THE PREPARATION AND BIOLOGICAL ACTIVITY OF SOME RIBOFLAVIN DERIVATIVES

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The quest for soluble derivatives of riboflavin has resulted in the description of several compounds of this nature. Relatively few of these have been well characterized, however, from the standpoint of biological or microbiological activity, due, in part, to the circumstance that some of these preparations were described before the advent of refined assay methods. Snell and Strong (1) reported careful microbioassays of a number of synthetic flavins, which had been prepared and bioassayed in various laboratories. They concluded that “insofar as the selection of active or inactive compounds is made the basis for comparison, the correlation between the rat assay and the present bacterial test seems rather close. A comparison of the degree of activity is at present rendered impossible by the paucity of data concerning the relative potency of the various active flavins on rats.” We have found no later efforts which attempt to relate these types of assay to flavin structure. The present report concerns such comparative measurements on a number of soluble riboflavin derivatives.

The riboflavin molecule offers two general possibilities for ready substitution, viz. in the ring at the imino nitrogen or in the ribose chain. The former possibility was explored by Kuhn and Rudy (2), who found that such compounds were biologically inactive and did not fluoresce. Substitution in the ribose chain, however, can result in biologically active derivatives. Those which have been described can be classified into two main groups, esters and acetals. Included among the esters which have been described are the phosphate (3–5), acetates (4, 6–8), borate (9), and tetrabenzoate (9). The phosphate and acetates were shown to be biologically active, but no tests for activity are recorded for the boric acid and tetrabenzoic acid esters. The present report presents data on the preparation and activity of succinic acid esters. Included among the acetals are the mono- and diacetone derivatives described by Kuhn et al. (10), who claimed that the diacetone compound is active (6). It will be shown in the present report that two other compounds of the acetal type, obtained by condensation of chloral and of levulinic acid with riboflavin, have no vitamin activity.

Preparation of Compounds—A detailed account of the methods of prep-
aration will be found in the experimental section. Essentially, the succinic acid esters were prepared by condensation of riboflavin with succinic anhydride in pyridine. The extent of substitution was controlled by varying the molecular ratio of succinic anhydride to riboflavin from 1 to 4.  

The acetics were made according to a procedure of Coles, Goodhue, and Hixon (11) for the preparation of chloraloses, in which a polyhydroxy compound (riboflavin) is treated in sulfuric acid solution with excess aldehyde or ketone (chloral, levulinic acid).

**Assay Methods**—All compounds were assayed by fluorometric (12), microbiological (13), and biological (rat) (14) procedures. For bioassay, Labco rice polish was substituted for the rice bran concentrate used by Street (14).

<table>
<thead>
<tr>
<th>treatment</th>
<th>method of measurement</th>
<th>mono</th>
<th>di</th>
<th>tri</th>
<th>tetra</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoclaved 20 min. at 15 lbs.</td>
<td>Turbidimetric</td>
<td>62</td>
<td>33</td>
<td>11</td>
<td>5.3</td>
</tr>
<tr>
<td>Filtered through glass</td>
<td>Titrimetric</td>
<td>63</td>
<td>33</td>
<td>12</td>
<td>5.5</td>
</tr>
</tbody>
</table>

In order to minimize hydrolysis of the compounds studied, the following precautions were observed.

The solutions of the compounds were prepared at room temperature. The final solutions used for microbioassay were prepared aseptically by filtration through fritted glass bacterial filters.  

Aliquots of these filtrates were then added by sterile pipette to the autoclaved medium. Comparative trials by this and the orthodox autoclave method (Table I) showed distinctly higher values by the latter, which can be ascribed only to hydrolysis.

The microbioassays were read both after 1 day at 37° (turbidimetric) and after 3 days (titrimetric) in order to ascertain whether the longer

1 This method of preparation provides no guarantee of the homogeneity of the di- and trisuccinates, but rather a predominance of one particular homologue. The presence of small amounts of other homologues does not vitally affect the validity of the vitamin assays by the various methods, but may exert considerable influence on the solubility measurements. Solubility data are presented nevertheless because replicate preparations of the compounds gave results of the same order of magnitude.

2 Seitz filters were tried first. It was found that considerable adsorption of the various flavins occurred on the Seitz filter pads. Filtration of large volumes of the solutions provided no relief, since the pads displayed an extensive capacity for adsorption.
incubation at 37° caused any hydrolysis. As shown in Table I, no appreciable effect was found for the succinate derivatives. These data also show that the rate of utilization of the active compounds by Lactobacillus casei was the same as for riboflavin, even though the extent of utilization did not reach theoretical values for any of them.

Activity of Riboflavin Succinates—Fig. 1 shows the levels of response obtained with the three assay methods in relation to the extent of substitution with succinic acid. The fluorometric values are in good agreement with the theoretical in all cases, and hence can serve as a measure of the concentration of these compounds. Bioassay showed the mono-

succinate to be fully active, the disuccinate partially active, the tri-succinate slightly active, and the tetrasuccinate completely inactive. The microbiological values ran parallel to but distinctly lower than the biological.

The biological inactivity of the tetrasuccinate, which we observed in both curative and prophylactic trials, may be contrasted with the reported

3The monosuccinate was found to be fully active both when administered by mouth and by intramuscular injection. The di- and trisuccinates were tested by feeding only, while the tetrasuccinate was found to be inactive both by mouth and by injection.
activity of the tetraacetate (15). The inactivity of the latter for lactic acid bacteria (1) suggests that its activity in the rat is due to hydrolysis in the animal. It would appear, therefore, that the tetrassuccinate is not hydrolyzed by the rat to the more active lower homologues or to riboflavin.

It is more difficult to interpret the low microbiological results, particularly with regard to the monosuccinate, which is completely active by bioassay. In an attempt to explain these data, the following aspects were studied on the monosuccinate but found not to influence the low values. (1) The age of the culture. Rapidly growing, young cultures, obtained by serial daily transfer for 5 days, gave the same response as an older culture transferred from stock 24 hours before use, the stock culture being transferred monthly. (2) The presence in the riboflavin standard of succinic acid in amounts equal to or twice as great as the amount which would be derived from the monosuccinate on complete hydrolysis. (3) Enzyme treatment with 2 per cent of clarase (16). However, complete hydrolysis leading to theoretical riboflavin values was achieved by heating in N HCl at 100° for 30 minutes. Complete hydrolysis was also obtained by heating in 0.1 N HCl at 15 pounds pressure (122°) for 30 minutes, which is the extraction procedure of the United States Pharmacopoeia (17).

Since we have been unable to elicit any analytical cause for the low microbiological responses, we must conclude for the present that these succinate derivatives cannot be utilized as fully by Lactobacillus casei as by the rat. There is some precedent for this finding in the work of Snell and Strong (1), who found that four of a group of thirteen flavins examined supported growth of Lactobacillus casei or Streptococcus faecalis as the sole source of flavin. Of these four, only one, however, 6-ethyl-7-methyl-9-(d,1'-ribityl)-isoalloxazine, approached the activity of riboflavin, while the other three showed lower quantitative responses. From the standpoint of bioassay, however, all four were classified as "active on rats." The similarity ends there, since none of the four had substituents in the ribose chain.

Solubility of Riboflavin Succinates—Solubility was determined by mechanical shaking with excess solute at 25° for 10 hours, filtering, and determining the concentration of solute in the filtrate by the fluorometric method; as previously noted, Fig. 1 shows that the fluorometric assay is adequate for this purpose. Fig. 2 demonstrates the marked increase in solubility in water that occurs as the number of succinate substituents increases. Since these compounds have free carboxyl groups, salts may be prepared. It was found that the sodium, monoethanolamine, and diethanolamine salts were even more soluble than the corresponding esters. Since riboflavin monosuccinate is the only member of the series which is fully active by bioassay, the solubility of this compound and its sodium
salt were studied more extensively. Representative data are shown in Table II.

**Acetals**—The condensation products of riboflavin with chloral and with levulinic acid were fluorescent but completely inactive by both microbiological and biological assays. The inactivity of these acetals may be con-

![Graph showing solubility of riboflavin succinates](http://www.jbc.org/)

**TABLE II**

*Solubility of Riboflavin Monosuccinate in Various Solvents at 25°*

All values are expressed in terms of riboflavin, mg. per 100 cc.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Riboflavin</th>
<th>Riboflavin monosuccinate</th>
<th>Sodium salt of riboflavin monosuccinate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>11</td>
<td>105</td>
<td>250</td>
</tr>
<tr>
<td>Ethanol (95%)</td>
<td>4.5</td>
<td>30</td>
<td>24</td>
</tr>
<tr>
<td>Glycerol</td>
<td>25</td>
<td>176</td>
<td>350</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>22</td>
<td>250</td>
<td>580</td>
</tr>
</tbody>
</table>

Contrasted with the claim (6) that diacetone riboflavin is biologically active, the latter being the only acetal for which activity has been reported, we were unable to find the data upon which Kuhn (6) based the claim for diacetone riboflavin, and hence must reserve judgment on relationships among acetals as a group.
RIBOFLAVIN DERIVATIVES

EXPERIMENTAL

Riboflavin Monosuccinate—1 gm. of riboflavin was refluxed with 300 mg. of succinic anhydride (1.1 moles) in 200 cc. of dry pyridine. The mixture became homogeneous after 2 to 2.5 hours; heating was continued for 1 hour thereafter. The pyridine was then distilled off in vacuo; the residue was completely freed of pyridine by drying in vacuo at 100°, and then recrystallized from water. 800 mg. of a yellow substance were obtained, melting at 245°, corrected, with decomposition.

Analysis—C$_{21}$H$_{29}$O$_4$N$_4$
Calculated. C 52.91, H 5.05, riboflavin 79.0
Found. " 52.55, " 5.19, " (fluorometric) 78

Riboflavin Disuccinate—2 gm. of riboflavin were refluxed with 1.2 gm. of succinic anhydride (2.2 moles) in 80 cc. of dry pyridine for 3 hours. The pyridine was distilled off in vacuo and the residue recrystallized from water. 2 gm. of yellow substance resulted, m.p. 223°, corrected, with decomposition.

Analysis—C$_{26}$H$_{32}$O$_6$N$_4$.
Calculated. C 52.06, H 4.90, N 9.72, riboflavin 65.2
Found. " 51.72, " 5.50, " 10.00, (fluorometric) 68

Riboflavin Trisuccinate—2 gm. of riboflavin were refluxed with 1.8 gm. (3.3 moles) of succinic anhydride in 30 cc. of dry pyridine for 3 hours. The pyridine was distilled off in vacuo, and the residue dissolved in absolute ethanol and precipitated with ether. 2.4 gm. of a yellow solid were obtained, which were redissolved in absolute ethanol and reprecipitated with ether. The substance melted at 120–132°.

Analysis—C$_{32}$H$_{38}$O$_6$N$_4$.
Calculated. Riboflavin 55.6
Found (fluorometric) 56

Riboflavin Tetrasuccinate—2 gm. of riboflavin were refluxed with 2.4 gm. (4.4 moles) of succinic anhydride in 20 cc. of dry pyridine for 2 hours. The pyridine was distilled off in vacuo and the residue taken up with absolute alcohol and precipitated with ether. 3.2 gm. of a light yellow solid were obtained and recrystallized from acetone; m.p. 112–115°, corrected.

Analysis—C$_{38}$H$_{44}$O$_6$N$_4$.
Calculated. C 50.91, H 4.88, N 7.22, riboflavin 48.5
Found. " 50.28, " 4.76, " 7.37, " (fluorometric) 48

4',5'-Trichloroethylidene Riboflavin—The method of Coles et al. (10) for the preparation of chloraloses was followed. 17 gm. of chloral hydrate were stirred mechanically with 30 cc. of concentrated sulfuric acid, while 10 gm. of riboflavin were added in small portions, the whole being
kept at 17°. Stirring was continued for 6 hours, and the reaction mixture was left in the refrigerator overnight. It was then poured into 250 cc. of ice water and again left in the refrigerator overnight. The precipitate was then filtered off, with a yield of 13.6 gm. of a yellow solid, which melted at 68–90°, was soluble in chloroform and acetone, and very soluble in methanol and ethanol. When boiled in ethanol or methanol, precipitation occurred, due probably to the conversion of the dichloral to the monochloral derivative. The precipitate decomposed above 265°. It was moderately soluble in dioxane, slightly soluble in ethanol and methanol, insoluble in water.

Analysis—C\textsubscript{12}H\textsubscript{15}O\textsubscript{5}N\textsubscript{4}Cl\textsubscript{2}

Calculated. C 44.92, H 3.78, Cl 20.96, riboflavin 74.1

Found. " 46.52, " 3.90, " 20.11, " (fluorometric) 76

46.41 4.20

The analytical data show that the product consists mainly of the monochloral derivative. The high carbon, hydrogen, and fluorometric values and the slightly low chlorine figure suggest small admixture of riboflavin, which may be due to further hydrolysis of the monochloral compound.

Condensation of Riboflavin with Levulinic Acid—10 gm. of riboflavin were stirred for 3 hours with 6.7 gm. of levulinic acid in 40 cc. of concentrated sulfuric acid. The solution was then poured into 1.5 liters of water and the sulfuric acid precipitated with excess barium carbonate. After standing overnight, the mixture was warmed to 70° and the precipitate filtered off. The filtrate containing the barium salt of the condensation product of levulinic acid with riboflavin, and some unchanged riboflavin was evaporated to dryness in vacuo. The residue was dissolved in hot water, whence, upon cooling, 2.8 gm. of a mixture of unchanged riboflavin and some barium levulinic riboflavin crystallized out and were filtered off. The filtrate was acidified with excess concentrated sulfuric acid and the precipitated barium sulfate filtered off. The filtrate was then adjusted to pH 2.7 with sodium carbonate, evaporated to dryness in vacuo, and the residue was extracted with hot methanol. On cooling, 3 gm. of an extremely watersoluble substance crystallized. As it still contained traces of inorganic salts, it was again recrystallized from methanol.

\begin{align*}
\text{Calculated for } & 4',5'-(\text{carboxymethylisopropylidene})\text{riboflavin}, \text{C}_{25}\text{H}_{26}\text{O}_{6}\text{N}_{4} & 11.81 & 79.3 \\
\text{Calculated for } & 2',3':4'5'-\text{di(carboxymethylisopropylidene)}\text{riboflavin}, \text{C}_{27}\text{H}_{30}\text{O}_{7}\text{N}_{4} & 9.79 & 66.7 \\
\text{Found} & & 10.53 & 73
\end{align*}
It is evident from these figures that the preparation represents a mixture of the mono and di compounds. Since the preparation was biologically inactive, no attempts were made to separate the components.

We are indebted to Dr. A. Steyermark and his staff for the microanalyses, and to Mr. E. De Ritter, Mr. L. Drekter, and Dr. R. L. Schuman for their cooperation in carrying out the other analyses.

SUMMARY

The preparation of two acetal derivatives of riboflavin and of several riboflavin succinates is described. All exhibit theoretical fluorescence values. Both the biological and microbiological activities of the succinates decrease in inverse relation to the extent of substitution, but the two sets of values are not equal, the microbiological being considerably lower. These results demonstrate that neither fluorescence nor microbiological response of Lactobacillus casei is necessarily a quantitative measure of vitamin activity in the mammal for such flavins.

The succinates exhibit remarkable increases in solubility with increasing substitution.

BIBLIOGRAPHY

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