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Synthetic Riboflavin-5′-Phosphate*

By E. De RITTER, J. SCHEINER, F. W. JAHNS, L. DREKTER, and S. H. RUBIN

Further confirmation of the full biological activity of synthetic riboflavin-5′-phosphate (FMN), including rat microbiological assays and human availability studies are described. Properties in relation to their effect on fluorometric and microbiological methods are presented. Synthetic FMN has full activity on a molar basis for growth of *L. casei* in the riboflavin assay.

THEORELL (1, 2) described the isolation of a riboflavin monophosphoric acid ester by a reversible splitting of protein from the yellow enzyme of Warburg and Christian (3). Following the synthesis of riboflavin by Karrer, *et al.* (4), and by Kuhn, *et al.* (5), a phosphate derivative of the vitamin was prepared by Kuhn and Rudy (6) and later shown (7) to combine with the protein group of the yellow enzyme in the same fashion as the natural riboflavin phosphate (flavin mononucleotide or FMN). By means of oxidation with periodic acid, the phosphate in FMN was shown by Karrer, *et al.* (8), to be in the 4′ or 5′-position in the riboflavin molecule. Forrest and Todd (9) prepared a synthetic product exhibiting the same paper chromatographic behavior as natural FMN. Periodate oxidation of the synthetic product indicated it to be the 5′-phosphate. A substance which also showed the same paper chromatographic properties was prepared by them in low yield via 5′-trityl,2′,3′,4′-triacetyl riboflavin according to the method of Kuhn, *et al.* (10), thereby providing additional confirmation that natural FMN is the 5′-phosphate.

FMN serves as the prosthetic group of a number of enzymes, as does flavin-adenine dinucleotide (FAD), which is formed from FMN by the addition of adenylic acid. The isolation of FAD has been described by Warburg and Christian (11) and its enzymatic synthesis from natural FMN and adenosine triphosphate by Schrecker and Kornberg (12). The biological importance of riboflavin-5′-phosphate is indicated by the fact that practically all of the riboflavin in many animal tissues occurs as the mononucleotide or to an even greater extent as FAD (13, 14).

Flexser and Farkas (15) have evolved a synthesis of FMN, which has made it available in large quantities. In addition to the free acid ester, both mono-diethanolamine and monosodium salts have been prepared. The biological activity of this synthetic FMN has been studied in various enzyme systems. For example, Kearney and Englard (16) found the synthetic product to be fully as active as natural FMN in its ability to activate the apo-enzyme of cytochrome *c* reductase of yeast according to the method of Haas, *et al.* (17). Riegel and Meyer (18) reported that synthetic FMN could replace a supernatant fraction of rat liver homogenate in tests of enzymatic inactivation of estradiol and diethylstilbestrol. Further confirmation of the full biological activity of the synthetic FMN is provided by the experiments described below, which include rat and microbiological assays and human availability studies. Properties of the FMN, some of which are of particular interest in relation to their effect on the usual fluorometric and microbiological methods for riboflavin assay, are described.

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RIBOFLAVIN ASSAYS

Rat Growth Tests.—Riboflavin assays by prophylactic and curative rat growth methods are given in Table I for two preparations of the monodiethanolamine salt of FMN. These assays were carried out according to a modification of the method of Street (19) with dosage by both oral and intramuscular routes. Twelve rats were used for each of the two levels of riboflavin standard and test material. A typical growth response in a prophylactic assay with oral dosage is shown in Fig. 1. Within the limits of error of the bioassay, these data indicate the FMN salt to have the same activity as riboflavin on a molar basis in all three types of bioassay.

TABLE I.—RAT-GROWTH ASSAYS OF MONODIETHANOLAMINE SALT OF RIBOFLAVIN-5′-PHOSPHATE (DIHYDRATE)

Lot No.	Type of Assay	Mode of Administration	Riboflavin Assay, % of Theoretical ± 2 S.E.
VI-101[a]	Prophylactic	Oral	95 ± 15
VI-116[b]	Curative	Oral	86 ± 15
VI-116	Curative	Intramuscular	101 ± 13

[a] Assayed 100% of theory fluorometrically and 99% microbiologically.
[b] Assayed 100% of theory fluorometrically and 98% microbiologically.

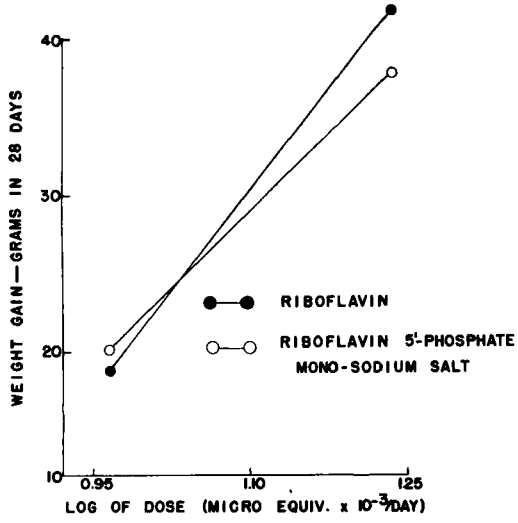

Fig. 1.—Rat curative assay of mono-sodium salt of riboflavin 5′-phosphate (dihydrate).

Microbiological Activity.—Riboflavin assays have been carried out according to the *L. casei* method of Snell and Strong (20). Because FMN is more sensitive than riboflavin to light destruction at the very low assay concentrations as detailed below, special precautions were taken to avoid exposure of test solutions. Operations were carried out as far as possible in a darkroom under Wratten Series OA filters and solutions were stored in amber or red flasks. Prior to autoclaving, the racks of assay tubes were placed in a covered metal box and kept therein until the forty-hour incubation was completed.

Synthetic preparations of FMN or its salts show the same activity for *L. casei* as riboflavin on a molar basis. To demonstrate this fact, a sample of mono-sodium salt (dihydrate) was assayed repeatedly to obtain a statistical average of both microbiological and fluorometric assays. In 13 tests of this preparation, which has a theoretical riboflavin content of 73%, the microbiological assays averaged 71.0% ± 2.4 (2 S.E.) and the fluorometric assays 71.0% ± 0.68 (2 S.E.). Prolonging the normal forty-hour incubation period in the microbiological assay to three, four, five, or seven days did not alter the relative activity of riboflavin and FMN.

Attempts by Theorell (2) to couple crude preparations of FMN with protein to form the yellow respiratory enzyme indicated that the position of the phosphate group in the molecule was of deciding importance. Linkage only at the 5′ or terminal position in the ribose side chain is essential for full micro-biological activity. Further phosphorylation in other positions of the ribose side chain may result in decreased activity for *L. casei* without affecting the fluorescence significantly. Hence, to assess the biological potency of FMN preparations, a biological or microbiological test is necessary. Such decreased activity upon esterification in other than the 5′-position has been reported by Furter, *et al.* (21). for mono-, di-, tri-, and tetra-succinates of riboflavin.

Fluorescence Measurements.—On a molar basis, the fluorescence of FMN prepared from natural sources has been reported by Bessey, *et al.* (13), to be equal to that of riboflavin. Similarly, the fluorescence of riboflavin in the synthetic product is unaffected by esterification in the 5′-position. Hence, estimation of the total percentage of riboflavin in preparations of FMN may be made conveniently by measurement of the fluorescence upon exposure to ultraviolet light. As noted above, however, the fluorescence may not indicate biological activity since it is not specific for the 5′-phosphate. Bessey, *et al.* (13), studied also the effect of *p*H between 1.3 and 6.6 on the fluorescence of riboflavin and natural FMN and found the latter to lose its fluorescence at a slightly higher *p*H than does the riboflavin. A similar study of the fluorescence of riboflavin and synthetic FMN between *p*H 2 and 11 is shown in Fig. 2. McIlvaine's phosphate citrate buffer was used from *p*H 2 to 8 with NaOH

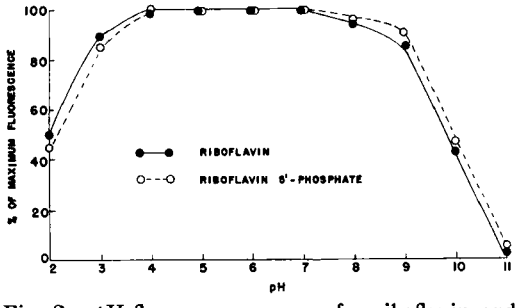

Fig. 2.—*p*H-fluorescence curves for riboflavin and riboflavin 5′-phosphate.

added to achieve *p*H levels above 8. The differences in fluorescence at the lower *p*H levels are very similar to those found by Bessey, *et al.* (13), for natural FMN. Between *p*H 4 and 7 both compounds show equal fluorescence on a molar basis. As the *p*H is increased above neutrality FMN again loses its fluorescence at a slightly higher *p*H than riboflavin.

Human Availability.—Relative urinary excretions of riboflavin after oral doses of riboflavin and FMN were determined according to the procedure described by Melnick, *et al.* (22), for testing physiological availability. Seven subjects participated in tests of both the free ester and its mono-diethanolamine salt (dihydrate), four taking in the first week a dose of 10 mg. of riboflavin and three an equivalent amount of the test substance. In the second week of the tests, the dose for each subject was reversed. Urinary excretions were measured fluorometrically by the double reduction method of Rubin, *et al.* (23), and microbiologically by the *L. casei* method noted above (20).

It was also of interest to determine whether FMN was excreted in the urine as such or as free riboflavin. For this purpose the benzyl alcohol extraction procedure of Burch, *et al.* (24), as applied to the macro assay of urine, was followed exactly except that all volumes were doubled to provide 15 ml. for fluorometric reading. By this method, riboflavin is extracted almost quantitatively by the benzyl alcohol as compared to only a few per cent of FMN or its salt. A summary of the excretion data is given in Table II. If the term "availability" is taken to denote the relative urinary excretion after FMN and riboflavin dosage, it is evident from the data in Table II that both the free ester and its diethanolamine salt are 100 per cent available. The fact that the average excretions determined by the benzyl alcohol technique are not significantly different from those found by the direct fluorometric and microbiological methods indicates that the excretion after both riboflavin and FMN ingestion, is largely, if not entirely in the form of free riboflavin. The sensitivity of this method is not sufficient to prove or disprove the presence of a small percentage of FMN. That the excretion in the form of FAD is negligible has been shown by Klein and Kohn (25) by means of the alanine test of Warburg and Christian (11).

PROPERTIES OF SYNTHETIC FMN

Sensitivity to U. V. Light.—While measuring the fluorescence of solutions of FMN at concentrations of about 0.15 μg. per ml., it was observed that the destruction of fluorescence on exposure to the ultraviolet light of the fluorophotometer was more rapid than in comparable solutions of riboflavin. This is illustrated in Fig. 3, which shows the relative rates of destruction of FMN and riboflavin during ten minutes exposure in the cuvettes of a Pfaltz and Bauer Model B Fluorophotometer with a Type H-3 mercury lamp, a primary filter combination having a peak at about 430 mμ, and a secondary filter to eliminate wave lengths below 520 mμ. Solutions of both compounds were compared in 0.015 *M* acetate buffer at *p*H about 5.9 and in 0.113 *M* phosphate + 0.033 *M* citrate buffer at *p*H about 6.0. In both buffers the losses of FMN are greater than those of riboflavin, the difference in rate of destruction being

TABLE II.—PHYSIOLOGICAL AVAILABILITY TO HUMANS OF RIBOFLAVIN-5′-PHOSPHATE, FREE ESTER AND MONO-DIETHANOLAMINE SALT (DIHYDRATE)

	% Recovery in Urine of Test Dose (equiv. to 10 mg. of riboflavin)					
	Fluorometric Assay				Microbiological Assay with *L. casei*	
	Direct Method (23)		In Benzyl Alcohol (24)			
Subject	Riboflavin	FMN Free ester	Riboflavin	FMN Free ester	Riboflavin	FMN Free ester
EM	58	70	60	63	65	60
IK	58	66	40	60	60	62
EDR	47	65	53	52	58	58
JS	71	68	65	65	75	69
WK	59	45	37	49	59	43
RW	42	42	44	35	44	41
MW	55	60	55	59	59	69
Av.	56	59	51	55	60	57
		Mono-diethanol-amine salt		Mono-diethanol-amine salt		Mono-diethanol-amine salt
EM	52	61	50	56	49	57
BM	65	58	63	65	59	60
NC	51	60	55	50	57	57
EDR	46	61	40	56	50	63
ES	53	56	54	58	54	61
RW	41	45	42	51	38	48
FWJ	47	48	40	48	46	53
Av.	51	56	49	55	50	57

	% Availability ± S.E.		
	Fluorometrically		
	In Aqueous Soln.	In Benzyl Alcohol	Micro-biologically
FMN, free ester	105 ± 10.0	108 ± 11.6	95 ± 8.9
FMN, mono-diethanolamine salt (dihydrate)	110 ± 7.9	112 ± 8.9	114 ± 7.2

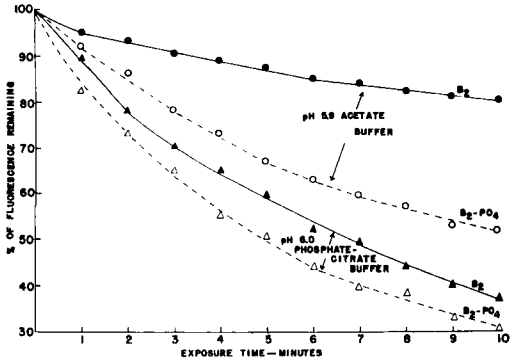

Fig. 3.—Relative rate of destruction of riboflavin and riboflavin-5′-phosphate by UV light.

most marked at the beginning of exposure. Higher losses were noted in the phosphate-citrate buffer, but the difference between FMN and riboflavin is greater in the acetate buffer. Because of this greater sensitivity of FMN in very dilute solutions, care must be taken to avoid exposure to light. The use of a photocell with a rapid response is desirable for precise measurements of fluorescence. The fluorescence may also be affected by the quality of the distilled water used for dilution. The use of ion-exchange purified water or prior addition of a chelating agent to the water will eliminate such difficulty. These special precautions are essential only at the very low concentrations involved in the analysis and not at normal pharmaceutical levels, which are usually at least a thousand-fold higher.

Enzymatic Hydrolysis.—The liberation of free riboflavin from FMN by treatment with an acid phosphatase (Clarase) was reported by Bessey, *et al.* (13). The rate of such hydrolysis of synthetic FMN (Fig. 4) has been determined by measurement

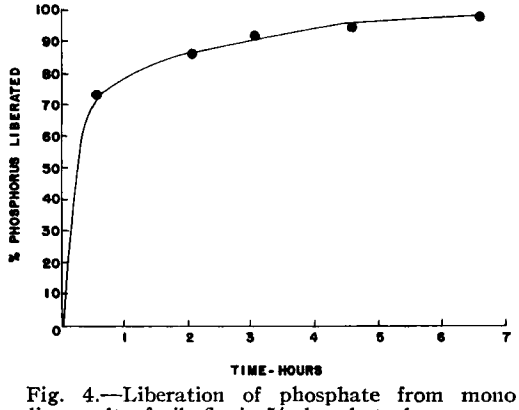

Fig. 4.—Liberation of phosphate from mono sodium salt of riboflavin-5′-phosphate by enzyme (Clarase) treatment at 45° and *p*H 4.5.

of the free phosphate produced, using a modification of the method of Lohmann and Jendrassik (26), which distinguishes between free and organically bound phosphate. Eight-milliliter aliquots containing 4 mg. of mono-sodium salt of FMN (dihydrate) in 0.05 *M* acetate buffer at *p*H 4.5 were digested at 45° with 10 mg. of Clarase for five-tenths to six and five-tenths hours, after which enzyme action was stopped by steaming for ten minutes and free phosphate determined. Parallel blank solutions were set up without FMN to correct for phosphate contributed by the enzyme. It is evident (Fig. 4) that the enzyme splitting of FMN is rapid, over 70% of the phosphate being liberated in the first half hour of incubation.

Acid Hydrolysis.—The directions for fluorometric and microbiological riboflavin assays by the U. S. P. method (27) call for autoclaving samples in 0.1 *N* HCl for thirty minutes at 121–123° in order to dissolve the riboflavin. In assaying riboflavin 5′-phosphate, application of this acid hydrolysis has been found to cause a 15% loss of microbiological riboflavin activity. Under the same conditions the fluorescence is decreased by only 2–3%. For this reason acid hydrolysis should be avoided, particularly in microbiological assays. Because of the high solubility of the phosphate in water, no difficulty is encountered in preparing assay solutions without such hydrolysis. Simple shaking, or in the case of tablets, blending in water with adequate protection from actinic light is sufficient.

SUMMARY

1. Synthetic riboflavin-5′-phosphate (FMN) is as active on a molar basis as riboflavin in promoting growth of weanling rats in curative assays with oral or intramuscular dosage or in a prophylactic test with oral dosage.

2. Synthetic FMN has full activity on a molar basis for growth of *L. casei* in the riboflavin assay. Since full microbiological activity is achieved only with substitution in the 5′-position, the microbiological assay is the method of choice for determining the purity of FMN preparations rather than the fluorometric assay which is less sensitive to phosphorylation at other points in the ribose chain.

3. Comparison of urinary excretion after oral doses of riboflavin and FMN shows the latter to be completely available to humans. Excretion of both substances is largely, if not entirely, in the form of free riboflavin.

4. The more rapid destruction by light of FMN than of riboflavin at the very low concentrations used in fluorometric and microbiological assays necessitates careful protection of such solutions from light exposure. Acid hydrolysis, which is also destructive, should not be applied in the assay of FMN.

5. The phosphate group is split rapidly from FMN by enzymatic hydrolysis with Clarase at *p*H 4.5 and 45°.

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The Relative Efficacy of Various Ointment Bases as Antiseptic Vehicles*

By CHARLES K. KOLSTAD and C. O. LEE†

This study indicates that the Beeler base, methyl cellulose base, and polyethylene glycol ointment are more efficient vehicles for ammoniated mercury, boric acid, and yellow mercuric oxide than the official white ointment. The Beeler base and polyethylene glycol ointment proved to be better vehicles for iodine than the official yellow ointment. Petrolatum-rose water ointment is a more efficient vehicle for ammoniated mercury and for yellow mercuric oxide than the official white ointment despite the incompatibility between the yellow mercuric oxide and the base.

IN THE USE OF OINTMENTS for the administration of antiseptics, two questions arise. First, which ointment base is best suited for the medicinals prescribed? Second, will a good antiseptic act as such, irrespective of the base in which it is incorporated?

So far as we know, no one has, as yet, proposed the base most suitable for efficient therapeutic action of the medicaments commonly used in the form of ointments. Investigators differ regarding the type of base to use in most instances. Some favor the use of bases which contain a high percentage of water (1, 2). Others maintain that the greasy bases are more efficient (3–6). Still others feel that one type of base is as good as another (7, 8). The ointments that are official in both the Pharmacopeia and the National Formulary are, for the most part, prepared with greasy bases.

A RÉSUMÉ OF PREVIOUS WORK

In 1929, Reddish and Wales (9) tested the antiseptic properties of 26 official ointments, 12 in the U. S. Pharmacopoeia X, and 14 in the National Formulary V. Of these, 5 of the U. S. P. and 6 of the N. F. ointments proved to be antiseptic when tested by the FDA method. Among those preparations which showed no antiseptic action was the official 2% phenol ointment. This observation, concerning phenol ointment, was confirmed by later workers (2, 10, 11).

Comprehensive studies of the action of antiseptics in a variety of ointment bases were made by Prout and Strickland (3) in 1937, Gershenfeld and Brillhart (1) in 1939, and Foley and Lee (8) in 1942. In 1945, Fiero and Loomis (12) and Neuroth and Lee (13) investigated the activity of a single antiseptic in a variety of bases. Hart and Huyck (14), in 1948 tested a series of quaternary ammonium compounds in several bases in an effort to determine the base most suitable as a vehicle for them.

The findings of these investigators substantiated the idea of Reddish, that no single base is wholly satisfactory as the vehicle for all antiseptics. In view of this fact, and for other reasons, this study was made in an effort to determine the relative antiseptic efficacy of four well-known chemicals using five of the newer ointment bases.

* Received July 21, 1954 from the Research Laboratories of the Purdue University School of Pharmacy, Lafayette, Ind.
† Present address: Ohio Northern University, College of Pharmacy, Ada.