Chromatographic Identification of a New Flavin Derivative in Plain Yogurt

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The presence of flavin derivatives in plain yogurt was assessed by high-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC). The total amount of flavins in yogurts produced by different companies was variable and oscillated between 150.0 and 218.8 μg/100 g. Riboflavin (RF) was the predominant flavin. Besides RF, flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), 7α-hydroxyriboflavin (7α-HRF), 4′- or 5′-D-riboflavin-β-D-galactoside (RFgal), and traces of 10-formylmethylflavin (10-FMF) and 10-hydroxyethylflavin (10-HEF) have been found. It is known that RFgal may be obtained using enzymes or cultures of different microorganisms, but its presence in foodstuffs has not been demonstrated, yet.

Keywords: *FAD; FMN; riboflavin; riboflavinyl galactoside; 7α-hydroxyriboflavin; 10-formylmethylflavin; 10-hydroxyethylflavin; yogurt*

INTRODUCTION

One of the naturally occurring riboflavin derivatives is riboflavinyl glucoside (5′-D-riboflavin-α-D-glucopyranoside). This compound was first obtained by Whitby (1950, 1952) when a rat liver enzyme was incubated with riboflavin. A few years later, this glucoside was found in rat urine (Ohkawa et al., 1983) and in cat (Kasai et al., 1972) and rat livers (Ohkawa et al., 1986). The enzyme responsible for the formation of riboflavinyl glucoside has also been found in pig liver (Uchida and Suzuki, 1974) and in some plant grains (Suzuki and Uchida, 1969).

Besides riboflavinyl glucoside, there are several other glycosidic and oligosaccharide derivatives of riboflavin. They may be formed by incubation of riboflavin and the appropriate sugar with cultures of different microorganisms and enzymes from various microorganisms (Suzuki and Katagiri, 1963a,b; Suzuki, 1965; Suzuki and Uchida, 1965, 1967; Tachibana, 1955a,b, 1971; Whitby, 1971). Generally, maltose, starch, glycogen, dextrin, and salicin are glucosyl donors for riboflavinyl glucoside. Sucrose serves as the fructosyl or glucosyl donor for riboflavinyl fructoside or riboflavinyl glucoside; lactose and melobiose are galactosyl donors for riboflavinyl galactosides with β- and α-configurations, respectively.

Up to now, riboflavinyl glucoside was the only known glycosidic derivative of riboflavin occurring naturally. Our results reveal that yogurt was the first tested source of riboflavinyl-β-galactoside. In this paper, we report the separation and identification of riboflavinyl-β-galactoside and other flavin derivatives found in plain yogurt.

MATERIALS AND METHODS

Reagents and Standards. The standards of riboflavin (RF) (Reanal, Budapest, Hungary), flavin adenine dinucleotide (FAD) (Boehringer Mannheim GmbH, Mannheim, Germany), and flavin mononucleotide (FMN) (Merck, Darmstadt, Germany) were used without additional purification, but for the quantitative analysis of flavins, the FMN and FAD concentrations were corrected for the impurities. The purity of commercial FAD was 96.9% and FMN 86.4%. 7α-Hydroxyriboflavin (7α-HRF) was a gift from K. Matsui (Division of Biology, Research Institute for Atomic Energy, Osaka University, Osaka, Japan). 8α-Hydroxyriboflavin (8α-HRF) was prepared by acid hydrolysis of 8α-bromo-2′,3′,4′,5′-tetraacetylriboflavin synthesized by the method of Walkner et al. (1972). 10-Formylmethylflavin (10-FMF) and 10-hydroxyethylflavin (10-HEF) were synthesized according to the method of Fall and Petering (1956). Riboflavinyl galactoside (RFgal) was prepared according to the method of Tachibana (1971) by incubation of riboflavin and lactose with Taka-Diastase powder (Sigma, St. Louis, MO) and purified using preparative TLC [solvent being *n*-butanol/glacial acetic acid/water (15:3:7, v/v)] and semipreparative HPLC (elution method B). Commercial sodium periodate (Aldrich, Steinheim, Germany), α- and β-galactosidases (Sigma), and α-glucosidase (Merck) were used. Plain yogurt and bioyogurt samples from different companies were bought at random in grocery stores.

Preparation and Analysis of Extracts. Flavins from 20 g samples were extracted by a method described previously (Gliszczyńska and Koziołowa, 1998). All HPLC preparations were performed on a Waters model 600E high-performance liquid chromatograph (Waters, Milford, MA) equipped with an Alphabond C_{18} column (4.6 mm × 300 mm, Alltech, Carnforth, Lancashire, U.K.) or an analytical Symmetry C_{18} column (3.9 mm × 150 mm, Waters) fitted with μBondapak C_{18} or Nova-Pak C_{18} precolumn inserts, respectively. For identification of flavins, three elution methods developed in our laboratory were used: (A) methanol/0.05 M ammonium acetate buffer (pH 6.0) and (B) methanol/demineralized water in the gradients described previously (Gliszczyńska and Koziołowa, 1998) (Alphabond C_{18} column) and (C) methanol/0.05 M ammonium acetate buffer (pH 6.0) with the gradient 25:75 v/v methanol/buffer, with a linear gradient to 70:30 v/v within 12 min at a flow rate of 0.6 mL/min, isocratic 70:30 v/v to 15 min at a flow rate of 0.8 mL/min, and isocratic 25:75 v/v to 25 min (Symmetry C_{18} column).

Semipreparative HPLC of plain yogurt's flavins was performed on a μBondapak C_{18} column (25 mm × 100 mm, 10 μm) fitted with a μBondapak C_{18} Guard-Pak insert (25 mm ×

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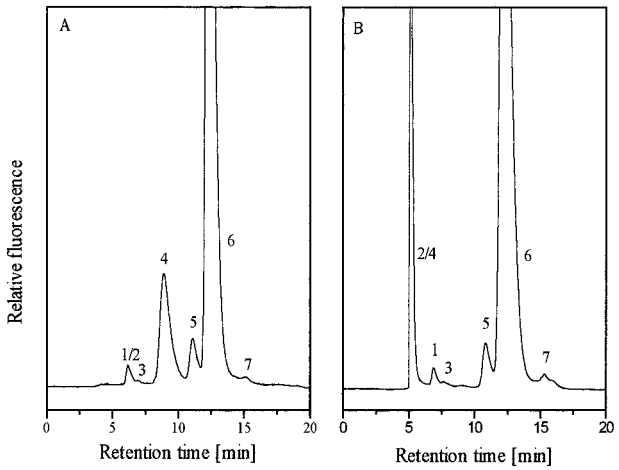

Figure 1. HPLC chromatogram of flavins extracted from plain yogurt: (**1**) 7α-HRF, (**2**) FAD, (**3**) 10-HEF, (**4**) FMN, (**5**) RFgal, (**6**) RF, and (**7**) 10-FMF. The conditions were as follows: an Alphabond C_{18} column (3.9 mm × 300 mm, 10 μm), a Waters model 474 scanning fluorescence detector with excitation and emission wavelengths of 450 and 530 nm, respectively. The eluent for method A was a mobile phase gradient of methanol/0.05 M ammonium acetate (pH 6.0); the eluent for method B was a mobile phase gradient of methanol/demineralized water.

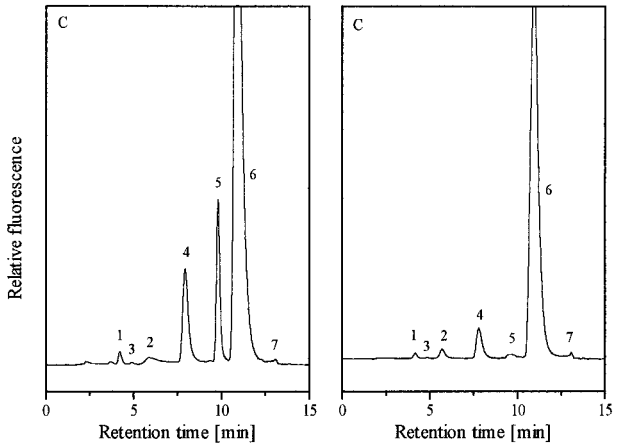

Figure 2. HPLC chromatogram of flavins extracted from two different kinds of plain yogurts: (**1**) 7α-HRF, (**2**) FAD, (**3**) 10-HEF, (**4**) FMN, (**5**) RFgal, (**6**) RF, and (**7**) 10-FMF. The conditions were as follows: a Symmetry C_{18} column (3.9 mm × 150 mm, 5 μm), a Waters model 474 scanning fluorescence detector with excitation and emission wavelengths of 450 and 530 nm, respectively. The eluent for method C was a mobile phase gradient of methanol/0.05 M ammonium acetate (pH 6.0).

10 mm, 10 μm) (Waters) using method B with a flow rate of 3 mL/min.

A Waters 474 scanning fluorescence detector was used at an emission wavelength of 530 nm with excitation at 450 nm for isoalloxazine derivatives and an emission wavelength of 430 nm with excitation at 380 nm for alloxazine derivatives with a slit width of 10 nm. Additionally, the Waters model 991 photodiode-array detector was used to differentiate flavins from others compounds on the basis of their absorption spectra.

The concentrations of FAD, FMN, and RF were determined from their corresponding standard curves obtained under the same chromatographic conditions (external standard method) using method C. Quantification of 7α-HRF, 10-FMF, 10-HEF, and RFgal was performed using the standard curve prepared for RF, but in the case of 7α-HRF, the concentration was corrected for the lower fluorescence intensity of this analogue (Ohkawa et al., 1986b).

Thin-Layer Chromatography (TLC). For analytical and preparative TLC purposes, concentrated flavin extracts were passed through a column packed with resorcinol-type resin R-15 synthesized and used according to the method of Koziołowa and Kozioł (1968) to remove all interfering non-flavin compounds. Analytical TLC was performed with silica gel plates (Kieselgel 60, 0.2 mm, E. Merck). For preparative purposes, 2 mm silica gel TLC plates were used (solvent I). Solvent systems used for thin-layer chromatography were as follows: (I) *n*-butanol/glacial acetic acid/water (2:1:1 v/v), (II) *n*-butanol/acetic acid/water (5:2:3 v/v), (III) chloroform/methanol/ethyl acetate (5:5:2 v/v), (IV) isoamyl alcohol/ethyl methyl ketone/glacial acetic acid/water (40:40:7:13 v/v), (V) 5% $Na_2HPO_4 \cdot 12H_2O$, (VI) *n*-butanol/ethanol/water (10:3:7 v/v), (VII) *n*-butanol/2-propanol/water/glacial acetic acid (30:50:10:2 v/v), (VIII) ethyl methyl ketone/acetic acid/methanol (3:1:1 v/v), and (IX) *n*-butanol/benzyl alcohol/glacial acetic acid (8:4:3 v/v).

Detection of sugars on TLC silica gel plates was achieved by spraying them with a mixture of 2% diphenylamine in acetone, 2% aniline in acetone, and 85% phosphoric acid in a volume ratio of 5:5:1 and heating the chromatograms at 100 °C over the course of 5−10 min.

Methods of Identification for Unknown Flavin (Riboflavinyl-β-galactoside). (1) For acid hydrolysis, equal volumes of unknown flavin and 4 M HCl were mixed and heated at 100 °C for 2 h. After hydrolysis, the mixture was neutralized, evaporated, dissolved in a small volume of distilled water, and analyzed using HPLC and TLC methods.

(2) For alkaline photolysis, equal volumes of unknown flavin and 1 M NaOH were mixed, left in light at least 4 h at room temperature, and analyzed on TLC plates.

(3) Periodate oxidation was performed by adding a double volume of 0.05 M sodium periodate to the solution of unknown flavin or flavin standard. The resulting solution was incubated in the dark for 4 h at room temperature, and oxidation products were analyzed using TLC and HPLC methods.

(4) For enzymatic hydrolysis of unknown flavin with α- and β-galactosidases, a reaction mixture containing equal volumes (0.1 mL) of an aqueous solution of the sample, 0.2 M acetate buffer, and α- or β-galactosidases (the same level of activity) was incubated at 37 and 25 °C (α- and β-galactosidase,

Table 1. HPLC Retention Times (t_R) of Flavins from Plain Yogurt

flavin	t_R (min) method A[a]	method B[a]	method C[b]
7α-HRF	6.17	6.87	4.18
FAD	6.17	5.13	5.77
10-HEF	7.00	7.58	4.88
FMN	8.88	5.13	7.86
unknown flavin	11.10	10.83	9.82
RF	12.27	12.20	10.90
10-FMF	15.20	15.32	13.09

[a] HPLC separation was performed on an Alphabond C_{18} column using a fluorescence detector and elution methods A with methanol/0.05 M ammonium acetate (pH 6.0) and B with methanol/demineralized water in appropriate gradients. [b] HPLC separation was performed on a Symmetry C_{18} column using a fluorescence detector and elution method C with a mobile phase gradient of methanol/0.05 M ammonium acetate (pH 6.0) as described in Materials and Methods.

Table 2. R_f Values on Silica Gel TLC of Flavin Standards and 7α-Hydroxyriboflavin (Compound 1) Isolated from Plain Yogurt[a]

flavin	II	III	VI	IX
FAD	0.23	0	0.37	0
FMN	0.41	0	0.37	0.04
RF	0.64	0.55	0.64	0.33
7α-HRF	0.57	0.32	0.53	0.20
compound **1**	0.57	0.32	0.53	0.20

[a] TLC was performed on silica gel, and the solvents were (II) *n*-butanol/acetic acid/water (5:2:3 v/v), (III) chloroform/methanol/ethyl acetate (5:5:2 v/v), (VI) *n*-butanol/ethanol/water (10:3:7 v/v), and (IX) *n*-butanol/benzyl alcohol/glacial acetic acid (8:4:3 v/v).

Table 3. R_f Values on Silica Gel TLC and HPLC Retention Times (t_R) with Elution Method A of the Unknown Flavin and Standards after Various Treatments

treatment	flavin	TLC[a] R_f II	TLC[a] R_f V	TLC[a] R_f IX	HPLC[b] t_R (min) A
standard	unknown flavin	–	0.55	0.07	11.09
	RF	0.59	0.40	0.29	12.30
	10-FMF	0.78	0.28	0.74	15.15
	LF	0.64	0.15	0.41	–
photolysis in alkaline solution	photolysis product of unknown flavin	0.64	0.15	0.41	–
	photolysis product of RF	0.64	0.15	0.41	–
hydrolysis with 4 N HCl	hydrolysis product of unknown flavin	0.59	0.40	0.29	12.28
hydrolysis with β-galactosidase	hydrolysis product of unknown flavin	0.59	0.40	0.29	12.33
hydrolysis with α-galactosidase	hydrolysis product of unknown flavin	–	0.55	0.07	11.11
periodate oxidation	oxidation product of unknown flavin	0.78	0.28	0.74	15.17
	oxidation product of RF	0.78	0.28	0.74	15.12

[a] TLC was performed on silica gel, and the solvents were (II) *n*-butanol/acetic acid/water (5:2:3 v/v), (V) 5% $Na_2HPO_4 \cdot 12H_2O$, and (IX) *n*-butanol/benzyl alcohol/glacial acetic acid (8:4:3 v/v). [b] HPLC analysis was performed on an Alphabond C_{18} column using a fluorescence detector and elution method A with a mobile phase gradient of methanol/0.05 M ammonium acetate (pH 6.0) as described in Materials and Methods.

respectively) for 4 h in the dark. The pH of acetate buffer was 6.5 for α-galactosidase and 7.2 for β-galactosidase.

(5) For enzymatic hydrolysis of samples with α-glucosidase, a reaction mixture containing equal volumes of unknown flavin solution, 0.2 M acetate buffer (pH 6.0), and enzyme solution was incubated at 37 °C for 4 h in the dark.

RESULTS AND DISCUSSION

Some differences were found in the effectiveness of two reversed-phase high-performance liquid chromatography columns used for the analysis of flavin derivatives present in plain yogurt. Methods A and B applied on an Alphabond C_{18} column [mobile phase gradient of methanol/0.05 M ammonium acetate buffer (pH 6.0) and mobile phase gradient of methanol/demineralized water] have some disadvantages. Method A does not allow separation of FAD and 7α-HRF and method B FAD and FMN (Figure 1). These methods have been used to confirm the identity of appropriate flavins isolated from flavin extract with the synthetic flavins used as external and internal standards. Method C [mobile phase gradient of methanol/0.05 M ammonium acetate buffer (pH 6.0)] applied on a Symmetry C_{18} column was the best for separation of all detected flavins (Figure 2 and Table 1). On the basis of retention times of standards and flavins found in yogurt, compounds **2**, **4**, **6**, and **7** were identified as FAD, FMN, RF, and 10-FMF, respectively. To ensure that compounds **1** and **3** were 7α-HRF and 10-HEF, respectively, these compounds were isolated on semipreparative HPLC and their retention times from all elution methods were compared with retention times of synthetic flavins applied as internal and external standards. Because of an insufficient quantity of 10-HEF, only the mobility of 7α-HRF on TLC plates was compared with the standard (Table 2).

Compound designated as **5** (Figures 1 and 2) exhibited chromatographic behavior quite different from that of all standard compounds that were used. Its absorption spectrum was typical for isoalloxazines with maxima at 370 and 445 nm. To determine the structure of "**5**", we performed a few different tests, the results of which are summarized in Table 3. The hydrolysis of the unknown flavin with 4 M HCl at 100 °C produced riboflavin. The product of the photolysis in alkaline solution was identified as lumiflavin (LF). These results indicate that the unknown flavin is an ester of riboflavin. The presence of microorganisms in yogurt suggested the possibility of an appearance of some kind of riboflavin glycoside. One of the known natural riboflavin glycosides is riboflavinyl-α-glucoside; however, the presence of lactose in milk suggested rather that unknown flavin could be riboflavinyl galactoside (RFgal). Enzymatic hydrolysis with α-glucosidase (results not shown) did not cause any changes in unknown flavin, which could exclude the presence of riboflavinyl-α-glucoside. To check the point at which unknown flavin is RFgal, we identified by TLC the sugar derived from the unknown flavin after HCl hydrolysis. As shown in Table 4, the R_f value of released sugar corresponds to those of galactose. To confirm the presence of the galactosidic bond and its configuration, flavin **5** was digested by α- and β-galactosidases. The digestion with α-galactosidase did not liberate riboflavin. β-Galactosidase liberated riboflavin, and the sugar part was again identified as

Table 4. R_f Values of the Sugar Part of the Unknown Flavin Obtained by HCl and Enzymatic Hydrolysis[a]

sugar part	R_f VII	R_f VIII	R_f IX
standards			
fructose	0.42	0.51	0.24
glucose	0.44	0.55	0.29
galactose	0.36	0.48	0.22
lactose	0.16	0.26	0.11
unknown flavin after acid hydrolysis	0.36	0.48	0.22
unknown flavin after enzymatic hydrolysis	0.36	0.48	0.22

[a] TLC was performed on silica gel, and the solvents were (VII) *n*-butanol/2-propanol/water/glacial acetic acid (30:50:10:2 v/v), (VIII) ethyl methyl ketone/acetic acid/methanol (3:1:1 v/v), and (IX) *n*-butanol/benzyl alcohol/glacial acetic acid (8:4:3 v/v).

Table 5. R_f Values on TLC of Flavin Standards and the Unknown Flavin[a]

flavin	I	IV	VII	VIII	IX
standards					
FAD	0.21	0	0	0	0
FMN	0.36	0.03	0.04	–	0.05
RFgal	0.56	0.06	0.21	0.18	0.10
RF	0.68	0.38	0.50	0.48	0.31
unknown flavin from yogurt	0.56	0.06	0.21	0.18	0.10

[a] TLC was performed on silica gel, and the solvents were (I) *n*-butanol/glacial acetic acid/water (2:1:1 v/v), (IV) isoamyl alcohol/ethyl methyl ketone/glacial acetic acid/water (40:40:7:13 v/v), (VII) *n*-butanol/2-propanol/water/glacial acetic acid (30:50:10:2 v/v), (VIII) ethyl methyl ketone/acetic acid/methanol (3:1:1 v/v), and (IX) *n*-butanol/benzyl alcohol/glacial acetic acid (8:4:3 v/v).

Table 6. Distribution of Flavins in Plain Yogurts and Bioyogurts[a]

sample	RF	FAD	FMN	RFgal	7α-HRF	10-HEF	10-FMF	total
yogurt 1	148.5 ± 6.9	16.0 ± 1.8	29.6 ± 0.8	14.2 ± 0.4	4.3 ± 0.2	traces	0.5 ± 0.1	214.0 ± 7.0
	69.4%	7.5%	13.8%	6.6%	2.0%	<0.1%	0.2%	
yogurt 2	186.8 ± 2.8	2.8 ± 0.6	20.6 ± 2.4	5.3 ± 0.1	3.1 ± 0.4	traces	traces	218.8 ± 3.5
	85.4%	1.3%	9.4%	2.4%	1.4%			
bioyogurt 3	160.3 ± 3.4	2.6 ± 0.1	32.3 ± 3.9	4.2 ± 0.2	3.5 ± 0.9	0.3 ± 0.1	0.2 ± 0.0	204.0 ± 7.5
	78.6%	1.3%	15.8%	2.1%	1.7%	0.2%	0.1%	
bioyogurt 4	127.4 ± 2.1	37.6 ± 4.9	13.1 ± 0.6	0.5 ± 0.3	2.1 ± 0.2	traces	0.6 ± 0.1	181.3 ± 3.8
	70.3%	20.7%	7.2%	0.3%	1.2%		0.3%	
bioyogurt 5	98.7 ± 1.6	18.3 ± 1.3	27.2 ± 2.2	2.0 ± 0.8	3.9 ± 0.9	traces	traces	150.0 ± 2.1
	65.7%	12.4%	18.5%	1.4%	2.6%			

[a] In units of micrograms per 100 g ± SD.

galactose (Table 4). These results established the structure of the unknown flavin as RFgal with the β-configuration.

Oxidation with sodium periodate resulted in a product identified as 10-FMF. It indicates that position 2′- and 3′-hydroxy groups of riboflavin could not be substituted by galactose. The galactosidic bond exists at the 4′- and 5′-positions. None of these positions can be unequivocally excluded; thus, the structure of this derivative is proposed to be 4′- or 5′-D-riboflavin-β-D-galactoside. To prove it, we have synthesized enzymatically riboflavinyl galactoside and compared its mobility on TLC (Table 5) and its chromatographic behavior as internal and external standards on HPLC with that of natural flavin isolated from plain yogurt.

A new flavin derivative found in yogurt was not found in cow's milk. Preliminary experiments in our laboratory indicate that traces of this derivative were also present in sour milk but absent in other fermented milk drinks such as kefir, acidophilus milk, and buttermilk. It is more than probable that RFgal is a product of the action of specific yogurt strains: *Streptococcus salivarius* ssp. *thermophilus* and/or *Lactobacillus delbrueckii* ssp. *bulgaricus* which are used in the technological process. The enzyme responsible for the formation of riboflavinyl galactoside may be β-transgalacosidase or β-galactosidase, which under certain reaction conditions can act as a transferase. Glycosidases typically catalyze the hydrolysis of glycosidic linkages, and they are also widely used for the enzymatic synthesis of several oligosaccharides or new glycoconjugates. β-Galactosidase has been used for galactosylation of some antibiotics using β-lactose as the galactosyl donor (Scheckermann et al., 1997) or to produce galactooligosaccharides, which are potentially useful as additives in milk for infants (Shin and Yang, 1994). Stivenson et al. (1993) reported the large-scale production of alkyl galactosides, which are suitable substrates for the lipase-catalyzed synthesis of surfactants and emulsifiers.

In the samples that were tested, we did not detect 8α-HRF, which has been found by Roughead and McCormick (1990) in cow's milk. They reported that only trace quantities of this analogue were available; hence, its identification is rather tentative.

In all the samples that were tested, we have not found derivatives with alloxazinic structure.

The flavin composition of the most common fermented milk drinks and the origin of the unknown flavin in yogurt are being studied in our laboratory.

Quantification of Flavins. The mean content of individual flavins and total flavin content are reported in Table 6. All values are arithmetic means of at least four measurements for three samples. The recovery of individual flavins was more then 95%. In general, the content of riboflavin in the samples that were analyzed was in agreement with that found in the literature (Souci et al., 1962; Piekarska and Łoś-Kuczera, 1983; Ashoor et al., 1983). Observed flavin amounts in tested samples are variable probably because milk companies apply different types of raw materials, strains of microorganisms, and technological parameters. The amounts of RFgal oscillate between 0.5 and 14.2 μg/100 g of yogurt, which constitutes 0.3−6.6% of the total flavin content, which is a relatively large quantity. No clear physiological function or role in animal and microorganism metabolism has been attributed to glycosidic and oligosaccharide derivatives of riboflavin. Joseph and McCormick (1995) reported that the nutritional efficiency of riboflavinyl glucoside is similar to that of the free vitamin. It is possible that riboflavinyl galactoside has also vitaminic character, but its true nutritional value requires further study.

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