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Phosphorylation of Biologically Active Analogs of Riboflavin[1] (39592)

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Two analogs of riboflavin (Fig. 1, I), 7-ethyl-8-methyl-flavin [7-ethyl-8-methyl-10-(1′-D-ribityl)isoalloxazine] (Fig. 1, II), and 7-methyl-8-ethyl-flavin [7-methyl-8-ethyl-10-(1′-D-ribityl)isoalloxazine] (Fig. 1, III) (1), are able to serve as replacements for riboflavin in the metabolism of the rat (2, 3), with respect to growth, survival, optimal physical appearance, and efficiency of food utilization. Although these three flavins are indistinguishable by the above criteria, their utilization shows some significant differences with respect to growth (2), and especially with respect to succinic acid dehydrogenase (EC 1.3.99.1) (SDH) activity of the heart, kidney, and liver (4, 5). However, in spite of the fact that the greatest differences in the SDH activity are found in the liver, the total flavin content and the quantity of the flavin found as FAD or analog-FAD in the liver mitochondria are the same for the three flavins (6).

A third analog of riboflavin, 7,8-diethyl-flavin [7,8-diethyl-10-(1′-D-ribityl)isoalloxazine] (Fig. 1, IV) (7) is a competitive inhibitor of riboflavin in the rat (8, 9), and as reasonably expected, it mimics riboflavin deficiency particularly with respect to its influence on the SDH activity of the above tissues (5). This flavin was the first analog of riboflavin shown to be phosphorylated *in vivo* in mammalian tissue (10).

The above three analogs of riboflavin are readily isolatable and identifiable in the form of free flavins or as nucleotides in the tissue of rats to which they are administered. This was not true of the fourth analog of riboflavin to be considered, namely 7-chloro-8-methyl-flavin [7-chloro-8-methyl-10-(1′-D-ribityl)isoalloxazine] (Fig. 1, V) (11), in which case only the free flavin was found in the livers of animals receiving it (12). In terms of growth response of the riboflavin-deficient rat (13) and the utilization of food (14), this is the most active of the analogs of riboflavin.

Since these four synthetic flavins are the only analogs of riboflavin possessing significant biological activity and since phosphorylation is the first step in the conversion of these flavins into their coenzyme forms, it was of interest to study their substrate specificity for comparative purposes and for clarification of mechanisms of action.

Materials and methods. The source of the flavokinase was the livers of female rats of the Wistar strain[4] weighing about 220 g. The livers of three such animals were taken sequentially, and after each was converted to a 20% homogenate in 0.05 *M* potassium phosphate buffer, they were pooled. The enzyme preparation used was one obtained by a procedure described in the literature (15), except for the following differences. The precipitate produced by 40% ammonium sulfate concentration was discarded. The fraction obtained between 40 and 55% saturation was the most active preparation, exceeding in specific activity by a factor of 2.2, the material obtained between 55 and 75% saturation. The precipitate obtained between 40 and 55% saturation was dissolved in 0.02 *M* glycine (pH 6.9) and dialyzed as described. This enzyme preparation may be frozen to preserve it for later use; however, it lost approximately 25% of its activity after it had been frozen for 8 days (−25°).

The protein content of the flavokinase

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[4] CFN rats; Carworth, Inc., New City, New York. Animals are now available from: Carworth Division, Charles River Breeding Laboratories, Wilmington, Mass. 01887.

solution was determined by the method of Lowry *et al.* (16), and the flavokinase was used as described for the phosphorylation of riboflavin. The FMN and analog-FMN derivatives were measured by the use of the differential extraction method of Burch *et al.* (17), as modified (18). The flavin phosphorylation was expressed as nanomoles of flavin phosphorylated in 60 min per milligram of enzyme protein.

Results. The rate of phosphorylation of riboflavin and the analogs of riboflavin are shown in Table I. The data need no restating nor elaboration. The figures in parenthesis following the nanomoles of flavin phosphorylated per hour per milligram of protein express the rate of phosphorylation in the percentage of the rate for riboflavin.

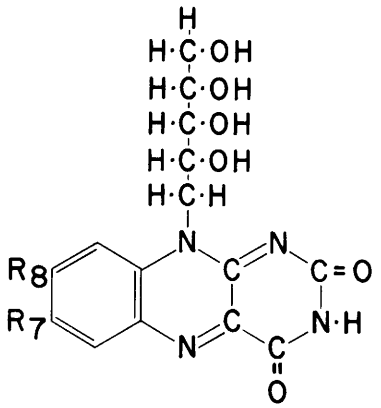

FIG. 1. Basic flavin structure.

No.	R_7	R_8	Trivial name
I	CH_3–	CH_3–	Riboflavin
II	C_2H_5–	CH_3–	7-Ethyl-8-methyl-flavin
III	CH_3–	C_2H_5–	7-Methyl-8-ethyl-flavin
IV	C_2H_5–	C_2H_5–	7,8-Diethyl-flavin
V	Cl–	CH_3–	7-Chloro-8-methyl-flavin

Discussion. There is no apparent positive correlation between the biological activity of the flavin analogs studied and their utilization by purified rat liver flavokinase. Both the 7-ethyl-8-methyl-flavin and 7-methyl-8-ethyl-flavin are potent vitamin-like replacements for riboflavin in the rat; however, the former is phosphorylated essentially as rapidly as riboflavin (88%) while the latter is phosphorylated very slowly (8%). It is of value to note that the flavokinase activity toward the synthetic substrates is in all likelihood not a determining factor in the biological action of these analogs, so long as the enzyme does present the animal with some amount of the coenzyme. This implies that the rate of coenzyme synthesis is not limiting for the animal and that the *in vivo* coenzyme levels are not heavily dependent on the rate at which the first flavin transformation takes place.

Phosphorylated 7,8-diethyl-flavin has been observed in animal tissue before (10), and it is phosphorylated very well (69%) by flavokinase. This is particularly interesting because it argues against the presence of the ethyl group in the eight-position as the responsible factor in the low rate of phosphorylation of 7-methyl-8-ethyl-flavin. The competitive inhibitory properties of 7,8-diethyl-flavin are probably due to the competition taking place at the coenzyme level; this would help to account for this analog's potency as an antagonist.

As stated above, 7-chloro-8-methyl-flavin is active as a vitamin-like substance and as

TABLE I. FLAVIN PHOSPHORYLATION BY PURIFIED RAT HEPATIC FLAVOKINASE.[a]

Flavin substrate	Number of assays	Flavin phosphorylated	
		Nanomoles[b]	Percentage[c]
I Riboflavin	8	26 ± 1	100 ± 3
II 7-Ethyl-8-methyl-flavin	6	23 ± 2	88 ± 7
III 7-Methyl-8-ethyl-flavin	5	2 ± 0[d]	8 ± 1
IV 7,8-Diethyl-flavin	6	18 ± 1[d]	69 ± 6
V 7-Chloro-8-methyl-flavin	8	27 ± 1	104 ± 5

[a] Four different enzyme preparations using three or four pooled livers for each preparation. Some preparations were used for two different assays.

[b] Nanomoles of flavin phosphorylated per 60 min per milligram of protein ± SE.

[c] The rate of riboflavin phosphorylation is expressed as 100% ± SE.

[d] For the following values of P (Student's t test), the symbol O! means P is 0.0001 or less. I vs II, III, IV, V = 0.21, O!, O!, 0.49; II vs III, IV, V = O!, 0.05, 0.10; III vs IV, V = O!, O!; IV vs V = O!, respectively. III and IV are each statistically different from all others.

an inhibitor of riboflavin in the rat. It is phosphorylated as rapidly (104%) as riboflavin. However, we have not found the coenzyme in the tissues of rats receiving this analog; we have found only traces of the free analog in such preparations. Clearly, the presence of the 7-chloro group does not influence the phosphorylation of this analog. It is interesting in this connection that 7,8-dichloroflavin is phosphorylated (15) as rapidly *in vitro* as riboflavin is, but it is devoid of biological activity in the rat and *L. casei* (19).

While it seems reasonable to expect that a flavin must be phosphorylated if it is to show activity as a vitamin-like substance or a potent inhibitor, the fact that a flavin can be phosphorylated by flavokinase in an *in vitro* system, gives us no information concerning whether it will or will not be active in the intact animal or the form of its activity.

Summary. Several analogs of riboflavin selected because of their exceptional biological activity in the rat have been studied as substrates for rat hepatic flavokinase. All of the analogs were phosphorylated, but the rate of phosphorylation showed no correlation with type of activity or potency for these activities. Phosphorylation of a flavin analog may be a prerequisite for significant biological activity but phosphorylation of a flavin does not insure that the substance will be biologically active in the whole animal.

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