesting to note that the data in Table III show an inhibition of a rather constant amount of riboflavin (about 0.019 $\gamma$) by 0.5 mg. of the diaminophenazine in each instance. Similarly, the inhibition of FAD

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### Table II

**Stimulation by Ribitylamino Compound and Alloxan of Utilization of Riboflavin for Growth of Lactobacillus casei**

<table>
<thead>
<tr>
<th>Ribitylamino compound</th>
<th>Alloxan monohydrate</th>
<th>Riboflavin-like activity found*</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\gamma$</td>
<td>$\gamma$</td>
<td>$0.00 \gamma$</td>
</tr>
<tr>
<td>200</td>
<td>$0.009$</td>
<td>0.036</td>
</tr>
<tr>
<td>400</td>
<td>$0.018$</td>
<td>0.044</td>
</tr>
<tr>
<td>50</td>
<td>$0.00$</td>
<td>0.025</td>
</tr>
<tr>
<td>25</td>
<td>$0.05$</td>
<td>0.05</td>
</tr>
<tr>
<td>25†</td>
<td>$0.12$</td>
<td>0.17</td>
</tr>
<tr>
<td>50</td>
<td>$0.10$</td>
<td>0.15</td>
</tr>
<tr>
<td>10</td>
<td>$0.00$</td>
<td>0.058</td>
</tr>
<tr>
<td>10</td>
<td>$0.00$</td>
<td>0.056</td>
</tr>
</tbody>
</table>

* The casein medium was used and the riboflavin-like activity was determined by pH measurements after 24 hours.

† In this experiment the ribitylamino compound and the alloxan were sterilized by filtration and added to the test after autoclaving.
Sample I by lumiflavin (presented in Table IV) is also constant in each series, and is what would be expected if 25 and 50 \( \gamma \) of lumiflavin counter-

### Table III

**Inhibition by Diaminophenazine Compound of Utilization of Riboflavin and Flavin Adenine Dinucleotide (FAD) for Growth of Lactobacillus casei**

<table>
<thead>
<tr>
<th>Riboflavin or FAD added*</th>
<th>Inhibition of growth in presence of 0.5 mg. diaminophenazine per tube†</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma )</td>
<td>Riboflavin</td>
</tr>
<tr>
<td>0.0</td>
<td>100‡</td>
</tr>
<tr>
<td>0.02</td>
<td>80</td>
</tr>
<tr>
<td>0.04</td>
<td>55</td>
</tr>
<tr>
<td>0.06</td>
<td>28</td>
</tr>
<tr>
<td>0.08</td>
<td>24</td>
</tr>
<tr>
<td>0.12</td>
<td>17</td>
</tr>
</tbody>
</table>

* The amounts of riboflavin and FAD are given in terms of riboflavin (microgram per tube) for comparison on a molecular basis.
† Diaminophenazine was sterilized by filtration and added after autoclaving. The peptone medium was used and growth measured by pH readings after 24 hours.
‡ The final pH of these cultures was the same as the uninoculated medium. Inoculated blank tubes in the absence of inhibitors show a pH change of 0.2 to 0.3.

### Table IV

**Stimulatory and Inhibitory Effects of Lumiflavin upon Utilization of Riboflavin and Flavin Adenine Dinucleotide (FAD) for Growth of Lactobacillus casei**

<table>
<thead>
<tr>
<th>Riboflavin or FAD added*</th>
<th>Activity in presence of lumiflavin†</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \gamma )</td>
<td>25 ( \gamma ) lumiflavin</td>
</tr>
<tr>
<td></td>
<td>Riboflavin</td>
</tr>
<tr>
<td>0.025</td>
<td>125</td>
</tr>
<tr>
<td>0.05</td>
<td>120</td>
</tr>
<tr>
<td>0.1</td>
<td>120</td>
</tr>
<tr>
<td>0.15</td>
<td>120</td>
</tr>
<tr>
<td>0.20</td>
<td>120</td>
</tr>
</tbody>
</table>

* The amounts of riboflavin and FAD are given in terms of riboflavin (microgram per tube) for comparison on a molecular basis.
† The casein medium was used and growth measured by pH readings after 24 hours.
‡ Sample I of FAD was kindly supplied by Dr. Margaret E. Greig of Vanderbilt University, Sample II by Dr. Oliver H. Lowry of The Public Health Research Institute of the City of New York, Inc.

acted the effect of 0.025 and 0.05 \( \gamma \) of FAD, respectively, at each level at which inhibition is obtained.
Lumiflavin (6,7,9-Trimethylisoalloxazine)—Lumiflavin was prepared from riboflavin by alkaline photolysis according to the method of Warburg and Christian (11). This compound has both an inhibitory and a stimulatory action upon the use of riboflavin or FAD for growth of Lactobacillus casei (Table IV). With both the casein and peptone media (24 or 72 hour readings) relatively large ratios of lumiflavin to riboflavin curtail acid production, whereas with smaller ratios (more riboflavin or less lumiflavin), lumiflavin has an augmenting effect. When FAD is supplied to the organism in place of riboflavin, lumiflavin inhibits growth at much lower ratios than are required for riboflavin inhibition (Table IV). This is not due to the inability of L. casei to use FAD as well as riboflavin, since in the absence of inhibitors L. casei utilizes equivalent amounts of riboflavin, riboflavin phosphate, and FAD equally well for growth and acid production.

The data in Table IV show the effect of lumiflavin upon the utilization of two samples of FAD. These were analyzed for riboflavin microbiologically and were found to contain not more than 2.5 and 10 per cent of FAD, provided all of the riboflavin present was bound as FAD. It is likely that some free riboflavin or riboflavin phosphate was present, especially in Sample I, since this was not inhibited by lumiflavin to the same extent as was Sample II. It was observed that the inhibition by lumiflavin decreased when solutions of FAD were kept in the refrigerator for a few weeks.

The greater inhibition by lumiflavin of the utilization of FAD than of riboflavin by Lactobacillus casei is analogous to the increase in inhibition by pyrithiamine when diphosphothiamine replaces thiamine as a growth factor for Lactobacillus fermenti (7) and suggests that similar mechanisms may exist for the phosphorylation of these vitamins. Since riboflavin is converted to riboflavin phosphate or to FAD for use in cellular enzymes (12), part of the inhibition of riboflavin activity may be due to blocking of the conversion of riboflavin to one of its conjugated forms. However, lumiflavin inhibits the utilization of FAD more than of free riboflavin. This suggests that lumiflavin competes with both riboflavin and FAD at the place of attachment of the riboflavin moiety to the protein portion of the enzymes, and that in the presence of lumiflavin, riboflavin has a greater affinity for these centers than does FAD. It also indicates that riboflavin can be converted to FAD after it is attached to an enzyme protein.

Hydrolysis of FAD—For assay of riboflavin by microbiological or fluorometric methods, samples are usually hydrolyzed first by dilute acid or digested enzymatically (13, 14). Similar values are obtained by both methods. Abraham (15) has shown that dilute acid hydrolysis splits FAD into riboflavin phosphate and adenyllic acid. Experiments with Lactobacillus casei were performed to determine the effect of lumiflavin upon the utilization of riboflavin phosphate prepared in this manner, and to ascertain
the extent of digestion of FAD by a mixture of papain and taka-diastase. The results in Table V show that acid-hydrolyzed FAD (riboflavin phosphate) is inhibited less by lumiflavin than is the original FAD. Mono-phosphothiamine is similarly inhibited less by pyrithiamine than is diphosphothiamine (7). Enzymatic digestion of FAD results in a preparation which responds to lumiflavin-like free riboflavin. Since riboflavin, riboflavin phosphate, and FAD are utilized equally well by Lactobacillus casei, and since the fluorescence of riboflavin and riboflavin phosphate is the same,\(^3\) riboflavin values obtained microbiologically or fluorometrically should be the same if complete extraction is obtained by acid or enzyme digestion. However, FAD provides only about 20 per cent of the fluorescence of riboflavin or of riboflavin phosphate\(^3\) at pH 5, and should be split by one of these methods before fluorometric analysis.

**DISCUSSION**

Although isoriboflavin competitively inhibits the utilization of riboflavin for the growth of rats (3), it is not oxidized at all by the riboflavin-oxidizing enzyme of *Pseudomonas riboflava*na (4) and it stimulates growth with riboflavin for *Lactobacillus casei*. Analogues of other vitamins have also been shown to differ in their effects upon different animals and bacteria (16, 17). The ribitylamino compound has an effect similar to that of isoriboflavin upon the growth of *L. casei*, but to a much smaller extent. This

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\(\text{\textsuperscript{3}}\) Personal communication from Dr. Oliver H. Lowry.
compound, however, is oxidized by *Pseudomonas riboflavina* at 63 per cent of the rate of riboflavin oxidation (4). Upon the addition of alloxan, *L. casei* increases its utilization of the ribitylamino compound from 0.003 per cent of that of riboflavin to 0.35 per cent, suggesting some synthesis of riboflavin from the ribitylamino compound and alloxan.

Banerjee, Dittmer, and du Vigneaud (18) have presented a method for assay of alloxan by condensation with an excess of the ribitylamino compound, and measurement of the riboflavin formed by microbiological or fluorometric techniques. According to the present findings, bacterial growth would be stimulated by the excess ribitylamino compound and by the riboflavin formed. The data of Table II suggest that the preliminary condensation may be omitted and that alloxan may be measured by the growth of *Lactobacillus casei* on a medium containing a known amount of the ribitylamino compound. With both methods adequate correction for the original riboflavin content of the samples would be necessary.

Isoriboflavin and the ribitylamino compound produce the same stimulatory effects when FAD is supplied to the organism in place of riboflavin. The diaminophenazine compound which inhibits the utilization of riboflavin by *Lactobacillus casei* (2) curtails the use of FAD to the same extent. It appears that these three compounds have their main effects upon the use of the riboflavin-containing enzymes and have little or no effect upon the conversion of riboflavin to FAD.

With different ratios of lumiflavin to riboflavin, there are found inhibitory and stimulatory effects of lumiflavin upon the growth of *Lactobacillus casei*. FAD is inhibited by lower concentrations of lumiflavin than is riboflavin. This is analogous to the stronger inhibition by pyridinesulfonic acid of the use of cozymase than of nicotinic acid or amide (19) and to the increased inhibition by pyrithiamine of the utilization of diphosphothiamine than of thiamine (7). All three of these inhibitors seem to compete with the linkage of the respective vitamin or of its coenzyme to the enzyme proteins.

Kuhn and Rudy (20) have postulated that riboflavin phosphate is attached to the enzyme protein at two places; namely, by the 3-imino group in the isoalloxazine ring and through the phosphoric acid group. Diphosphothiamine also appears to be attached to its protein at two points; namely at the 6-amino group of the pyrimidine ring and a phosphoric acid group (21). On the basis of the greater inhibition of the utilization of diphosphothiamine than thiamine by pyrithiamine and by 6-aminopyrimidines, it has been suggested that thiamine is attached to its apoenzyme before being phosphorylated (7). The inhibition of riboflavin and FAD by lumiflavin is similar to the thiamine inhibition and suggests that riboflavin is also attached to its enzyme protein at the 3-imino position before it
is converted to riboflavin phosphate or to FAD. If riboflavin were converted to FAD before being attached to its protein, the utilization of riboflavin should be inhibited by lumiflavin to an equal or greater extent than is found for FAD. Since lumiflavin inhibits the use of FAD more than it does of riboflavin, it appears that the 3-imino group of riboflavin may have a greater affinity for the enzyme proteins than does that of FAD. Although Kuhn and Rudy (20) have shown that riboflavin phosphate is bound to its enzyme more firmly than is free riboflavin, this is due to its two bindings to the protein. Lumiflavin competes only with the linkage of the 3-imino group to the enzyme, and it is the affinity of this group that may differ in the three forms of riboflavin.

In the case of carboxylase, Westenbrink et al. (21) have shown that the naturally occurring form contains diphosphothiamine which is tightly bound to protein, whereas in the reconstructed enzyme (formed by addition of diphosphothiamine to alkaline washed yeast) the linkage is highly dissociable. The intact enzyme is also more active than the reconstituted form (21). Ratner, Nocito, and Green (22) have isolated a flavoprotein enzyme, glycine oxidase, in an undissociated form which is about 4.5 times as active (on the basis of FAD) as the reconstructed enzyme. These authors conclude that the kinetics of combination determine the rate of activity (22). These further similarities between riboflavin and thiamine enzymes make it appear likely that riboflavin, like thiamine, is attached to an enzyme protein before it is converted to riboflavin phosphate or to FAD.

SUMMARY

The effects of various analogues of riboflavin upon the utilization of suboptimal amounts of riboflavin and flavin adenine dinucleotide (FAD) by Lactobacillus casei have been studied.

Isoriboflavin and 1-ribitylamino-2-amino-4,5-dimethylbenzene possess little riboflavin-like activity by themselves, but are able to stimulate the utilization of riboflavin and FAD by Lactobacillus casei. In the presence of alloxan the riboflavin-like activity of the ribitylamino compound is markedly increased.

The diaminophenazine analogue of riboflavin competitively inhibits the utilization of riboflavin and FAD to the same extent.

Lumiflavin can either inhibit or stimulate the use of riboflavin or FAD by Lactobacillus casei, depending upon the relative amounts of lumiflavin present. The inhibition by lumiflavin is much greater when FAD is supplied as a growth factor in place of riboflavin. The data suggest that riboflavin may be attached to an apoenzyme before it is converted to riboflavin phosphate or to FAD.
Lactobacillus casei utilizes riboflavin, riboflavin phosphate, and FAD equally well for growth and acid production. FAD is hydrolyzed to riboflavin phosphate by weak acid, and to riboflavin by enzymatic digestion.

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THE EFFECT OF RIBOFLAVIN ANALOGUES UPON THE UTILIZATION OF RIBOFLAVIN AND FLAVIN ADENINE DINUCLEOTIDE BY LACTOBACILLUS CASEI
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