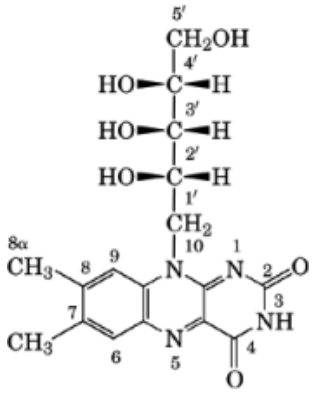
Kirk-Othmer Encyclopedia of Chemical Technology.

RIBOFLAVIN (B_2)

Riboflavin [83-88-5] (vitamin B_2, vitamin G, lactoflavin, ovoflavin, lyochrome, hepatoflavin, uroflavin) has the chemical name 1-deoxy-1-(3,4-dihydro-7,8dimethyl-2,4-dioxobenzo[*g*]pteridin-10(2*H*)-yl)-D-ribitol, 7,8-dimethyl-10-D-ribitylisoalloxazine, $C_{17}H_{20}N_4O_6$ **(1)**, mol wt 376.37.



(1)

In 1933, R. Kuhn and his co-workers first isolated riboflavin from eggs in a pure, crystalline state (1), named it ovoflavin, and determined its function as a vitamin (2). At the same time, impure crystalline preparations of riboflavin were isolated from whey and named lyochrome and, later, lactoflavin. Soon thereafter, P. Karrer and his co-workers isolated riboflavin from a wide variety of animal organs and vegetable sources and named it hepatoflavin (3). Ovoflavin from egg, lactoflavin from milk, and hepatoflavin from liver were all subsequently identified as riboflavin. The discovery of the yellow enzyme by Warburg and Christian in 1932 and their description of lumiflavin (4), a photochemical degradation product of riboflavin, were of great use for the elucidation of the chemical structure of riboflavin by Kuhn and his co-workers (5). The structure was confirmed in 1935 by the synthesis by Karrer and his co-workers (6), and Kuhn and his co-workers (7).

For therapeutic use, riboflavin is produced by chemical synthesis, whereas concentrates for poultry and livestock feeds are manufactured by fermentation using microorganisms such as *Ashbya gossypii* and *Eremothecium ashbyii*, which have the capacity to synthesize large quantities of riboflavin.

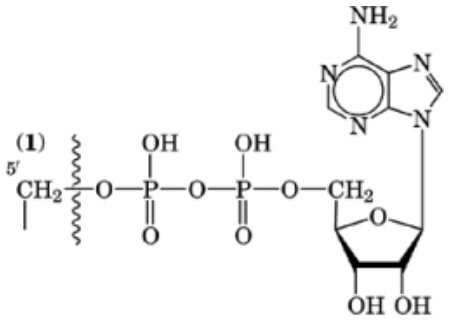
In the free form, riboflavin occurs in the retina of the eye, in whey, and in urine. Principally, however, riboflavin fulfills its metabolic function in a complex form. In general, riboflavin is converted into flavin mononucleotide (FMN, riboflavin-5′-phosphate) and flavin–adenine dinucleotide (FAD) **(2)**, which serve as the prosthetic groups (coenzymes), ie, they combine with specific proteins (apoenzymes) to form flavoenzymes, in a series of oxidation–reduction catalysts widely distributed in nature. In several riboflavin coenzymes, the apoenzyme is covalently attached to $C-8\alpha$ through a linkage to the nitrogen of a histidine imidazolyl group,

Table 1. Riboflavin Content of Various Foods[a]

Food	mg/100 g	Food	mg/100 g
fruits		fish	
apple, raw	0.01	cod, haddock, raw	0.17
banana, raw	0.04	salmon, raw	0.17
citrus, grapefruit, orange	0.03–0.04	salmon, canned	0.12
strawberry	0.03	tuna, canned	0.13
vegetables		whitefish, herring, halibut	0.17–0.29
broccoli, raw	0.27	grain	
cabbage, raw	0.05	corn, entire	0.10
fresh green peas	0.14	wheat, entire	0.10
mushroom	0.57	wheat, germ	0.6
parsley	0.24	rice, entire	0.06
potato, raw	0.03	rye, entire	0.20
sweet corn	0.14	cereal products	
sweet potato, raw	0.05	refined	
tomato, raw	0.03	bread	0.07–0.10
meat		cereal	0.10–0.15
beef muscle	0.16–0.32	soda cracker	0.02–0.10
pork muscle	0.19–0.33	whole grain and enriched	
chicken muscle	0.10–0.31	bread	0.12–0.25
liver, beef, pork	3.00–3.60	cereal	0.20–1.25
		dairy products	
		cheese	0.33–0.68
		eggs	0.48
		milk	0.15–0.18

[a] Averages from several sources, often encompassing a wide range of analytical results; should be regarded as working estimates which vary with geography, season, and preparative method.

to the sulfur of a cysteine residue, or to the oxygen of a tyrosine moiety (see Amino acids). Riboflavin is not a nucleotide, since it is derived from D-ribitol rather than D-ribose, and therefore FMN and FAD are not truly nucleotides; yet this designation has been accepted overwhelmingly and continues to be used.



(2)

As a coenzyme component in tissue oxidation–reduction and respiration, riboflavin is distributed in some degree in virtually all naturally occurring foods. Liver, heart, kidney, milk, eggs, lean meats, malted barley, and fresh leafy vegetables are particularly good sources of riboflavin (see Table 1). It does not seem to have long stability in food products (8).

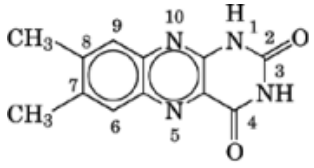
Riboflavin is widely used in the pharmaceutical, food-enrichment, and feed-supplement industries. Riboflavin USP is administered orally in tablets or by injection as an aqueous solution, which may contain nicotinamide or other solubilizers. As a supplement to animal feeds, riboflavin is usually added at concentrations of 2–8 mg/kg, depending on the species and age of the animal (seeFeeds and feed additives).

1. Properties

Riboflavin forms fine yellow to orange-yellow needles with a bitter taste from 2 *N* acetic acid, alcohol, water, or pyridine. It melts with decomposition at 278–279°C (darkens at ca 240°C). The solubility of riboflavin in water is 10–13 mg/100 mL at 25–27.5°C, and in absolute ethanol 4.5 mg/100 mL at 27.5°C; it is slightly soluble in amyl alcohol, cyclohexanol, benzyl alcohol, amyl acetate, and phenol, but insoluble in ether, chloroform, acetone, and benzene. It is very soluble in dilute alkali, but these solutions are unstable. Various polymorphic crystalline forms of riboflavin exhibit variations in physical properties. In aqueous nicotinamide solution at pH 5, solubility increases from 0.1 to 2.5% as the nicotinamide concentration increases from 5 to 50% (9).

In aqueous solution, riboflavin has absorption at ca 220–225, 226, 371, 444 and 475 nm. Neutral aqueous solutions of riboflavin have a greenish yellow color and an intense yellowish green fluorescence with a maximum at ca 530 nm and a quantum yield of $\Phi_f = 0.25$ at pH 2.6 (10). Fluorescence disappears upon the addition of acid or alkali. The fluorescence is used in quantitative determinations. The optical activity of riboflavin in neutral and acid solutions is $[\alpha]_D^{20} = +56.5 - 59.5^\circ$ (0.5%, dil HCl). In an alkaline solution, it depends upon the concentration, eg, $[\alpha]_D^{25} = -112 - 122^\circ$ (50 mg in 2 mL 0.1 *N* alcoholic NaOH diluted to 10 mL with water). Borate-containing solutions are strongly dextrorotatory, because borate complexes with the ribityl side chain of riboflavin; $[\alpha]_D^{20} = +340^\circ$ (pH 12).

Photochemical decomposition of riboflavin in neutral or acid solution gives lumichrome **(3)**, 7,8-dimethylalloxazine, which was synthesized and characterized by Karrer and his co-workers in 1934 (11). In alkaline solution, the irradiation product is lumiflavin **(4)**, 7,8,10-trimethylisoalloxazine; its uv–vis absorption spectrum resembles that of riboflavin. It was prepared and characterized in 1933 (5). Another photodecomposition product of riboflavin is 7,8-dimethyl-10-formylmethylisoalloxazine (12).



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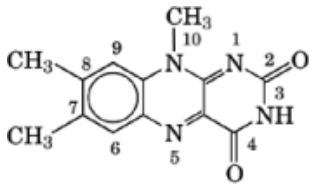


(4)

Riboflavin is stable against acids, air, and common oxidizing agents such as bromine and nitrous acid (except chromic acid, $KMnO_4$, and potassium persulfate). Upon reduction by conventional agents such as sodium dithionite, $Na_2S_2O_4$, zinc in acidic solution, or catalytically activated hydrogen, riboflavin readily takes

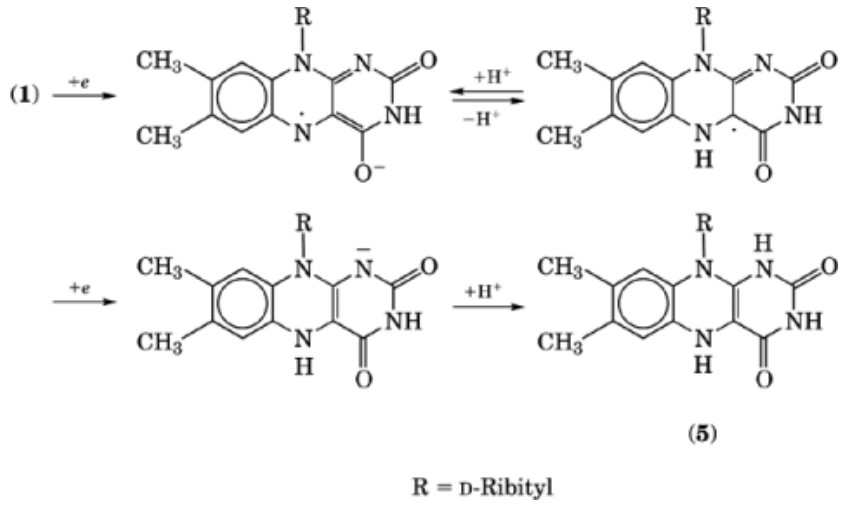


Fig. 1. Formation of dihydroriboflavin.

up two hydrogen atoms to form the almost colorless 1,5-dihydroriboflavin 1 (Fig. 1), which is reoxidized by shaking with air. This oxidation–reduction system has considerable stability, a normal potential of -0.208 V (referred to as the normal hydrogen electrode), and is probably responsible for the physiological functions of riboflavin. The flavins are reduced to dihydroflavins through intermediate semiquinone radicals (13, 14), which have been directly observed by electron spin resonance (esr) (15–18) and electron double resonance (endor) (19).

Riboflavin forms a deep-red silver salt (1). The strong bathochromic shift of the spectra of riboflavin analogues occurring by interaction with Ag^+ can also be obtained with Cu^+ and Hg^{2+} complexes (20). These complexes contain the flavin and the metal ligand anion in a ratio of 1:1. Their color is the result of a charge transfer between the metal and flavin (21). The chelates with Fe(II/III), Mo(V/VI), Cu(I/II), and Ag(I/II) belong to this group; the last two are stable in the presence of water. Another group of metal complexes, radical chelates, are formed with Mn(II), Fe(II), Co(II), Ni(II), Zn(II), and Cd(II); in these cases, the radical character of the ligand is conserved (22).

2. Chemical Synthesis

In 1935, Karrer (6) and Kuhn (7) each proved independently that riboflavin was 7,8-dimethyl-10-D-ribitylisoalloxazine by total synthesis (see Fig. 2). These syntheses are essentially the same and involve a condensation of 6-D-ribitylamino-3,4-xylidine 2 with alloxan 2 in acid solution. Boric acid as a catalyst increases the yield considerably (23). The intermediate (2) was prepared by a condensation of 6-nitro-3,4-xylidine 2 with D-ribose 2, followed by catalytic reduction of the riboside 2. The yield based on D-ribose was increased (24) by using *N*-D-ribityl-3,4-xylidine 2, which was prepared by the condensation of 3,4-xylidine 2 with D-ribose 2, followed by catalytic reduction. The reduced product 2 was coupled with *p*-nitrophenyldiazonium salt to give 4,5-dimethyl-2-*p*-nitrophenylazo-1-D-ribitylaminobenzene 2, which was reduced to 2 and treated with alloxan 2 (25) to give riboflavin. Replacement of 2 by 5,5-dichlorobarbituric acid (26), 5,5-dibromobarbituric acid, or 5-bromobarbituric acid (27) in the above syntheses yields riboflavin.

More conveniently, compound 2 was directly condensed with barbituric acid 2 in acetic acid (28) or in the presence of an acid catalyst in an organic solvent (29). The same azo dye intermediate 2 and alloxantin

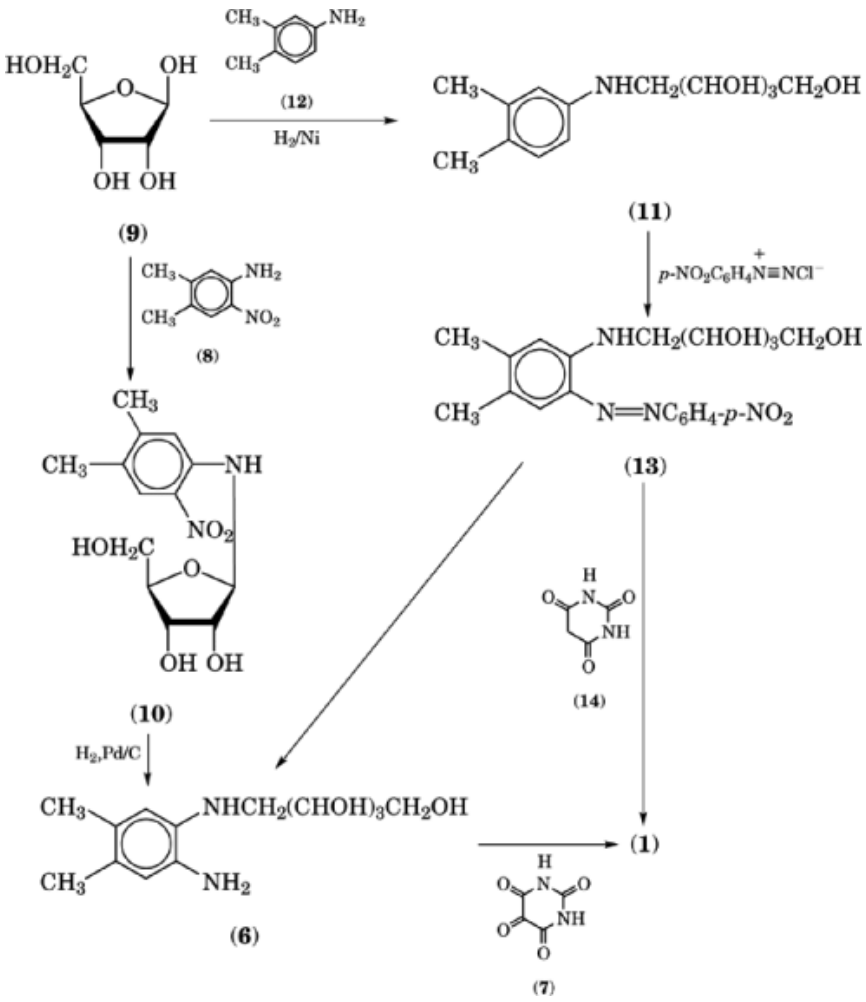


Fig. 2. Syntheses of riboflavin.

give riboflavin in the presence of palladium on charcoal in alcoholic hydrochloric acid under nitrogen. This reaction may involve the reduction of the azo group to the *o*-phenylenediamine by the alloxantin, which is dehydrogenated to alloxan (see Urea) (30).

Although it is not suitable for large-scale manufacture, the synthesis of riboflavin from lumazine derivatives is interesting in connection with the biosynthesis of riboflavin (see Fig. 3). Thus, 5-amino-6-D-ribitylaminouracil 3 was condensed with a dimeric or trimeric aldol of biacetyl to give riboflavin **(1)** through the formation of intermediary 6,7-dimethyl-8-D-ribityllumazine 3 (31). A variation of the above synthesis involves the condensation of monomeric biacetyl and preformed 3 prepared by the condensation of 3 with biacetyl (32). The condensation of 3 with 4,5-dimethyl-1,2-benzoquinone 3 is another pathway to riboflavin, although in low yield (33).

Later, a completely different and more convenient synthesis of riboflavin and analogues was developed (34). It consists of the nitrosative cyclization of 6-(*N*-D-ribityl-3,4-xylidino)uracil 4, obtained from the condensation of *N*-D-ribityl-3,4-xylidine 2 and 6-chlorouracil 4, with excess sodium nitrite in acetic acid, or the

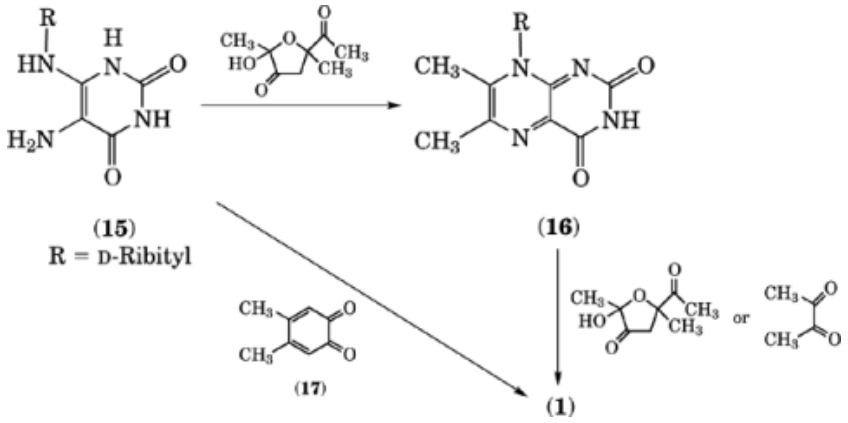


Fig. 3. Syntheses of riboflavin from a lumazine.

cyclization of 4 with potassium nitrate in acetic in the presence of sulfuric acid, to give riboflavin-5-oxide 4 in high yield. Reduction with sodium dithionite gives **(1)**. In another synthesis, 5-nitro-6-(*N*-D-ribityl-3,4-xylidino) uracil 4, prepared *in situ* from the condensation of 6-chloro-5-nitrouracil 4 with *N*-D-ribityl-3,4-xylidine 2, was hydrogenated over palladium on charcoal in acetic acid. The filtrate included 5-amino-6-(*N*-D-ribityl-3,4-xylidino)uracil 4 and was maintained at room temperature to precipitate **(1)** by autoxidation (35). These two pathways are suitable for the preparation of riboflavin analogues possessing several substituents (Fig. 4).

The chemistry of flavins, including several synthetic methods for the preparation of *N*-D-ribityl-3,4-xylidine 2 is reviewed in Reference 36.

3. Microbial Synthesis

3.1. Biosynthetic Mechanism

Riboflavin is produced by many microorganisms, including *Ashbya gossypii*, *Asperigillus sp*, *Eremothecium ashbyii*, *Candida* yeasts, *Debaryomyces* yeasts, *Hansenula* yeasts, *Pichia* yeasts, *Azotobactor* sp, *Clostridium* sp, and *Bacillus* sp.

These organisms have been used frequently in the elucidation of the biosynthetic pathway (37, 38). The mechanism of riboflavin biosynthesis has formally been deduced from data derived from several experiments involving a variety of organisms (Fig. 5). Included are conversion of a purine such as guanosine triphosphate (GTP) to 6,7-dimethyl-8-D-ribityllumazine 3 (39), and the conversion of 3 to **(1)**. This concept of the biochemical formation of riboflavin was verified *in vitro* under nonenzymatic conditions (40) (see Microbial transformations).

3.2. Fermentative Manufacture

Throughout the years, riboflavin yields obtained by fermentation have been improved to the point of commercial feasibility. Most of the riboflavin thus produced is consumed in the form of crude concentrates for the enrichment of animal feeds. Riboflavin was first produced by fermentation in 1940 from the residue of butanol–acetone fermentation. Several methods were developed for large-scale production (41). A suitable carbohydrate-containing mash is prepared and sterilized, and the pH adjusted to 6–7. The mash is buffered

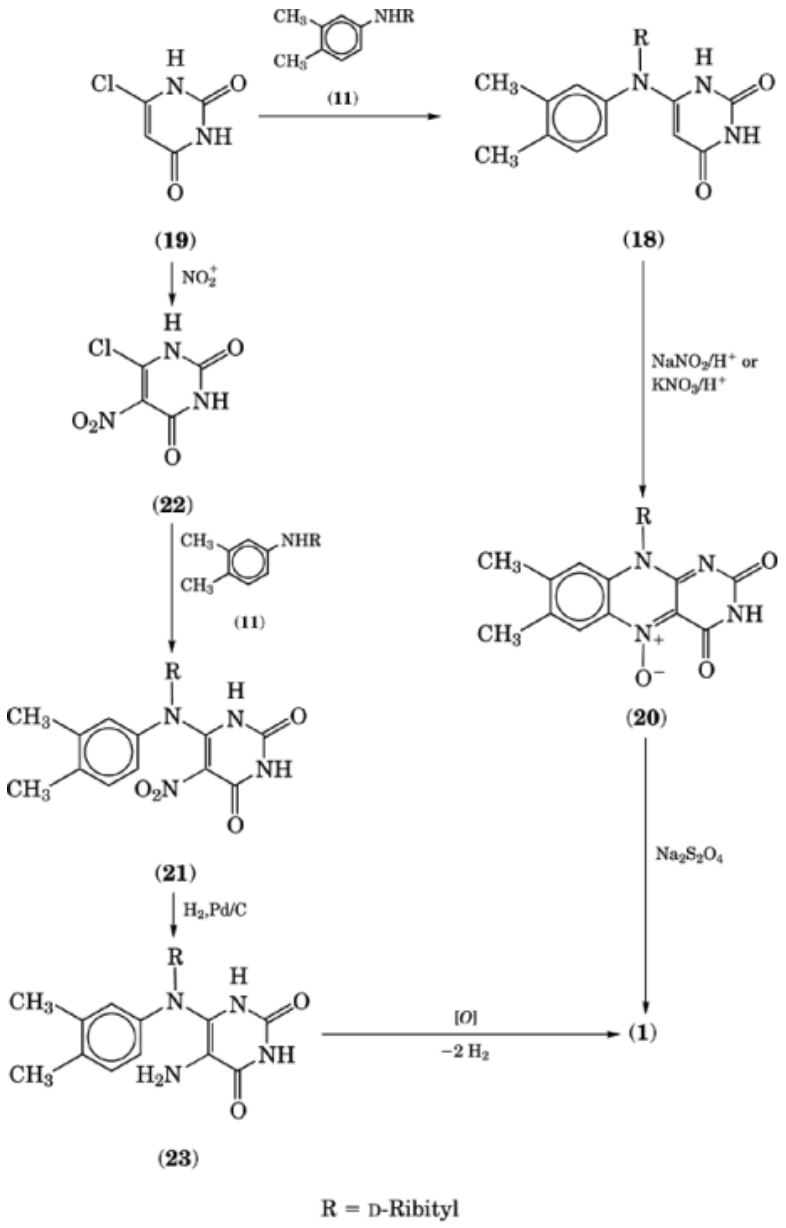


Fig. 4. Alternative syntheses of riboflavin.

with calcium carbonate, inoculated with *Clostridium acetobutylicum*, and incubated at 37–40°C for 2–3 d. The yield is ca 70 mg riboflavin/L (42) (see Fermentation).

Most varieties of *Candida* yeasts produce substantial amounts of riboflavin when glucose is the carbon source. Particularly, *Candida guilliermondia* and *Candida flaveri* produce high yields on a simple synthetic medium of low cost. Some modifications employing several *Candida* yeasts have been patented; eg, *C. intermedia var. A*, a newly isolated microorganism assimilating lactose and ethanol, gave yields of 49.2 mg riboflavin/L

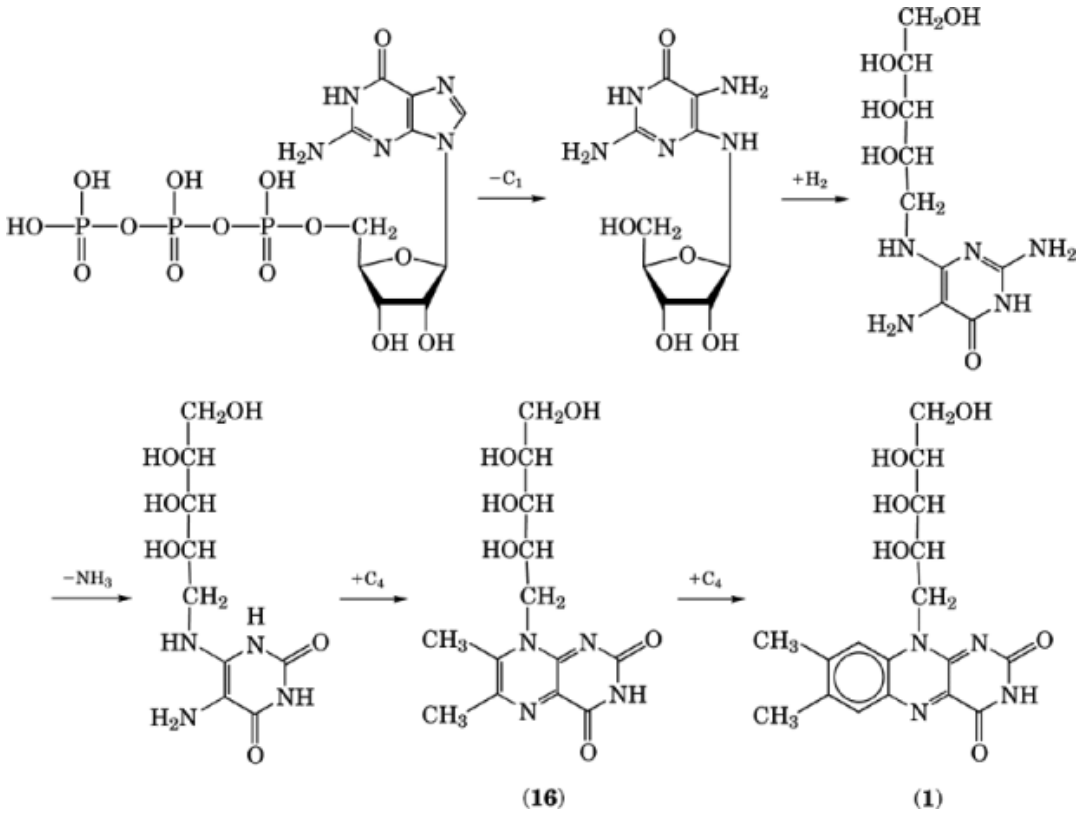


Fig. 5. Biosynthesis pathway to riboflavin.

from ethanol in the presence of biotin (43). *Candida T-3*, assimilating methanol, produces riboflavin by this method from 50 g methanol (44). *Candida* bacteria have the advantage of extremely low iron tolerance. Chelating agents, such as 2,2′-dipyridyl, are recommended to control the iron content (45).

Most of the commercial riboflavin production by aerobic fermentation is obtained by biosynthesis with the yeastlike fungus *Eremothecium ashbyii*. Many variations for the production of riboflavin by *E. ashbyii* have been patented. Employing *E. ashbyii* grown on a yeast medium, riboflavin production on an industrial scale is said to have reached 11.6 g/kg from dried powder (46). Riboflavin is also obtained by fermentation with *E. ashbyii* preserved on Difco-treated millet seeds. The culture, kept for 7–8 d at 32°C on the medium containing crude collagen, corn extracts, unrefined plant oil, glucose, KH_3PO_4, trace elements, and H_2O at pH 7–8, gave 4.5–5.0 g/L (47). In another procedure, *E. ashbyii* was cultivated on a culture medium containing sources of assimilable nitrogen, essential minerals, and growth factors, along with sources of assimilable carbohydrates, unsaturated fatty acids, saccharides, and amino acid or their salts. Incubation at 29°C for 6 d on a rotary shaker with aeration gave average yields of 3.8 g/L (48). *Eremothecium ashbyii* grown in a culture medium containing the oil cake obtained after extraction of lipids from the biomass grown on hydrocarbons gives an even better yield (49).

In operations similar to the *E. ashbyii* procedures, the closely related fungus *Ashbya gossypii* gave similar yields. Thus, a yield of 7.3 g/L was obtained with a lyophilized culture in a medium containing fat, leather glue, and corn extracts (50), and 6.420 g/L with bone or hide fat, alone or in a mixture with other plant or animal

fats as the carbon source (51). The yield from immersed cultures of *A. gossypii* was increased to 6.93–7.20 g/L by use of waste fats or technical cod-liver oil (52).

Riboflavin is also made by aerobic culturing of *Pichia guilliermondii* on a medium containing n-C_{10}–C_{15} paraffins in a yield of 280.5 mg/L (53). A process employing *Pichia* yeasts, such as *P. miso*, *P. miso Mogi*, or *P. mogii*, in a medium containing a hydrocarbon as the carbon source, has been patented (54).

Processes employing *Torulopsis xylinus* (55), *Hansenula polymorpha* (56), *Brevibacterium ammoniagenes* (57), *Achromobactor butrii* (58), *Micrococcus lactis* (59), *Streptomyces testaceus* (60), and others have also been patented. These procedures yield, at most, several hundred milligrams of riboflavin per liter.

Manufacturing procedures of riboflavin have also appeared using *Saccharomyces* bacteria, eg, fermentation with a purine-independent *S*. reverse mutant (61) and with *S. cerevisiae* NH-268 (62) produced 2.79 g/L and 4.9 g/L, respectively.

Further efficient fermentative methods for manufacture of riboflavin have been patented; one is culturing *C. famata* by restricting the carbon source uptake rate, thereby restricting growth in a linear manner by restriction of a micronutrient. By this method, productivity was increased to >0.17 g riboflavin/L/h (63). The other method, using *Bacillus subtilis* AJ 12644 low in guanosine monophosphate hydrolase activity, yielded crude riboflavin 0.9 g/L/3 days, when cultured in a medium including soy protein, salts, and amino acids (64).

In recent (ca 1997) years, fermentative manufacturing methods using recombinant microorganisms have been developed. The mutant clone *GA18Y8-6#2*, prepared by fusion and mutagenation of *C. flaveri* mutants A22 and GA18, produced riboflavin 7.0–7.5 g/L/6 d, when cultivated in 4B medium with a supplement of $FeCl_3$, yeast extracts, peptone, and malt extracts (65). Riboflavin overproducing bacteria prepared by expression of the cloned *rib* operon of *Bacillus subtilis* showed increases in riboflavin manufacture of up to a hundredfold (up to 0.7 g riboflavin/L/48 h). The *rib* operon was cloned as series of overlapping genes using an oligonucleotide derived from the amino acid sequence of the riboflavin synthase β subunit (66). Culturing recombinant *Corynebacterium ammoniagenes KY13313* harboring gene for at least *guanidine triphosphate cyclohydrolase* and riboflavin synthase produced riboflavin ~ 30-fold higher than that with the controlled bacteria (67).

4. Industrial Aspects

For the industrial production of riboflavin as pharmaceuticals, the traditional methodology comprising the direct condensation of 2 with 2 in an acidic medium with continuous optimization of the reaction conditions is still used (28). A great part of riboflavin manufactured by fermentative methods is used for feeds in the form of concentrates. The present world demand of riboflavin may be about 2500 t per year. Of this amount, 60%, 25%, and 15% are used for feeds, pharmaceuticals, and foodstuffs, respectively. The main producers are Hoffmann-La Roche, BASF, Merck & Co., and others.

5. Analytical Methods

Riboflavin can be assayed by chemical, enzymatic, and microbiological methods. The most commonly used chemical method is fluorometry, which involves the measurement of intense yellow-green fluorescence with a maximum at 565 nm in neutral aqueous solutions. The fluorometric determinations of flavins can be carried out by measuring the intensity of either the natural fluorescence of flavins or the fluorescence of lumiflavin formed by the irradiation of flavin in alkaline solution (68). The later development of a laser–fluorescence technique has extended the limits of detection for riboflavin by two orders of magnitude (69, 70).

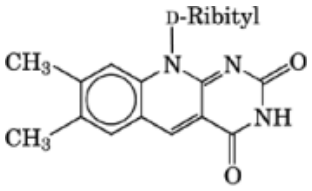
Polarography is applied in the presence of other vitamins, eg, in multivitamin tablets, without separation. The polarography of flavins is reviewed in Reference 71.

The microbial assay is based on the growth of *Lactobacillus casei* in the natural (72) or modified form. The lactic acid formed is titrated or, preferably, the turbidity measured photometrically. In a more sensitive assay, *Leuconostoc mesenteroides* is employed as the assay organism (73). It is 50 times more sensitive than *L. casei* for assaying riboflavin and its analogues (0.1 ng/mL vs 20 ng/mL for *L. casei*). A very useful method for measuring total riboflavin in body fluids and tissues is based on the riboflavin requirement of the protozoan cliate *Tetrahymena pyriformis*, which is sensitive and specific for riboflavin. This method can be applied to large-scale nutrition studies.

Although riboflavin can be assayed more readily by chemical or microbiological methods than by animal methods, the latter are preferred for nutritional studies and as the basis of other techniques. Such assays depend upon a growth response; the rat or chick is the preferred experimental animal. This method is particularly useful for assaying riboflavin derivatives, since the substituents frequently reduce or eliminate the biological activity.

An enzymatic method for assessing riboflavin deficiency in humans has been developed (74). It is based on the fact that NADPH-dependent glutathione reductase of red cells reflects riboflavin fluctuations.

High pressure liquid chromatography (hplc) has been extensively used for the riboflavin determinations. This method is usually automated and more rapid and sensitive than the microbial method. It has been used in combination with fluorometric detection for the riboflavin assay in foods (75), meat and meat products (76), and enriched and fortified foods (77), as well as in a simple assay for animal tissues (78). A rapid and efficient reversed-phase hplc method is described for the quantitative separation of flavin coenzymes and their structural analogues such as 5-deazaflavin [19342-73-5] **(24)** and 8-hydroxy-7-demethyl-5-deazariboflavin [37333-48-5] **(25)** (F_{420} chromophore) (79). Comprehensive reviews of the analytical methods for riboflavin are given in References 80 and 81.



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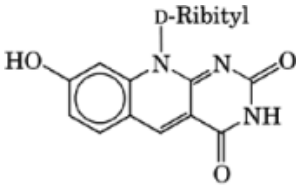

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6. Biological Function

In biological systems, riboflavin functions almost exclusively in the form of flavoproteins, in which the FMN or FAD is generally bound as prosthetic group or coenzyme to specific proteins. These enzymes catalyze oxidation–reduction reactions (Table 2). The flavin group of the oxidized coenzyme is reduced chemically or enzymatically to 1,5-dihydroflavin coenzyme, probably in two one-electron steps, each involving the addition of a single

Table 2. Some Reactions Catalyzed by Flavoproteins[a]

Enzyme	Electron donor	Product	Coenzyme and other components	Electron acceptor
D-amino acid oxidase	D-amino acids	α-keto acids $+NH_3$	2FAD	$O_2 \longrightarrow H_2O_2$
L-amino acid oxidase (liver)	L-amino acids	α-keto acids $+NH_3$	2FAD	$O_2 \longrightarrow H_2O_2$
L-amino acid oxidase (kidney)	L-amino acids	α-keto acids $+NH_3$	2FMN	$O_2 \longrightarrow H_2O_2$
L(+)-lactate dehydro-genase (yeast)	lactate	pyruvate	1FMN; 1heme (*cyt* b_5)	respiratory chain
glycolate oxidase	glycolate	glyoxylate	FMN	$O_2 \longrightarrow H_2O_2$
NADH-cytochrome *c* reductase	NADH	NAD^+	FMN; 2Mo, NHI	cytochrome c_{ox} respira-tory chain
NADH-cytochrome b_5 reductase	NADH	NAD^+	FAD; Fe	cytochrome b_5
aldehyde oxidase (liver)	aldehydes	carboxylic acids	FAD; Fe, Mo	respiratory chain
α-glycerol phosphate dehydrogenase	glycerol 3-phos-phate	dihydroxy-acetone phosphate	FAD; Fe	respiratorychain
succinate dehydro-genase	succinate	fumarate	FAD; Fe, NHI	respiratory chain
acyl-CoA(C_6–C_{12}) dehydrogenase	acyl-CoA	enoyl-CoA	FAD	electron-transferring flavoprotein
nitrate reductase	NADPH	$NADP^+$	FAD; Mo, Fe	nitrate
nitrite reductase	NADPH	$NADP^+$	FAD; Mo, Fe	nitrite
xanthine oxidase	xanthine	uric acid	FAD; Mo, Fe	O_2
lipoate dehydrogenase	reduced lipoic acid	oxidized lipoic acid	2FAD	NAD^+
dehydroorotate dehydrogenase	dihydroorotic acid	orotic acid	2FMN; 2FAD, 4Fe	undetermined

[a] Ref. 82.

electron. Stable semiquinone radicals are formed as intermediates, because the unpaired electron is highly delocalized by the conjugated isoalloxazine structure.

In contrast to the nicotinamide nucleotide dehydrogenases, the prosthetic groups FMN and FAD are firmly associated with the proteins, and the flavin groups are usually only separated from the apoenzyme (protein) by acid treatment in water. However, in several covalently bound flavoproteins, the enzyme and flavin coenzymes are covalently affixed. In these cases, the flavin groups are isolated after the proteolytic digestion of the flavoproteins.

Many flavoproteins react directly with molecular oxygen to produce hydrogen peroxide. Some flavoproteins, such as the flavin-containing monooxygenase, give water instead of hydrogen peroxide. In these cases, one atom of oxygen is introduced into a substrate to undergo hydroxylation, whereas the other oxygen atom is released as water. Several flavoproteins include metal complexes where these reactions take place. A number of reviews of the preparation, properties, and mechanism of action of these enzymes have been published (83, 84).

7. Deficiency, Requirements, and Toxicity

Riboflavin is essential for mammalian cells. A lack of riboflavin in the human diet causes characterized deficiency syndromes, such as sore throat, hyperemia, cheilosis, angular stomatitis, glossitis (magenta tongue), a generalized seborrheic dermatitis, scrotal and vulval skin changes, and a normocytic anemia. Because riboflavin is essential to the functioning of vitamins B_6 and niacin, some symptons attributed to riboflavin deficiency are actually due to the failure of systems requiring these other nutrients to operate effectively (85).

The 1989 Recommended Dietary allowances (RDA) of the Food and Nutrition Board (86) are 0.6 mg riboflavin per 239 kJ (1000 kcal) for essentially healthy people of all ages. This leads to the ranging from 0.4 mg/day for early infants to 1.8 mg/day for young males. For elderly people and others whose daily calorie intake may be less than 478 kJ (2000 kcal), a minimum of 1.2 mg/day is recommended. During pregnancy, an additional riboflavin intake of 0.3 mg/day is recommended in view of the increased tissue synthesis for both fetal and maternal development. For the lactating woman, the requirement is assumed to increase by an amount at least equal to that excreted in milk, which has a mean riboflavin content of 35 μg/100 mL. At an average milk production of 750 mL/day and 600 mL/d during the first and second 6 months of lactation, riboflavin secretion is 0.26 mg/d and 0.21 mg/d, respectively. Because the utilization of the additional riboflavin for milk production is assumed to be 70%, and the coefficient of variation of milk production is 12.5%, an additional daily intake of 0.5 mg is recommended for the first 6 mo of lactation and 0.4 mg thereafter.

Riboflavin is essentially nontoxic. The LD_{50} values in mice and rats by intraperitoneal injection are 340 mg/kg (87) and 560 mg/kg (88), respectively. The oral administration of 10 g/kg to rats or 2 g/kg to dogs showed no toxic effects (89).

8. Derivatives

8.1. Riboflavin-5′-Phosphate

Riboflavin-5′-phosphate [146-17-8] (vitamin B_2 phosphate, flavin mononucleotide, FMN, cytoflav), $C_{17}H_{21}N_4O_9P$, mol wt 456.35, is a microcrystalline yellow solid, mp 195°C, $[\alpha]_D^{28}$ = +44.5° (2% soln in conc HCl) with biological and enzymatic activity. It is prepared by phosphorylation of riboflavin with chlorophosphoric acid (90), pyrophosphoric acid (91), metaphosphoric acid (92), or catechol cyclic phosphate (93). It is soluble in water to the extent of 3 g/100 mL at 25°C as the sodium salt but tends to gel. Because of the high sensitivity of FMN to uv, it must be preserved in dark, tight containers.

Flavin mononucleotide was first isolated from the yellow enzyme in yeast by Warburg and Christian in 1932 (4). The yellow enzyme was split into the protein and the yellow prosthetic group (coenzyme) by dialysis under acidic conditions. Flavin mononucleotide was isolated as its crystalline calcium salt and shown to be riboflavin-5′-phosphate; its structure was confirmed by chemical synthesis by Kuhn and Rudy (94). It is commercially available as the monosodium salt dihydrate [6184-17-4], with a water solubility of more than 200 times that of riboflavin. It has wide application in multivitamin and B-complex solutions, where it does not require the solubilizers needed for riboflavin.

8.2. Riboflavin-5′-Adenosine Diphosphate

Riboflavin-5′-adenosine diphosphate [146-14-5] (flavin–adenine dinucleotide, FAD), $C_{27}H_{33}N_9O_{15}P_2$ **(2)**, mol wt 785.56, was first isolated in 1938 from the D-amino acid oxidase as its prosthetic group (95), where it was postulated to be flavin–adenine dinucleotide. The structure was established by the first synthesis in Todd's laboratory (96); the monosilver salt of FMN was condensed with 2′,3′-isopropylidene-adenosine-5′-benzylphosphorchloridate, followed by removal of protective groups. It was also synthesized directly from FMN and adenosine-5′-monophosphate (AMP) with di-*p*-tolylcarbodiimide as the condensation agent (97). Another direct synthesis was achieved by dehydration between FMN and AMP with trifluoroacetic acid anhydride (98). A 40% yield was obtained by condensation of adenosine-5′-phosphoramidate and FMN using a mixture of pyridine and *o*-chlorophenol as the solvent (99). Condensation of AMP with FMN in ethoxyacetylene gives a 10–15% yield; by-products such as riboflavin-4′,5′-cyclic phosphate are avoided (100). An efficient procedure was developed, which transforms the free acid of both nucleotides into their tri-*n*-butyl-ammonium salts and uses a reactive carbodiimide for the coupling reaction. The yield of FAD can be as high as 70% (101). In

addition to D-amino acid oxidase, FAD is the prosthetic group for the other flavoproteins including glucose oxidase, lycine oxidase, fumarate hydrogenase, histaminase, and xanthine oxidase.

8.3. Covalently Bound Flavins

The FAD prosthetic group in mammalian succinate dehydrogenase was found to be covalently affixed to protein at the 8 α-position through the linkage of 3-position of histidine (102, 103). Since then, several covalently bound riboflavins (104, 105) have been found successively from the enzymes listed in Table 3. The biosynthetic mechanism, however, has not been clarified.

8.4. 6-Hydroxyriboflavin

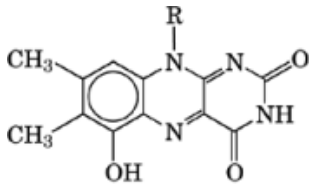
This compound [86120-61-8] **(26)** was isolated as a green coenzyme of the NADH dehydrogenase from *Peptostreptococcus elsdenii* and also from glycolate oxidase of porcine liver. It is not fluorescent, and its structure was established by synthesis (106). The 5′-monophosphate serves as a cofactor for glycolate oxidase from pig liver.

8.5. 8-Nor-8-hydroxyriboflavin

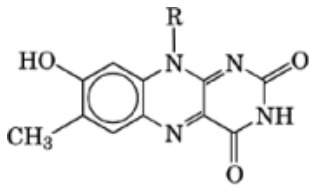
A prosthetic group of red color has been isolated from NADH dehydrogenase of the electron-transferring flavoprotein of *Peptostreptococcus elsdenii*. Its structure [52134-62-0] **(27)** has been established as the FAD derivative of 8-hydroxy-7-methylisoalloxazine. Proof has been obtained by the synthesis of 8-hydroxy-7-methylisoalloxazine models and stepwise degradation of the naturally occurring compound (107).

8.6. Roseoflavin

Roseflavin [51093-55-1], $C_{18}H_{23}N_5O_6$, **(28)**, mol wt 405.41, mp 274–297°C, $[\alpha]_D = -320^\circ$ (0.1*M* NaOH), was isolated from a culture medium of *Streptomyces davawensis* as dark, reddish-brown fine needles (from ethanol); the 8-methyl group of riboflavin is substituted by a dimethylamino group. This structure was confirmed by the synthesis. Roseflavin shows antimicrobial activity against gram-positive bacteria (108).

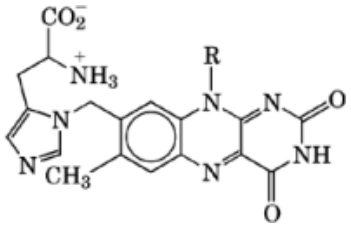
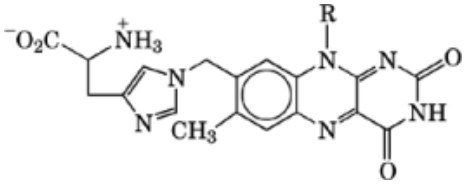
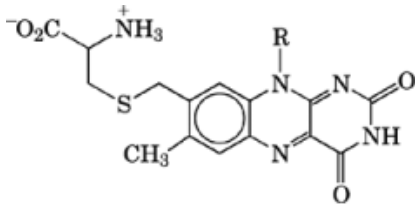
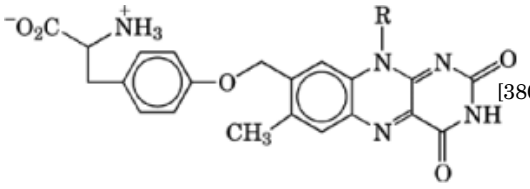
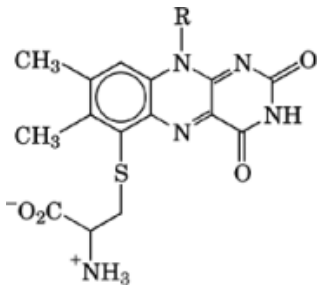


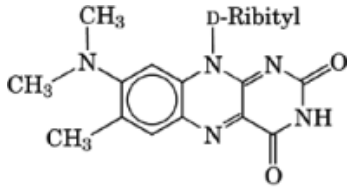
(26)



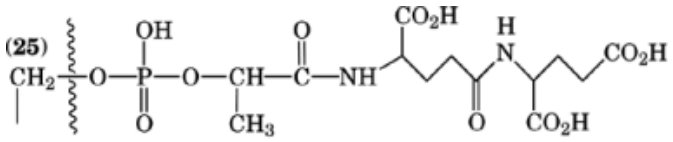
(27)

Table 3. Covalently Bound Flavins

Prosthetic group (R = D–Ribityl)	CAS Registry Number	Formula	Enzyme
 8 α-(N^1-histidyl)riboflavin	[58525-92-1]	$C_{23}H_{27}C_7O_8$	thiamine dehydrogenaseβ-cyclopiazonate oxidocyclaseL-gulono-γ-lactone oxidaseL-galactonolactone oxidasecholesterol oxidase
 8 α-(N^3-histidyl)riboflavin	[37854-44-7]	$C_{23}H_{27}N_7O_8$	succinate dehydrogenasefumarate reductasesalcosine dehydrogenasesalcosine oxidasedimethylglycine dehydrogenaseD-hydroxynicotine oxidasecholine oxidaseD-gluconate dehydrogenase
 8 α-*S*-cysteinylriboflavin	[35836-22-7]	$C_{20}H_{25}N_5O_8S$	monoamine oxidase Amonoamine oxidase B*Chlorobium* cytochrome C_{552}*Chlomatium* cytochrome C_{553}
 8 α-*O*-tyrosylriboflavin	[38065-74-6]	$C_{26}H_{29}N_5O_9$	*p*-cresol methylhydroxylase
 6-*S*-cysteinylriboflavin	[73647-60-6]	$C_{20}H_{25}N_5O_8S$	trimethylaminedehydrogenase-dimethylaminedehydrogenase



(28)



(29)

8.7. 5-Deazariboflavin

In 5-deazariboflavin **(24)**, the N-5 of riboflavin is replaced by CH; it serves as cofactor for several flavin-catalyzed reactions (109). It was first synthesized in 1970 (110); improved synthetic processes were reported later (111).

A low potential electron carrier, the fluorescent factor F_{420} [37333-48-5, 64885-97-8] **(29)** (it absorbs maximally at 420 nm), possessing a 5-deazaflavin moiety, was isolated from methane-producing bacteria (112). F_{420} is an obligate intermediate for passage of an electron from H_2 to $NADP^+$ to generate NADPH. The structure of F_{420} was proposed as an 8-hydroxy-7-demethyl-5-deazaflavin derivative **(25)** (113) and confirmed as compound **(29)** by the total synthesis (114).

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