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Induction of Hepatic and Intestinal Flavokinase After Oral Administration of Riboflavin to Riboflavin-Deficient Rats[1] (40248)

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Flavokinase (ATP: riboflavin-5′-phosphotransferase, EC 2.7.1.26) catalyzes the conversion of riboflavin to the coenzyme riboflavin-5′-phosphate (FMN). FMN formation is required for activation of the FMN-dependent flavoproteins, as an intermediate step in the production of flavin-adenine dinucleotide (FAD), the major coenzymic form of riboflavin, and possibly for trapping intestinally absorbed riboflavin in an ionic form (1). Flavokinase is present in most mammalian tissues, the largest amount being in liver (2).

Rats fed a riboflavin-deficient diet for 3 months have at most 50% of the flavokinase activity of animals fed a normal level of this vitamin (3). Since flavokinase is crucial for the utilization of riboflavin, the present study was undertaken to determine if a single *per os* administration of riboflavin would alter the hepatic and intestinal levels of this enzyme for the deficient rat.

Materials and methods. Male Sprague-Dawley rats were maintained in wire-bottom cages and were fed a commercially prepared (Teklad Test Diets) riboflavin-deficient diet for 50 days after weaning. Each rat then received *per os* 50 μg (about twice the daily requirement for the rat (4)) of riboflavin (Aldrich) in 1 ml of water. One animal was killed by decapitation at each designated time interval after administration, except at early times (0 and 2 hr) when two animals were used. The livers were removed, blotted, weighed, and chilled on ice while the whole small intestines were excised and gently washed with a 0.9% saline solution at 37° to remove the contents. Each organ was minced in a fourfold volume of cold 0.02 *M* potassium phosphate buffer, pH 7.0, (intestines were estimated to weigh 5 g) and homogenized by six strokes in a hand-held Ten-Broeck apparatus (Arthur Thomas Co.). The homogenates were centrifuged for 20 min at 30,000*g* and the supernatants filtered through glass wool. Liver supernatants were further centrifuged for 1 hr at 100,000*g*.

Flavokinase activities were determined by a modification of the method of McCormick (5). Each assay contained 0.1 m*M* [2-^{14}C]riboflavin (Amersham/Searle, 31 mCi/mmol diluted to 11.1 μCi/mmol with unlabeled riboflavin), 1 m*M* ATP (Sigma), 1 m*M* $ZnCl_2$, 75 m*M* potassium phosphate buffer, pH 8.0, and from 25 to 100 μg protein for a total volume of 0.2 ml. After 0, 15, 30, and 60 min of incubation with shaking at 37°, 5 μl aliquots were applied to Whatman No. 1 chromatographic paper, dried to terminate the reaction, and approximately 5 μg unlabeled FMN was applied to each spot. The chromatograms were developed with descending *n*-butanol–acetic acid-water (2:1:1), air-dried, and the FMN-containing regions detected under uv light. These were counted in 2 ml of scintillation fluid (PCS, Amersham/Searle, diluted 2:1 with xylene). Initial velocities were used to calculate flavokinase activities. One enzyme unit produces one nmol of FMN per hr under these assay conditions. With longer incubation times or at higher enzyme concentrations, the apparent production of FMN decreased considerably due to product hydrolysis by phosphatases (2). Negligible FAD was formed under these conditions.

The amount of phosphatase contamination was determined by conducting 2 ml assays with FMN instead of riboflavin and omitting ATP. After one and 2 hr of incubation, one

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ml was shell frozen and lyopholized, the residue redissolved in 0.1 ml of water, and 25 μl of solution applied to Whatman 3MM paper. After development and location of the spots as described above, the riboflavin was extracted from the paper with 3.0 ml of 0.02 *M* potassium phosphate buffer (pH 7.0). The extracts were filtered and the riboflavin contents determined by comparing the fluorescence (λ_{ex} 270 mm, λ_{em} 520 mm) with that for known amounts of riboflavin treated similarly.

Protein was determined by the biuret method (6) with bovine serum albumin as the standard.

All operations were performed in dim light to minimize photodegradation of the flavins. Tissue extracts were kept on ice and assayed immediately after preparation.

Results. At the time of sacrifice, the riboflavin-deficient animals weighed 137 ± 6 (SEM) g, the liver weights were 6.4 ± 0.3 g, and the total protein content for each tissue preparation was 146 ± 4 mg for intestine and 476 ± 20 mg for liver. The animals fed standard chow weighed 250 ± 12 g, with liver weights of 13.3 ± 0.9 g, and liver and intestine protein contents of 803 ± 10 mg and 221 ± 50 mg, respectively.

The flavokinase activities of liver and intestine, expressed as units per mg protein, are given in Fig. 1. The specific activity for both organs increased rapidly after riboflavin administration, nearly doubling within 6–8 hr. This response is also shown in Fig. 2 where flavokinase is expressed as units per total organ and per 100 g body wt.

For comparison, flavokinase activities were determined for four animals of approximately the same age as those used for this study, but which were fed standard lab chow. Two of these animals were administered 50 μg of riboflavin 5 hr before sacrifice. Hepatic activities for these animals were 6.2 ± 0.2 units/mg protein, 4980 ± 310 units/liver, and 1990 ± 250 units/100 g body wt; intestinal activities were 13.4 units/mg protein, 2960 ± 250 units/intestine, and 1180 ± 100 units/100 g body wt. No significant difference in activity was found between the normal animals administered riboflavin and those without.

The amount of FMN phosphatase activity associated with the liver preparations from riboflavin-deficient rats with or without riboflavin administration averaged 0.75 ± 0.08 mmol FMN hydrolyzed/hr/mg protein, compared to 0.65 ± 0.05 for normal animals. Intestinal activity was less consistent from preparation to preparation, averaging 2.2 ± 0.4 mmol FMN hydrolyzed/hr/mg, compared to 1.7 ± 0.1 for normal animals. No correlation was found between the observed flavokinase activity and the levels of phosphatase, which would be expected if the phosphatase were interferring with the flavokinase assay, as has been observed (2) with the stan-

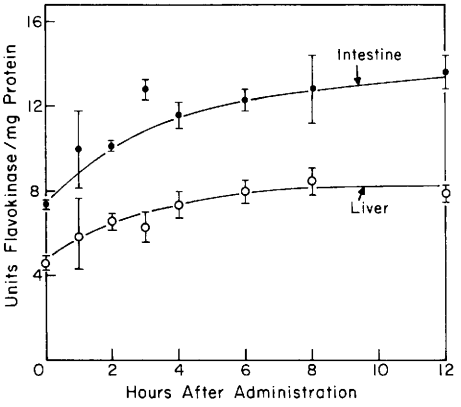

FIG. 1. Hepatic (○) and intestinal (●) flavokinase activities after *per os* administration of riboflavin to the riboflavin-deficient rat. Each value represents the mean ± SE of the mean for triplicate determinations. Activities at 6 and 8 hr were significantly different ($P < 0.005$) from those at 0 time as determined by the students' *t* test.

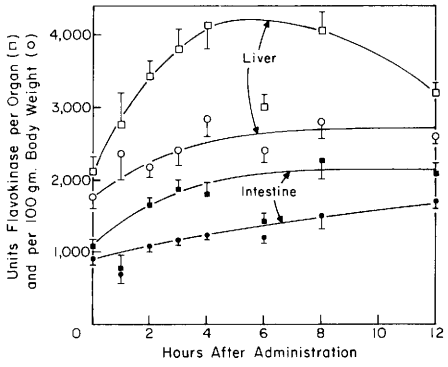

FIG. 2. Total flavokinase activities of liver (○) and intestine (●) and activities per 100 g body weight (□, ■) after administration of riboflavin to the riboflavin-deficient rat. Each value represents the mean + or − SE of the mean for triplicate determinations. Activities at 6 and 8 hr were significantly different ($P < 0.005$) from those at 0 time as determined by the students' *t* test.

dard flavokinase assay.

Discussion. The levels of flavokinase found in the present work are greater than those observed previously (2, 7), especially for the intestine. This is partially due to removal of a large portion of the phosphatase activity by centrifugation. Use of [^{14}C]-labeled riboflavin enables assay of smaller samples and, furthermore, increases the reliability of the assay by enabling the detection of small amounts of FMN.

The intestinal level of flavokinase should be relatively high if phosphorylation is necessary for trapping riboflavin. For the rat, riboflavin is absorbed by passive diffusion (1, 8) and appears in the intestine first in the nonphosphorylated form, subsequently as FMN, then as FAD (9, 10). Only very low concentrations of the vitamin are accumulated against a concentration gradient (11). Additionally, the intestinal flavokinase is at least partially responsible for forming FMN that appears in plasma (1).

Rats maintained on a riboflavin-deficient diet for 50 days have hepatic flavokinase levels 25% lower than control animals when units/mg protein are compared, but only 11% lower levels when activity is expressed as units/100 g body wt. Intestinal levels are much lower for the deficient rat, both in units/mg protein (45%) and units/100 g body wt (25%).

The flavokinase activities of liver and intestine increase twofold after *per os* administration of riboflavin to the deficient rat, reaching levels slightly higher than those for the normal animal. This increase probably promotes more efficient utilization of the vitamin. The nearly parallel increases in activity for both organs may be due to simple substrate-induced synthesis of new enzyme, stabilization or activation of enzyme already present (12), or may indicate hormonal involvement (3).

Summary. Use of [^{14}C]riboflavin to assay flavokinase of rat liver and intestine yields apparent activities which are higher than those previously reported. Rats fed a riboflavin-deficient diet for 50 days after weaning have lower intestinal and hepatic flavokinase activities than animals fed a complete diet. Within 6–8 hr after *per os* administration of 50 μg of riboflavin to the deficient rat, the flavokinase levels of both organs have nearly doubled. Hence, the riboflavin-deficient rat rapidly recovers the capacity to phosphorylate riboflavin after the vitamin is reintroduced into the diet.

1. Jusko, W. J., and Levy, G., *in* "Riboflavin" (R. S. Rivlin, ed.) p. 99. Plenum Press, New York (1975).
2. McCormick, D. B., Proc. Soc. Exp. Biol. Med. **107**, 784 (1961).
3. Rivlin, R. S., Menendez, C., and Langdon, R. G., Endocrinology **83**, 461 (1963).
4. McCormick, D. B., Nutr. Rev. **30**, 75 (1972).
5. McCormick, D. B., J. Biol. Chem. **237**, 959 (1962).
6. Gornall, A. G., Bardawill, C. J., and David, M. M., J. Biol. Chem. **177**, 751 (1949).
7. Hasegawa, E., Ando, O., and Nose, Y., J. Vitaminol. **14**, 303 (1968).
8. Christensen, S., Acta Pharmacol. Toxicol. **32**, Supplement II (1973).
9. Yagi, K., J. Biochem. **41**, 757 (1954).
10. Chen, C., and Yamaguchi, K., J. Vitaminol. **6**, 247 (1960).
11. Meinen, M., Aeppli, R., and Rehner, G., Nutr. Metabol. **21** (Suppl. 1), 264 (1977).
12. Rivlin, R. S., and Langdon, R. G., *in* "Advances in Enzyme Regulation" (G. Weber, ed.), Vol. 4, p. 45 (1966).

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