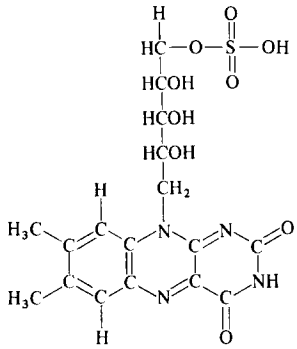
[149] Riboflavin 5′-Monosulfate



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Riboflavin 5′-monosulfate is a sulfate analog of FMN. It inhibits D-amino acid oxidase in competition with the FMN part of FAD.[1] This analog shows an antivitamin action for riboflavin in bacteria such as *Lactobacillus casei* and *Streptococcus faecalis*[2] as well as in mammals,[3] though the action in mammals is very weak. The method of preparation and some properties of riboflavin 5′-monosulfate are described below.

Chemical Synthesis and Purification[4]

In a 300-ml round-bottomed three-necked flask fitted with an air-tight stirrer, a dropping funnel, and a reflux condenser closed with a calcium chloride tube, dried riboflavin (200 mg, 0.54 millimole) is dissolved in 200 ml of dried pyridine by heating at 80°. The solution is cooled to 30°, then a mixture composed of 1 ml of chlorosulfonic acid (15.3 millimoles) and 3 ml of dried chloroform is added dropwise with vigorous stirring, and heated at 40° for 30 minutes.[5] The solution is evaporated *in vacuo* at 40°. The residue is dissolved in a minimal volume of water. Unreacted riboflavin, which is less soluble, is filtered off and washed with cold water. Filtrate and washings are combined and neutralized with $CaCO_3$. Calcium sulfate

[1] F. Egami and K. Yagi, *J. Biochem.* **43,** 153 (1956).

[2] F. Egami, M. Naoi, M. Tada, and K. Yagi, *J. Biochem.* **43,** 669 (1956).

[3] K. Yagi and S. Yamada, *Acta Biochim. Polon.* **11,** 315 (1964).

[4] N. Takahashi, K. Yagi, and F. Egami, *J. Chem. Soc. Japan, Pure Chem. Sect.* **78,** 1287 (1957).

[5] By heating at 60°–70° for 1 hour after the addition of chlorosulfonic acid, all the alcoholic hydroxyl groups in the ribitol chain of riboflavin are esterified to produce riboflavin 2′,3′,4′,5′-tetrasulfate.

formed is removed by filtration. A further small amount of $CaCO_3$ is added to the filtrate, and the filtrate is concentrated *in vacuo* until the odor of pyridine is eliminated. The residue is dissolved in water and made up to 50 ml, and insoluble materials are filtered off. Barium chloride (0.13 g) is added to the filtrate, and $BaSO_4$ formed is removed by centrifugation. The supernatant is condensed *in vacuo*, and the crude calcium salt of riboflavin 5′-monosulfate is precipitated by adding excess ethanol. The precipitate is collected by centrifugation and washed with ethanol.

Since the crude preparation is contaminated with a small amount of unreacted riboflavin and other sulfuric acid esters of riboflavin,[6] further purification is needed to obtain pure riboflavin 5′-monosulfate. For this purpose, cellulose column chromatography using a mixture of benzyl alcohol–ethanol–water (3:2:1, v/v/v) as a mobile phase is recommended. After riboflavin 5′-monosulfate is clearly separated from the above-mentioned contaminating materials on the column,[7] the fraction corresponding to riboflavin 5′-monosulfate is taken out, and eluted with water. The eluate is washed with ethyl ether to eliminate benzyl alcohol and condensed under reduced pressure. From the condensate, the calcium salt of riboflavin 5′-monosulfate is precipitated by adding excess ethanol. It is crystallized from water.

Properties

Solubilities[4] of riboflavin 5′-monosulfate in various solvents are similar to those of FMN: insoluble in benzene, chloroform, or ethyl ether; slightly soluble in methanol or ethanol; but easily soluble in water. The visible absorption spectrum[4] of riboflavin 5′-monosulfate in water is identical with that of FMN, having absorption peaks at 375 and 445 mμ. In paper chromatography[2] using solvents such as the upper layer of *n*-butanol–acetic acid–water (4:1:5, v/v/v), benzyl alcohol–ethanol–water (3:2:1, v/v/v), or *n*-propanol–pyridine–water (5:3:2, v/v/v), riboflavin 5′-monosulfate can be separated from riboflavin, FMN, and FAD and migrates between FMN and riboflavin.

By kinetic analysis of the enzymatic reaction of D-amino acid oxidase,

[6] Usually a small amount of riboflavin 2′,3′,4′,5′-tetrasulfate and a trace amount of riboflavin tri- and/or disulfate are found. To check these substances, silica-gel G thin-layer chromatography using the upper layer of *n*-butanol–acetic acid–water (4:1:5, v/v/v) as a mobile phase is useful. In this case, R_f values of riboflavin 2′,3′,4′,5′-tetrasulfate, riboflavin 5′-monosulfate, and riboflavin are 0.03, 0.23, and 0.47, respectively. Riboflavin tri- and disulfate migrate between tetra- and monosulfate.

[7] The mobilities of these substances can be judged by paper chromatography using the same solvent; R_f values of riboflavin 2′,3′,4′,5′-tetrasulfate, riboflavin 5′-monosulfate, and riboflavin are 0.03, 0.30, and 0.46, respectively. Also in this case, riboflavin tri- and disulfate migrate between tetra- and monosulfate.

riboflavin 5′-monosulfate has been shown to compete with the FMN part of FAD, resulting in inhibition of this enzyme.[1] The dissociation constant between riboflavin 5′-monosulfate and the D-amino acid oxidase apoprotein is evaluated to be 3.2×10^{-5} M.

Using this substance as a specific indicator, the mode of action of an inhibitor which inhibits a flavin enzyme in competition with FAD can be analyzed.[8] In the presence of this indicator, it can be elucidated which moiety of FAD actually competes with the inhibitor to be tested.

In contrast with the case of D-amino acid oxidase, however, riboflavin 5′-monosulfate cannot combine with the apoprotein of the old yellow enzyme, showing no inhibitory action.[9]

As expected from the chemical structure, riboflavin 5′-monosulfate can act as an antivitamin B_2 when examined with *Lactobacillus casei* and *Streptococcus faecalis*.[2] This sulfate inhibits the growth of these bacteria, which is a bacteriostatic action.

In nutritional experiments with mammals,[3] riboflavin 5′-monosulfate, when injected, shows an antivitamin B_2 effect; this cannot, however, be shown when it is administered orally. Riboflavin 5′-monosulfate, when injected, is detected in the liver, kidney, heart, and small intestine at 30 minutes after the administration and almost disappears after 12 hours. Most of the injected riboflavin 5′-monosulfate is excreted in urine so rapidly that it shows no serious toxicity. After injection of riboflavin 5′-monosulfate, the amounts of FAD, FMN, and riboflavin in the liver, kidney, heart, and small intestine are not changed significantly, indicating that the physiologically existing flavin in these organs might not be exchanged easily for the injected riboflavin 5′-monosulfate.

Determination of Riboflavin 5′-Monosulfate in Animal Tissues[10]

Riboflavin 5′-monosulfate can be analyzed using both the lumiflavin fluorescence method and paper chromatography.[11] Analytical procedures, e.g., warm-water extraction, photodecomposition, fluorometric assay of lumiflavin formed, and paper chromatography, are essentially the same as those for the separate determination of the physiologically existing flavins, except that in this case the use of the mixture of *n*-butanol–acetone–acetic acid–water (5:2:1:3, v/v/v/v) is recommended as a suitable mobile phase for paper chromatography. R_f values of FAD, FMN, riboflavin 5′-monosulfate, and riboflavin are 0.08, 0.15, 0.25, and 0.42, respectively.

[8] K. Yagi and T. Ozawa, *Biochim. Biophys. Acta* **42,** 381 (1960).
[9] H. Theorell, K. Yagi, G. D. Ludwig, and F. Egami, *Nature* **180,** 922 (1957).
[10] K. Yagi and S. Yamada, *Nagoya J. Med. Sci.* **25,** 228 (1963).
[11] K. Yagi, this volume, [134].