Regulation of riboflavin-metabolizing enzymes in riboflavin deficiency

STEVEN FASS AND RICHARD S. RIVLIN

Department of Medicine and Institute of Cancer Research, Francis Delafield Hospital,
College of Physicians and Surgeons of Columbia University, New York City 10032

Fass, Steven, and Richard S. Rivlin. Regulation of riboflavin-metabolizing enzymes in riboflavin deficiency. Am. J. Physiol. 217(4): 988-991. 1969.—The effects of riboflavin deficiency in rats on the hepatic activities of flavokinase, FAD pyrophosphorylase, and FMN phosphatase, three enzymes involved in riboflavin metabolism, have been measured and compared to the hepatic concentrations of FAD, FMN, riboflavin, and protein. Of the enzymes involved in FMN and FAD biosynthesis, the activity of the first, flavokinase, is depressed in deficient animals to 60% of normal, whereas the activity of the second, FAD pyrophosphorylase, is elevated to 150% of normal. The FMN-degrading enzyme, FMN phosphatase, is unaffected by riboflavin deficiency. Refeeding of riboflavin to deficient animals restores the activities of both flavokinase and FAD pyrophosphorylase to normal. The data demonstrate that riboflavin deficiency has selective effects upon the activities of liver enzymes involved in riboflavin metabolism. It is suggested that these effects may constitute a possible adaptive mechanism for conserving FAD in riboflavin deficiency.

RIBOFLAVIN DEFICIENCY characteristically results in diminished hepatic concentrations of flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD), the two coenzymes derived from riboflavin. Hepatic activities of enzymes with FMN or FAD as a cofactor, such as xanthine oxidase and d-amino-acid oxidase, are also depressed in riboflavin deficiency (3, 16).

The present study was undertaken to determine whether, in riboflavin-deficient rats, the diminished levels of FMN and FAD may be related to, or mediated by, changes in the activities of enzymes involved in their biosynthesis. Measurements were made in normal and in riboflavin-deficient animals of the activities of three enzymes involved in riboflavin metabolism: 1) hepatic flavokinase which catalyzes the initial conversion of riboflavin to FMN, 2) FAD pyrophosphorylase which converts FMN to FAD, and 3) FMN phosphatase which degrades FMN to riboflavin. The activities of these enzymes were then compared to the hepatic concentrations of riboflavin, FMN, and FAD.

MATERIALS AND METHODS

Animals. Male Wistar rats were used in all experiments. One month after birth, male littermates were equally divided into two groups. One group was fed Purina chow ad libitum. This diet, according to the manufacturer's specifications, contains approximately 8.3 mg riboflavin per kilogram of diet, or 16 times the minimum daily requirements for the vitamin. The second group of animals, individually housed in wire-bottom cages, was fed a riboflavin-deficient diet (Nutritional Biochemicals Corp., riboflavin-deficient test diet). This diet utilized casein hydrolysate as the source of protein.

Animals were sacrificed at intervals after being placed on riboflavin-deficient diets. After approximately 30 days of deficiency, animals showed evidence of severe riboflavin lack, including weight loss, and ocular and dermatological changes. The mortality rate was very high in animals deficient for more than 30–60 days, and only a few animals survived 150 days. In the riboflavin-deficient animals, as a whole, at the time of sacrifice, body weights averaged 69 g compared to 290 g in the age-matched controls. Riboflavin deficiency was also associated with an increase in liver weight relative to body size. Liver-to-body weight ratios of 4.9% were observed in deficient rats, compared to 3.6% in control animals.

In subsequent experiments, deficient animals were repleted with riboflavin. This was accomplished by placing the animals, for 4–6 days ad libitum, on a diet specially prepared by Nutritional Biochemicals Corp., which was identical in composition to the riboflavin-deficient diet except for the addition of 22 mg riboflavin per kilogram of diet. These animals also received daily intraperitoneal injections of 0.4 mg riboflavin in isotonic saline. Controls for these experiments were animals deficient for the same period of time and receiving injections of isotonic saline. During the period of realimentation, a rapid gain in weight occurred, and signs of riboflavin deficiency were observed to decrease.

All animals were sacrificed by decapitation followed by exsanguination. Livers were immediately removed, washed, and placed on ice. A single specimen of liver was frequently employed for the assay of several enzymes and coenzymes. All assays were performed on fresh liver, with the exception of several determinations of flavokinase activity which were performed on samples stored for 1–2 months in liquid nitrogen. Storage under these conditions did not decrease flavokinase activity.

Procedures. Hepatic flavokinase activity was assayed as previously described (11, 19). Formation of FMN was...
measured by the differential extraction method of Burch et al. (2), as modified by Kearney and Englard (9). Flavokinase activity was expressed as millimicromoles FMN formed per milligram liver protein per hour.

The assay of hepatic FAD pyrophosphorylase activity was according to DeLuca and Kaplan (5). Aliquots of the supernatant solutions were analyzed for FAD employing \( \alpha \)-amino-acid apoproteinase, (6), as previously described in detail (18). Enzyme activity was expressed as millimicromoles FAD formed per milligram liver protein per hour. Standard curves with known amounts of FAD were obtained with each day's experiment.

The activity of hepatic FMN phosphatase was determined by the method of McCormick and Russell (13). Flavin mononucleotide hydrolysis during the reaction was measured by the same differential extraction method used in the assay of flavokinase (11). Phosphatase activity was expressed as millimicromoles FMN hydrolyzed per milligram liver protein per 30 min.

Protein concentration in liver was measured in duplicate employing the biuret reagent (7).

In the same specimen of liver FMN, FAD, and riboflavin were simultaneously assayed by the method of Burch (1). Data were obtained as micrograms per gram of liver wet weight and have been expressed in Fig. 3 in terms of the percentages of the concentration recorded in normal animals.

RESULTS

The results of assay of hepatic flavokinase, FAD pyrophosphorylase, and FMN phosphatase in rats which were riboflavin-deficient for various periods of time are given in Fig. 1. For purposes of comparison, all activities have been expressed on the same scale, namely as the percentage of activity recorded in normal animals. It is apparent that riboflavin deficiency exerts selective effects upon the three enzymes. As reported previously from this laboratory (19), the activity of hepatic flavokinase is depressed in riboflavin deficiency. The present study demonstrates that reduced activity occurs after animals have fed for 30 days on a deficient diet, and that a modest further reduction occurs in animals which are deficient for longer periods of time.

The activity of FAD pyrophosphorylase exhibits a striking contrast. Activity is significantly elevated above normal values at all the time periods studied, as shown in Fig. 1. It appears that FMN phosphatase is the only enzyme entirely unaffected by riboflavin deficiency. Even in animals which had been deprived of riboflavin for more than 140 days, no alteration in enzyme activity occurs.

In order to determine whether the changes observed in deficient rats in the activities of flavokinase and of FAD pyrophosphorylase are irreversible, subsequent experiments were conducted in which deficient animals received oral and parenteral riboflavin, as described above, daily for several days. Results of these experiments are shown in Fig. 2. Riboflavin refeeding is completely successful in elevating the diminished activity of flavokinase to normal, and in decreasing the elevated activity of FAD pyrophosphorylase to normal. With respect to both enzymes, values obtained after administration of riboflavin to deficient rats do not differ from values obtained in control animals without treatment.

The observed changes in enzyme activities can be compared with the tissue levels of the flavin coenzymes obtained...
in this group of animals. As shown in Fig. 3, no consistent change in hepatic protein concentration is demonstrable as the duration of riboflavin deficiency increases. The hepatic levels of FAD, FMN, and riboflavin are all substantially decreased in deficient animals. After 20 days of deficiency, levels of FAD and FMN are reduced to approximately one-half of normal levels; with a longer duration of deficiency, greater reductions in both FMN and FAD levels occurred. It is important to note that the tissue levels of FMN are consistently reduced more than are the levels of FAD. This relationship has been observed previously (16), with even greater reductions in FMN levels after nearly 50 days of deficiency. This finding is compatible with the suggestion that there is an apparent lower limit in the hepatic flavin levels compatible with life (16).

The reduction in the hepatic concentrations of riboflavin in deficient animals is relatively greater than that either of FMN or FAD. Hepatic levels of riboflavin observed after 2 and 3 months of deficiency do not differ from one another.

**DISCUSSION**

The present study demonstrates that the activities of enzymes involved in the metabolism of riboflavin are themselves modified by riboflavin deficiency. The effects of riboflavin deficiency upon the three enzymes studied are selective, depressing the activity of one enzyme, elevating another, and failing to affect the third.

The finding that hepatic flavokinase activity is depressed in deficient animals is compatible with our previous finding that this enzyme in vitro is unstable in the absence of riboflavin (17). When liver extracts are incubated at 37 C in buffer, a rapid loss of enzyme activity occurs. After 30 min to 1 hr of this relatively bland procedure, less than one-fifth of the original activity is preserved. When the substrate of the enzyme, riboflavin, is added in vitro, a dramatic preservation of flavokinase activity occurs: after 1 hr of incubation, 70% of enzyme activity is retained. The other substrate, ATP, also preserves enzyme activity against inactivation in this system. Whether the protective effects of riboflavin and of ATP are entirely those of substrate stabilization is unknown, since certain compounds which are not substrates, such as ADP, AMP, and GTP, also exhibit some degree of effectiveness (2). Regardless of the mechanism of stabilization of flavokinase by riboflavin, however, it is apparent that both in vivo and in vitro flavokinase activity is diminished in the absence of riboflavin.

One factor contributing to the decrease in flavokinase activity in vivo, therefore, may be due to a direct lack of riboflavin available for stabilization.

The mechanisms accounting for the elevation of FAD pyrophosphorylase activity cannot be described at the present time. McCormick (12) has observed that this enzyme is inhibited to some extent by riboflavin and its analogues. It is of interest, therefore, that the elevated activity in deficient rats can be decreased completely to normal by administration of riboflavin. It is not known whether stress or adrenal cortical hormones increase the activity of this enzyme.

The fact that the enzyme activities observed are not all reduced below normal provides further evidence that riboflavin deficiency does not necessarily produce a generalized depression of liver functions. Although in riboflavin-deficient rats there are reductions in the activities of a number of the flavoprotein enzymes, notably glycolic acid oxidase, TPN cytochrome C reductase, xanthine oxidase, D- and L-amino-acid oxidases (16, 19), and of enzymes involved in folic acid metabolism (15), other enzymes are not necessarily similarly affected. The hepatic activities of several transaminases are elevated in deficient rats (4, 14). Recent findings further indicate that lactic dehydrogenase is unaffected by riboflavin deficiency, and that the activity of glutamic dehydrogenase is elevated threefold in deficient animals (8).

It is important to note further that the changes which occur in deficient rats in the activities of the two FMN and FAD biosynthetic enzymes are actually in opposite directions. The result is that the normal ratio of hepatic flavokinase to FAD pyrophosphorylase activity is reduced from approximately 3.5 to 1.6 (Fig. 2) in livers of deficient rats, decreased in riboflavin-deficient rats. Concentration of FMN in livers of deficient rats may be expected to decline as a result of diminished flavokinase activity combined with elevated activity of FAD pyrophosphorylase.
animals. These ratios are based upon the present methods of tissue preparation which differ for each enzyme. It has been suggested that the conversion of riboflavin to FMN catalyzed by flavokinase is the faster step, and the further conversion of FMN to FAD by pyrophosphorylase the slower step (21). The reduction in flavokinase activity by itself could contribute to the decreased tissue levels of FMN and FAD, imposing a further restriction upon the utilization of the already diminished supply of riboflavin. This would not, however, account for the finding that FMN levels are decreased to a greater extent than are FAD levels. Explanation for this phenomenon may well reside in the elevated activity of FAD pyrophosphorylase.

As shown in Fig. 4, diminished flavokinase activity combined with elevated activity of FAD pyrophosphorylase would be expected to deplete FMN levels particularly. The elevation in FAD pyrophosphorylase activity may represent an adaptive mechanism for conserving FAD at the expense of FMN in the deficient rat.

The finding that FMN phosphatase activity is unaltered even in extreme deficiency suggests that the degradation of FMN may be relatively independent of the riboflavin status of the animal. Since the degradation of FAD to FMN by nucleotide pyrophosphatase (10) was not measured in these experiments, one can only speculate on its role in total riboflavin economy in the deficient rat.

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REFERENCES