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Serum Triiodothyronine Concentrations in Riboflavin-Deficient, Diabetic and Normal Mice (40124)

A. S. REDDI

Department of Medicine, Diabetes and Metabolism Division, New York Medical College, 1249 Fifth Avenue, New York, N.Y. 10029

Certain investigations have demonstrated a direct relationship between thyroid hormone and riboflavin metabolism (1, 2). Hepatic concentrations of flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN) and free riboflavin are significantly decreased in hypothyroid and increased in hyperthyroid rats (1). Similarly, hepatic activity of flavokinase, which converts riboflavin to FMN, is decreased in hypothyroid animals (1). Administration of thyroid hormone produces nearly a twofold increase in enzyme activity of these animals (1). A similar hepatic profile as in hypothyroidism with regard to concentrations of flavin coenzymes and flavokinase activity was demonstrated in riboflavin-deficient rats (1). Consequently, a biochemical similarity between hypothyroidism and riboflavin deficiency was proposed (3).

An additional interrelationship between thyroid hormone and riboflavin metabolism was described by Galton and Ingbar (4). These authors reported a significantly decreased hepatic deiodination of thyroxine in riboflavin-deficient rats. The deiodination of thyroxine was not affected in the kidney, muscle or brain. In addition, these authors reported that the urinary excretion of ^{131}I and the serum concentration of protein-bound iodine were similar in normal and riboflavin deficient animals. By contrast, Nolte *et al.* (5) reported a significant decrease in serum immunoreactive thyroxine concentration in riboflavin deficient rats. Since spontaneous riboflavin deficiency was reported to occur in genetically diabetic KK mice (6), the present study was undertaken to investigate whether or not the serum triiodothyronine concentrations are altered in these mice.

Material and methods. The Swiss albino (SA) and KK (genetic diabetic) mice have been inbred in our laboratory for several generations. They were fed the Old Guilford mouse laboratory chow (The Emory Morse Co., Guilford, CN) *ad libitum*. Both groups of mice were maintained under similar laboratory conditions. The animals in this part of the study were males between 8 and 9 months of age, and were tested for riboflavin nutritional status.

Riboflavin deficiency was produced in 40-day-old male SA mice. They were purchased from Camm Research Institute, Wayne, NJ. The mice were divided into two groups. One group was placed on a riboflavin deficient diet (ICN Life Sciences Group, Cleveland, OH) for 50 days. The other group was fed the Old Guilford mouse laboratory chow for a similar period of time. Both groups of mice were fed the respective diets *ad libitum*. The mice on riboflavin deficient diet lost weight and showed signs of the deficiency.

All groups of mice were maintained in plastic cages with sanichips as bedding. Six KK mice were given 140 μg of riboflavin per mouse per day s.c. in 0.9% saline for 20 days and then tested for riboflavin nutritional status.

Prior to the study, the mice were fasted for 18 hr with free access to water. Blood was drawn from the orbital plexus. Serum was collected by centrifugation of blood at 10,000*g* for 10 min. All serum samples were stored at −25° until analyzed for T_3 concentrations.

The riboflavin nutritional status of each animal was assessed by determining the glutathione reductase activity in erythrocyte hemolysates (7). The hemolysates were prepared as follows: about 0.3–0.35 ml of whole blood was transferred into tubes containing 0.1 ml of ACD solution (7.3 g of anhydrous citric acid, 22.0 g of sodium citrate and 24.5 g of glucose made up to 1 liter with water) and centrifuged at 200*g* in a refrigerated centrifuge for 10 min. The plasma and buffy coats were removed with a Pasteur pipette, and the red cells were mixed with 1.0 ml of

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0.9% sodium chloride. The suspension was centrifuged at 200*g* for 10 min, and the supernatant solution was removed and discarded. The red cells were mixed again with saline and centrifuged as before. In this way, the red cells were washed 3 times. To prepare the hemolysate, 0.1 ml of erythrocyte suspension was diluted to 2.0 ml with cold distilled water and allowed to stand at 4° for 45 min. The suspension was centrifuged at 800*g* for 15 min in a refrigerated centrifuge and the supernatant solution was separated and stored at −25° until assayed.

Glutathione reductase activity in the hemolysate was determined by the method of Tillotson and Sauberlich (7). The assay medium contained 2.0 ml of 100 mM potassium–sodium phosphate buffer, pH 7.4, 0.1 ml of hemolysate, 0.1 ml of 7.4 m*M* oxidized glutathione (GSSG), 0.05 ml of 80 m*M* EDTA-Na_2, 0.1 ml of 1.9 m*M* NADPH, 0.1 ml of 0.27 m*M* FAD and distilled water to a final volume of 2.5 ml. Two tubes were used for each assay, one containing FAD and the other without FAD. Blanks for these tubes contained the respective reactants except hemolysates. All the reactants except NADPH were mixed thoroughly and incubated at 37° in a water bath for 8 min. Following the preincubation, the reaction was started by the addition of NADPH, and the initial absorbance was measured at 340 nm in a Beckman DB spectrophotometer. The blanks were used to zero the instrument. The reaction mixture was transferred into a tube and incubated again at 37° for 10 min. Following this incubation period, the reduction in the absorbance (oxidation) of NADPH was measured. The results were expressed as activity coefficients (AC), calculated by dividing the amount of reduction of absorbance of NADPH in the presence of FAD for 10 min by the amount of reduction of absorbance of NADPH without added FAD for 10 min. AC values from 0.9 to 1.3 were considered normal, and those greater than 1.3 were regarded as evidence of riboflavin deficiency (8). Based upon AC values, both SA and KK mice were classified as normal (non-deficient) or riboflavin-deficient.

Triiodothyronine (T_3) concentrations were determined in 0.1 ml of serum by a radioimmunoassay, with an RIA-Mat circulating ^{125}I–T_3 kit. The method is specific for measuring total circulating serum T_3 levels, because of the use of antibodies which react *in vitro* with T_3 but not with thyroxine or mono- and diiodotyrosines. Duplicate determinations were performed only in 13 samples, since sufficient amount of serum was not available in the remaining samples. The difference between the duplicate samples varied from 3 to 7.5%. In order to determine the reproducibility of the assay, the T_3 levels were determined in five samples 20 days after the original determination. The standards and the reagents were used from the same kit. Five to ten percent decrease in the T_3 levels was found. The method is, therefore, accurate and reproducible in our hands. The T_3 levels were expressed as ng per 100 ml of serum. Determinations of T_3 levels were done in a blind fashion.

Student's *t* test for unpaired samples was applied to calculate the significance of the difference between means of observations. *P* values greater than 0.05 were considered nonsignifcant.

Chemicals. GSSG, NADPH, FAD and riboflavin were obtained from Sigma Chemical Co., St. Louis, MO, and ^{125}I–T_3 kit including standards from Mallinckrodt-Nuclear, St. Louis, MO.

Results. Table I shows the mean AC values and serum T_3 levels in aged SA and KK mice. The AC values of riboflavin-deficient SA and KK mice did not differ significantly from each other. When six deficient KK mice were supplemented with riboflavin, their AC values returned to normal. There was no significant difference in T_3 concentrations between normal and riboflavin-deficient SA mice. Also no significant difference in the T_3 level was found between normal KK and deficient KK mice. Furthermore, administration of riboflavin to deficient KK mice did not alter the T_3 concentrations. No statistically significant differences in T_3 levels were found between normal SA and normal KK or between riboflavin-deficient SA and riboflavin-deficient KK mice.

Induced riboflavin deficiency in young SA mice also caused no difference in the serum T_3 concentration (Table II). Although the mean T_3 level was 30% higher in riboflavin-deficient mice, the increase was not statisti-

TABLE I.[a]

	SA		KK		Deficient KK + Riboflavin
	Normal	Deficient	Normal	Deficient	
AC	1.24 ± 0.02 (8)	1.60 ± 0.06 (5)	1.00 ± 0.02 (3)	2.01 ± 0.40 (5)	1.15 ± 0.03 (6)
T_3	49.00 ± 6.00 (8)	58.00 ± 5.00 (5)	77.00 ± 17.00 (3)	69.00 ± 13.00 (5)	49.00 ± 9.00 (6)

Significance (T_3)
Normal SA vs. Deficient SA : NS
Normal KK vs. Deficient KK : NS
Normal KK vs. Deficient KK + riboflavin : NS
Normal SA vs. Normal KK : NS

[a] Activity coefficients (AC) for glutathione reductase activity in erythrocyte hemolysates and serum triiodothyronine (T_3) concentrations (ng/100 ml) in Swiss albino (SA) and genetically diabetic (KK) mice aged 8–9 months. Values shown are means ± SE. Numbers in parentheses indicate number of animals.

TABLE II.[a]

Normal	Riboflavin-deficient
73.00 ± 15.00 (7)	95.00 ± 12.00 (6)[b]

[a] Serum triiodothyronine levels (ng/100 ml) in Swiss albino mice fed control and riboflavin deficient diets for 50 days. Each value is the mean ± SE. Numbers in parentheses represent number of animals.
[b] Normal vs riboflavin deficient : p = NS.

cally significant because of the variability of the data.

There was no difference in the fasting blood sugar levels between normal and spontaneously developed riboflavin-deficient SA or KK mice. However, in mice with induced riboflavin deficiency a significant decrease ($P < 0.001$) in the fasting blood sugars was found when compared to mice fed a normal diet *ad libitum* or pair-fed to the riboflavin-deficient group.

Discussion. It has been shown that determination of the erythrocyte glutathione reductase, an FAD-containing enzyme, is an accurate procedure for assessing the riboflavin nutritional status in rats (7) and humans (9–11). In the deficient state, enzyme activity is stimulated *in vitro* by FAD (12, 7) and *in vivo* by riboflavin intake (13). The extent of stimulation of the erythrocyte glutathione reductase *in vitro* is inversely related to the FAD saturation of the enzyme protein, which in turn is dependent on the availability of riboflavin. Glatzle *et al.* (10) have reported the percentage of stimulation of the NADPH-dependent erythrocyte glutathione reductase in the presence of added FAD as a measurement of the riboflavin status in man. They referred to this stimulatory effect as activity coefficient (AC). We employed the erythrocyte glutathione reductase assay system to assess the riboflavin nutritional status of aged normal control SA and genetically diabetic KK mice.

The KK mice are an inbred strain of animals with spontaneous hereditary diabetes. They develop impaired tolerance to oral glucose (after an 8 hr fast) between 2 and 3 mon of age. This impairment becomes more pronounced at 9 mon of age. Since these mice demonstrate mainly impaired tolerance to glucose with normal fasting blood sugars, they are classified as chemical diabetics (14).

The present report demonstrates that spontaneous riboflavin deficiency occurs in aged SA and KK mice on a chow diet. This deficiency was not due to a low caloric or riboflavin intake, because these mice consumed a regular quantity of food (the intake of food was measured) which had the required amount of riboflavin (3 mg/lb). This deficiency has been found to be more common among KK than among SA mice (6). Administration of riboflavin to deficient KK mice returned the AC values to normal. A biochemical similarity between hypothyroidism and riboflavin deficiency has been demonstrated in that flavin coenzymes are decreased in the liver in both conditions (3). In view of this similarity, it is of interest that serum T_3 levels remain normal in mice with spontaneous or induced riboflavin deficiency.

Galton and Ingbar (4) found no difference in serum protein-bound iodine levels between normal and riboflavin-deficient rats. On the other hand, Nolte *et al.* (5) reported significantly decreased levels of serum thyroxine in riboflavin-deficient rats. Nevertheless, and in spite of the previous observation that deiodination is impaired in riboflavin deficiency (4), the serum T_3 levels were unaltered in

riboflavin-deficient mice. Furthermore, supplementation of riboflavin to deficient KK mice did not alter the T_3 levels. Additional studies including measurements of thyroxine levels are indicated. The failure to observe changes in circulating T_3 levels in riboflavin deficiency, associated either with genetic diabetes or dietary restriction, suggests that thyroid function remains normal in this condition. This is in spite of the fact that hypothyroidism and riboflavin deficiency have similar effects on hepatic flavin coenzyme levels (1).

Summary. The riboflavin nutritional status was assessed by activity coefficient (AC) of glutathione reductase in erythrocyte hemolysates of normal Swiss albino (SA) and genetically diabetic (KK) mice aged 8–9 months. AC values greater than 1.3 were considered as evidence of riboflavin deficiency. Based upon AC values, both SA and KK mice were divided into normal and riboflavin-deficient groups. In some young SA mice, riboflavin deficiency was produced by feeding of a riboflavin-deficient diet. In both normal and riboflavin-deficient mice, the serum L-triiodothyronine (T_3) concentrations were determined by radioimmunoassay. The results indicate that the T_3 concentration is not affected either in riboflavin deficiency or in genetic diabetes.

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