The Effect of Riboflavin Deficiency on Key Gluconeogenic Enzyme Activities in Rat Liver (37393)

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The sequel of riboflavin deficiency, as it is now known, may be initiated by a selective decrease in the activity of several flavoproteins involved in cellular oxidation (1–3). This in turn may affect protein turnover and lead to an increased pool of free amino acids (4) which alone, or in conjunction with altered endocrine activity (5), may stimulate the activity of several enzymes involved in amino acid metabolism (6–8). It has been hypothesized that the stimulation of alanine transaminase activity by this mechanism may be responsible for greater fasting liver glycogen levels (6) and *in vivo* ^{14}C-alanine incorporation into liver glycogen of riboflavin deficient rats than in pair-fed controls (9). However riboflavin deficiency is also associated with a large decrease in mitochondrial respiration and energy production (1). Since these factors would be expected to inhibit several key steps in the gluconeogenic pathway, it was of interest to study some of the key gluconeogenic enzyme activities which are known to be rate limiting under a variety of conditions. These enzymes activities are increased in many situations where gluconeogenesis is increased such as fasting, diabetes, high fat feeding, and glucocorticoid administration (10–15). Phosphoenolpyrurate carboxykinase (PEPck) activity is especially well correlated with gluconeogenesis (16). The regulation of these enzyme activities is a control system which appears to operate in intact liver under physiologic conditions (16). We sought to determine if the increased glyconeogenesis reported in riboflavin deficient rat liver was associated with an increased activity of one or more of these enzymes.

Materials. Animals and Diet. Male Sprague–Dawley weanling rats were used in these studies. Animals weighed 50–60 g at the start of the experiment and these were randomly divided into three initial groups: Group A was fed a commercial riboflavin-deficient diet (Nutritional Biochemicals Co., Cleveland, OH) *ad libitum*; Group B was pair-fed the same diet supplemented with 30 mg of riboflavin/kg of diet; Group D was fed the control diet *ad libitum*. The casein used in this diet preparation contained 0.5 μg of riboflavin/g of casein. Records of food consumption and weight gain were kept. Rats were kept on the diet for 6 wk, and housed individually in wire bottom cages. At this time, the riboflavin-deficient group showed marked signs of deficiency, *i.e.*, dermatitis, alopecia, weakness and failure of growth. After 4 wk, one-half of the original Group A was randomly selected as Group C, removed from the deficient diet and then fed the control diet, pair-fed with Group A for 2 wk. The other half of the original Group A was fed as before.

The windowless animal quarters, constant 73°F, were on a regulated light schedule (dark from 6 PM–6 AM; light from 6 AM–6 PM). Pair-fed controls were fed between 5–6 PM every day. All rats were fed from aluminum food cups with stainless steel tops (Wahman Manufacturing Co., Baltimore, MD). All animals had tap water *ad libitum*.

Methods. Rats were fasted overnight, killed by decapitation, and profuse bleeding was encouraged. The abdominal cavity was opened, the liver was removed, weighed and placed in ice-cold homogenization medium containing 0.25 *M* sucrose, 1 m*M* EDTA, 1 m*M* reduced glutathione (GSH), 20 m*M* Tris, pH 7.4. The preparation of homogenates

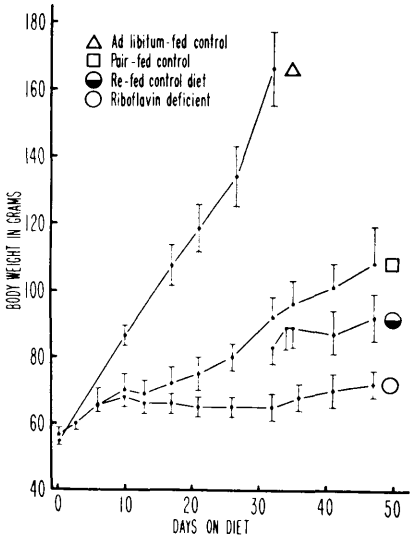

FIG. 1. The effect of the experimental diets on growth. The riboflavin-deficient group was allowed continuous access to a commercial riboflavin-deficient diet. The pair-fed group was fed daily the same diet, supplemented with riboflavin, in amounts equal to that eaten by their counterpart from the *ad libitum*-deficient group. The *ad libitum*-fed control group was allowed continuous access to the supplemented diet. The refed control diet group, which had been previously riboflavin deficient, was fed daily the supplemented diet in amounts equal to that eaten by their counterpart from the riboflavin-deficient group.

and enzyme assays were completed as previously cited (17).

Results and Discussion. Figure 1 shows the growth rates for each of the experimental groups. Weanling rats grew for 10 days after initiation of feeding the riboflavin-deficient diet (Group A). Thereafter, the deficient rats had no significant change in body weight. Although Group B received the same amount of food as Group A, they continued to grow from Day 17 to the end of the experiment. This effect, probably due to decreased food utilization by riboflavin deficient rats (18), has been previously reported. There was an increased body weight in the riboflavin-deficient rats refed control diet (Group C).

Table I shows the key gluconeogenic enzyme activities contained in liver homogenates of each experimental group. The activities of these enzymes have been expressed in three ways.

Expression of enzyme activities per milligram of protein allows us to determine if the enzyme in question is being synthesized at the same or different rate as liver proteins in general (19). Expression of enzyme activities per 100 g body weight best shows the total liver function based on the total metabolic requirements (19). Since the metabolic need for glucose is partly dependent on animal size, an increased activity per 100 g body weight is the only true indication that the increase was due to a metabolic adaptation. An increase in the activity per gram of liver or per milligram of protein may only reflect an increased body size.

When activities are expressed per g of liver, pyruvate carboxylase (EC 6.4.1.1), glucose-6-phosphatase (EC 3.1.3.9) and fructose-1,6-diphosphatase (EC 3.1.3.11) showed no significant differences among the 4 experimental groups. However, phosphoenolypyruvate carboxykinase (EC 4.1.1.32) was increased more than twofold by riboflavin deficiency. Subsequent feeding of the control diet returned the activity to control values (Table I). Pyruvate carboxylase (PC) is the only enzyme of the four enzymes measured that is primarily of mitochondrial origin.

Consistent with the observations of other workers (18), we have found a large increase in the mitochondrial protein content of riboflavin-deficient rat liver. Riboflavin-deficient rats had an average of 138% as much mitochondrial protein/g of wet tissue as did the controls. The mean, SD, and SEM for control was 32.48, 4.02, and 1.80 mg mitochondrial protein/g wet weight liver, respectively; for the deficient rats the values were 44.82, 5.64, and 2.52, respectively. Analysis of paired data gave a mean difference of 12.38 mg of protein/g wet weight with a t value greater than 15.8. Since the overall liver protein concentration remains unchanged in riboflavin deficiency (20, 21), this implies that the nonmitochondrial liver protein must decrease. It is possible that some of the increased mitochondrial protein might be due to *de novo* synthesis of enzyme proteins, either pyruvate carboxylase or other mitochondrial

TABLE I. Key Gluconeogenic Enzyme Activities in Rat Liver Homogenates.[a]

Enzyme	Activity expressed per	Group A Pair-fed deficient	Group B Pair-fed control	Group C Re-fed control diet	Group D *Ad libitum* control
Pyruvate carboxylase[b]	g liver	16.46	19.51	16.28	15.65
	mg protein × 10^2	7.49	8.24	7.23	7.27
	100 g body wt	71.52	57.29	54.71	49.45
Phosphoenol pyruvate carboxykinase[b]	g liver	3.52	1.60	1.35	1.35
	mg protein × 10^2	3.80	1.45	1.46	1.39
	100 g body wt	14.97	4.89	4.41	4.10
Fructose[c] diphosphatase	g liver	3.37	3.97	3.23	3.79
	mg protein × 10^2	1.67	1.81	1.49	1.80
	100 g body wt	16.12	12.02	11.16	12.21
Glucose-6[c] phosphatase	g liver	26.81	28.58	20.29	27.42
	mg protein × 10^2	12.90	12.40	9.30	13.00
	100 g body wt	137.89	74.74	70.16	88.40

[a] Statistical comparisons are found in Table II.

[b] Activity: μmoles CO_2 fixed/min; [c] μmoles P_i liberated/min under assay conditions.

enzyme systems. Whether or not the new enzymes are metabolically active, we would expect a change in their specific activities, unless the particular enzyme maintained a constant percentage of the total increased protein. The specific activity of PC is slightly decreased, indicating that there is a decrease in the percentage of liver protein with PC activity in riboflavin-deficient rat liver.

Fructose-1,6-diphosphatase (FDPtase) and glucose-6-phosphatase (G6Ptase) specific activities were unchanged, even though the total amount of nonmitochondrial protein was apparently decreased. This implies that the percentage of cytosolic protein with FDPtase and G6Ptase activity is increased in riboflavin-deficient rat liver. Phosphoenolpyruvate carboxykinase (PEPck) specific activity is consistent with the activity expressed per gram of liver, indicating that a significantly greater percentage of cytosolic protein has PEPck activity in riboflavin-deficient rat liver.

Riboflavin-deficient rats have a decreased body weight and an increased ratio of liver wt/body wt. Representative values are 5.3 × 10^{-2} for riboflavin deficient, 4.2 × 10^{-2} for pair-fed control, and 2.9 × 10^{-2} for *ad libitum* control. This implies that to maintain normal liver function, the riboflavin-deficient rat must have an increased metabolic capacity to overcome decreased coenzyme levels (1, 2, 22). It either takes more liver to serve the same metabolic function or the liver activity increases to compensate for an increased demand for some hepatic function. If, with a decreased body weight, there is a decreased metabolic need for glucose, expression of gluconeogenic enzyme activities per 100 g body weight will be consistent with activity per gram of liver. On this basis (activity per 100 g body weight), all four key enzymes are significantly increased in riboflavin-deficient rats, as compared to pair-fed control diet (Table I). The increase in activities of the gluconeogenic enzymes is consistent with an increased metabolic production of glucose, and with the hypothesis that this is the result of a metabolic adaptation induced by riboflavin deficiency. An increased production of glucose could occur in response to an increased metabolic need, or to a secondary nonspecific effect of increased amino acid pools (4) or to altered hormonal balance produced by the deficiency (4, 23, 24). We conclude that although increased activities do not prove that the increased liver glycogen reported was due to increased gluconeogenesis, it is an additional piece of evidence that favors that hypothesis.

TABLE II. Group Comparison and Level of Significance for Key Gluconeogenic Enzyme Activities in Rat Liver Homogenates.

Enzyme	Activity expressed per	p Values[a]; group comparisons[b]: A vs B	A vs C	A vs D	B vs C	B vs D	C vs D
PC	g liver	NS	NS	NS	<0.05	<0.05	NS
	mg protein	<0.001	NS	NS	<0.01	NS	NS
	100 g body wt	<0.01	<0.02	<0.01	NS	<0.05	NS
PEPck	g liver	<0.001	<0.001	<0.001	NS	NS	NS
	mg protein	<0.01	<0.01	<0.001	NS	NS	NS
	100 g body wt	<0.001	<0.001	<0.001	NS	NS	NS
G6Ptase	g liver	NS	NS	NS	<0.001	NS	<0.01
	mg protein	NS	<0.02	NS	<0.01	NS	<0.01
	100 g body wt	<0.02	<0.01	<0.02	NS	NS	<0.05
FDPtase	g liver	NS	NS	NS	NS	NS	NS
	mg protein	NS	<0.05	NS	<0.01	NS	NS
	100 g body wt	<0.01	<0.01	<0.05	NS	NS	NS

[a] A p value ≥ 0.05 is considered not significant (NS), as determined by Students' t test.
[b] A = riboflavin-deficient; B = pair-fed control; C = riboflavin-deficient re-fed control diet; D = *ad libitum* control.

Pair-feeding leads to restricted food intake in control rats because riboflavin-deficient rats may eat less than normal rats, at least in early stages (24). We found that the rats fed the deficient diet *ad libitum* ate only 5–6 g/day through 7 wk. This is considerably less than the amount eaten by rats fed a control diet (25). Since starvation is known to stimulate gluconeogenic enzyme activities, this food restriction caused by pair-feeding might stimulate these enzyme activities. Table II shows that only PC activity was increased by pair-feeding compared to *ad libitum* feeding. In all cases, refeeding the control diet for 2 wk restored the enzyme activities toward control values expressed per 100 g body weight. Table II gives a list of the significance of possible group comparisons obtained by the use of Students' t test.

The glucose metabolism is apparently dependent both on the degree of deficiency and the amount of stress to which the animal is submitted. The increased glycogen levels and possible increased gluconeogenic rate are seen in early or moderate deficiency. The final collapse, coma, and death sometimes observed in severely riboflavin-deficient dogs (26) swine (27), rats (26) and perhaps humans (28) has been associated with a failure of the glucose supply. Forker and Morgan (29) showed that glyconeogenesis in severely riboflavin-deficient rat liver was impaired when rats were simultaneously subjected to starvation and anoxic anoxia. There was also an apparent decrease in fasting liver glycogen levels of riboflavin-deficient rats at normal atmospheric pressure.

Summary. Key gluconeogenic enzyme activities were measured in liver homogenates from rats which were either riboflavin-deficient, pair-fed control, *ad libitum* control, or initially riboflavin-deficient and then re-fed the control diet. It is concluded that: (a) the method of expressing the results has implications in interpretation; (b) the increased activity per 100 g body weight of key gluconeogenic enzymes is consistent with the increased rate of liver glycogen synthesis previously reported in riboflavin deficiency; and (c) PEPck activity showed the largest increase irrespective of the method of expression. Although activities *in vivo* may be substantially different than optimal capacities measured *in vitro, in vitro* assays can provide some insight concerning the physiologic condition (30).

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