Riboflavin and riboflavin-derived cofactors in adolescent girls with anorexia nervosa[1,2]

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ABSTRACT

Background: Thyroid hormones, riboflavin, riboflavin cofactors, and organic acids were assessed in girls with anorexia nervosa.

Objective: The objective was to examine the effect of malnutrition and low thyroid hormone concentrations on erythrocyte and plasma riboflavin metabolism and their relation with urinary organic acid excretion.

Design: Seventeen adolescent girls with anorexia nervosa [body mass index (BMI; in kg/m^2): 14.8 ± 2.2] and 17 age-matched, healthy girls (control subjects; BMI: 20.5 ± 2.2) took part in the feeding study. Erythrocyte and plasma riboflavin as well as riboflavin cofactors (flavin mononucleotide and flavin adenine dinucleotide) were assessed by HPLC, whereas urinary organic acids were assessed by gas chromatography–mass spectrometry.

Results: Anorectic patients who began a feeding program had higher erythrocyte riboflavin (3.5 ± 2.2 compared with <0.1 nmol/mol hemoglobin; $P < 0.001$), lower plasma flavin adenine dinucleotide (57.8 ± 18.5 compared with 78.5 ± 54.3 nmol/L; $P < 0.05$), and higher urinary ethymalonic acid (7.12 ± 4.39 compared with 1.3 ± 2.8 μmol/mmol creatinine; $P < 0.001$) and isovalerylglycine (7.65 ± 4.78 compared with 3.8 ± 0.9 μmol/mmol creatinine; $P < 0.05$) concentrations than did control subjects. Triiodothyronine concentrations were low and negatively correlated with plasma riboflavin concentrations ($r = -0.69$, $P < 0.01$). Not all patients showed improvements in these biochemical indexes after 30 d of refeeding.

Conclusions: The low triiodothyronine concentrations observed in anorexia nervosa could alter the extent of riboflavin conversion into cofactors, thus leading to high erythrocyte riboflavin concentrations, low plasma flavin adenine dinucleotide concentrations, and high rates of ethylmalonic acid and isovalerylglycine excretion. Am J Clin Nutr 1999;69:672–8.

KEY WORDS Anorexia nervosa, riboflavin, ethylmalonic acid, flavin mononucleotide, isovalerylglycine, triiodothyronine, adolescent girls, vitamin deficiency, flavin adenine dinucleotide

INTRODUCTION

Anorexia nervosa is a psychiatric disorder manifested mainly in adolescent girls and that may lead to micronutrient deficiencies. Deficiencies in vitamins A, E, and D and riboflavin have been reported (1–6) along with evidence of hypothalamic and thyroid dysfunction (7–12). Triiodothyronine (T_3) is implicated in the conversion of riboflavin into flavin mononucleotide (FMN) by enhancing the expression of riboflavin kinase (13, 14), whereas the flavocoenzymes flavin adenine dinucleotide (FAD) and FMN are involved in mitochondrial energy metabolism and metabolic pathways involving the monoamine neurotransmitter. In this latter pathway, amine oxidase (flavin containing) was shown to contain FAD in an 8 α-thioether linkage (15, 16), which was ultimately synthesized for proof of structure and delineation of properties (17, 18). Until now, however, no study has evaluated the status of riboflavin and riboflavin cofactors (FAD and FMN) in anorexia nervosa. The aim of the present study, therefore, was to examine riboflavin and riboflavin cofactor concentrations in erythrocytes and in plasma as well as related organic acid concentrations in urine, and to compare our findings with the simultaneously assessed thyroid hormone profile.

SUBJECTS AND METHODS

Subjects

Plasma thyroid hormones; erythrocyte and plasma FAD, FMN, and riboflavin concentrations; as well as urinary organic acids were assessed in 17 adolescent girls ranging in age from 11 to 23 y (median: 16 y) who had anorexia nervosa and severe malnutrition. Fifteen of the subjects had a body mass index (BMI; in kg/m^2) below the 3rd percentile and 2 had a BMI between the 3rd and 10th percentiles of the corresponding reference for the French population (19). Anorectic patients were admitted to the

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Psychiatric Department of the Nancy University Children's Hospital. No case of bulimia was recorded. Patients were not given the opportunity to perform exercise; however, normally accepted levels of movement within the department were allowed.

Criteria for diagnostic and refeeding methods were described previously (20). All patients had reached the age of menarche but were amenorrheic because of malnutrition and endocrine dysfunction (7). Two of the patients were at the P3 stage of puberty and 15 were at the P4 or P5 stage according to the definition of Tanner (21). Anorectic patients were allowed to leave the hospital when they reached 90 ± 5% of their theoretical weight-for-height (19); they were still amenorrheic at that time. Urine and fasting blood samples were obtained on the morning after admission (day 0) and on days 15 and 30 of the refeeding period.

All anorectic patients were permitted ad libitum feeding during the day and 4 patients were provided an additional intake by constant-infusion nasogastric tube feeding at night. In 3 of these patients the enteral solution contained medium-chain triacylglycerols. Carbohydrate, lipid, and protein provided 46–51%, 35–40%, and 14–16%, respectively, of the daily energy intake via ad libitum feeding (6798 ± 2592, 8368 ± 3187, and 13372 ± 830 kJ on days 0, 15, and 30, respectively), whereas the riboflavin intake was 2.5 ± 0.5 mg/d. For the enteral feeding program, carbohydrate, lipid, and protein provided 49–51%, 35%, and 14–16%, respectively, of the energy intake (6276–6610 kJ/L) and the riboflavin intake ranged from 0.9 to 1.1 mg/L. Three patients left the refeeding program before day 30 because they had reached their goal weight within 21 d (BMI: 18–18.6). As such, n = 17 on days 0 and 15, whereas n = 14 on day 30.

Seventeen healthy, young girls aged 10–17 y (median: 13 y) with no history of any eating disorder were used as control subjects; they had been hospitalized for 24 h for benign orthopedic surgery. Three control subjects were at the P3 stage of puberty and 14 were at the P4 or P5 stage (21). The control subjects had BMIs within normal limits, above the 25th percentile of the French population (19), with 8 having a BMI <20. An assessment of the nutritional value of their food intake showed that the control subjects consumed normal diets, with carbohydrate, protein, and lipid accounting for 50–55%, 30–35%, and 10–15%, respectively, of the daily energy intake (9205 ± 1775 kJ/d). Riboflavin intake was 2.5 ± 0.5 mg/d. Blood and urine samples were collected before surgery. The study was conducted according to the Declaration of Helsinki 1989.

Blood and urine collection

Fasting venous blood was collected into 5-mL heparin-containing tubes (wrapped in aluminium foil) and centrifuged at 2200 × g (CR 4.11; Jouan, Winchester, VA) for 5 min at 4°C. Erythrocytes were washed 3 times with a 0.9%-NaCl solution. Plasma and erythrocytes were immediately stored at –20°C until analyzed. The first urine voided in the morning was collected, placed on ice with no preservatives, and stored immediately at –20°C until analyzed.

Blood vitamin extraction

A vitamin extraction procedure modified from that described by Bötticher and Bötticher (22) was used to detect FAD, FMN, and riboflavin in the same analytic run. All experiments were carried out under low-intensity light. A 0.5-mL volume of hemolysate or 1 mL plasma was used for vitamin extraction, whereas an equal volume of 0.2 mol sodium acetate/L was added and incubated at 60°C for 15 min to release FAD and FMN from apoenzymes present in the medium. A 0.5-mL aliquot of 10% trichloroacetic acid solution was added to precipitate proteins. The tube was left for 5 min at 4°C in the dark and then centrifuged at 3200 × g (MR 14.11; Jouan) for 15 min. The supernate was desalted on a C_{18} Sep-Pak cartridge (Waters, Milford, MA) conditioned previously with 2 mL methanol (Prolabo, Paris) and 2 mL solution A (10 mmol potassium dihydrogen phosphate/L and 5 mmol hexane sulfonic acid/L, brought to a pH of 3 with orthophosphoric acid) and was rinsed with 2 mL solution A. Vitamins were then eluted with 2 mL solution B (a 1:1 mixture of methanol and solution A). The vitamin extract was injected into the HPLC column for analysis. Standard solutions of 265 nmol riboflavin/L (Merck, Darmstadt, Germany), 240 nmol FAD/L, and 220 nmol FMN/L (Sigma, St Louis) were used for calibration, with or without preincubation with vitamin B-2–deficient plasma. The extraction recovery (ER) of each vitamin was estimated according to the following formula:

$$ER = 100 \times (C_f - C_i/C_s) \quad (1)$$

where C_f is the concentration of riboflavin, FAD, or FMN obtained from the enriched plasma; C_i is the initial concentration of riboflavin, FAD, or FMN obtained from the plasma; and C_s is the concentration of riboflavin, FAD, or FMN in the added standard solution.

Separation and identification of riboflavin, FAD, and FMN

The procedure used to separate and identify compounds by HPLC was adapted from the method of Batey and Eckert (23). Analyses were carried out on a C_{18} reversed-phase column (250 × 4 mm, 5 μm internal diameter; Interchim, Montluçon, France) under isocratic conditions by using 15% acetonitrile in solution A at a flow rate of 1 mL/min. The HPLC system was composed of 2 model 501 pumps (Waters) connected to an RF 535 fluorescence HPLC monitor and a CR6A Chromatopac integrator (both from Shidmadzu Corporation, Kyoto, Japan). The spectrofluorometer was set at 445 nm for the excitation wavelength and 530 nm for the emission wavelength.

Urinary organic acid and plasma amino acid analyses

Glutaric, ethylmalonic, suberic, and adipic acids and isovalerylglycine in urine from anorectic patients and control subjects were analyzed by using gas chromatography–mass spectrometry (model 5971 A; Hewlett-Packard, Palo Alto, CA) as described previously (24). Aliquots of urine containing 1 mg creatinine were used for the extraction of these compounds. The content of creatinine in the urine was determined according to the Jaffé reaction (25).

Plasma amino acid concentrations in anorectic patients and control subjects were analyzed with a Pharmacia Biochrom 20 amino acid analyzer equipped with a cation-exchange resin column (Pharmacia Biotech, Uppsala, Sweden) according to the method of Modino et al (26). Amino acids were identified and quantified by using EZ chromatography software (Scientific Software Inc, San Ramon, CA).

Body fat mass and lean mass

Body fat mass and lean mass in anorectic patients were assessed with a Norland XR-26 Marks II/HS 2015 dual-energy X-ray absorptiometry scanner (Norland Corporation, Fort Atkin-

son, TX) as described by Orphanidou et al (27). The influence of hydration on body composition as measured by dual-energy X-ray absorptiometry was discussed previously (28, 29).

Plasma thyroid hormones

Plasma thyroid hormones in anorectic patients and control subjects were assessed by using time-resolved fluoroimmunoassay (30) with a DELFIA kit and an LKB Wallac 1230 Arcus fluorometer (Wallac, Oy, Finland). Values typically ranged from 1.3 to 2.5 nmol/L, 69 to 141 nmol/L, 4.6 to 7.8 pmol/L, 7.64 to 19.7 pmol/L, and 0.3 to 3.8 mU/L for T_3, thyroxine (T_4), free T_3 (FT_3), free T_4 (FT_4), and thyrotropin, respectively.

Statistical analyses

Statistical analyses of the data were performed by using STATVIEW 4.02 software (Abacus Concepts, Inc, Berkeley, CA). Because the data were not normally distributed, a Wilcoxon paired test and a Mann-Whitney U test were used to compare data between anorexia nervosa patients on days 0 and 30 and between anorexia nervosa patients and control subjects, respectively. Data are given as means ± SDs and ranges. Bonferroni-adjusted P values were used to compare the significance of the results, with significance set at $P < 0.05$. Linear regression analyses and correlation coefficients were used to assess the relations between riboflavin, riboflavin cofactors, and thyroid hormones, with correlations considered significant at $P < 0.05$.

RESULTS

BMIs measured in the control group were within normal ranges reported for the French population (19) and were significantly higher than BMIs recorded for the anorectic patients at the time of entry (day 0) to the treatment program (**Table 1**). The weight, lean body mass, and fat body mass of anorectic patients increased quickly over the refeeding period, with body-composition indexes after the refeeding program being close to values observed by Goulding et al (31) in healthy 10–14-y-old girls in stage P3 (amenorrheic stage) of puberty (lean mass: 31.2 ± 2.4 kg; fat mass: 11.5 ± 4.2 kg; and BMI: 19.3 ± 1.9) and lower than values in 11–15-y-old girls in stage P4 of puberty (lean mass: 35.5 ± 2.4 kg; fat mass: 14.73 ± 5.76 kg; BMI: 20.3 ± 1.9). After refeeding, the anorectic patients had preadolescent-like body-composition indexes.

Hemoglobin concentrations in control subjects (122.0 ± 2.0 g/L) and in anorectic patients on day 0 (128.0 ± 7.0 g/L) were within ranges typically seen in the laboratory (120–160 g/L); the concentration was slightly depressed (113.0 g/L) in just one anorectic patient. Plasma protein concentrations in control subjects (73.6 ± 3.6 g/L) and in anorectic patients on day 0 (71.1 ± 8.2 g/L) were also within the range of typical laboratory values (60–75 g/L), as were plasma creatinine concentrations: 70.2 ± 11.4 μmol/L for control subjects and 89.5 ± 13.7 μmol/L for anorectic patients (typical laboratory values range from 38 to 114 μmol/L). There was no correlation between urinary creatinine concentration and weight gain during the refeeding period (Table 1).

Plasma albumin, transthyretin, and retinol binding protein concentrations in control subjects and anorectic patients were within the range of values typically measured in our laboratory (37–46 g/L, 0.18–0.28 g/L, and 20–60 mg/L, respectively) (Table 1). The ratio of nonessential to essential amino acids (32, 33) in anorectic patients decreased with refeeding ($P < 0.01$) and by day 30 was close to the value in control subjects (Table 1). Whitehead and Dean's (32, 33) definition of the normal limit is <2 for this ratio; 2 anorectic patients in this study had an amino acid ratio of 1.9 on day 0 and 3 had an amino acid ratio >2 after the refeeding period.

Thyroid hormone concentrations in plasma; FAD, FMN, and riboflavin concentrations in erythrocytes and plasma; and organic acids and isovalerylglycine concentrations in urine are summarized in **Table 2** for anorectic patients and control subjects. FT_4, T_4, FT_3, and T_3 concentrations were lower in anorectic patients than in control subjects at all time points. FT_4 and T_4 decreased significantly during the refeeding period. No significant difference in thyrotropin concentration was observed between the anorectic patients and control subjects. The most emaciated patient (BMI: 10.2) had a very low T_3 concentration before treatment (0.7 nmol/L), which increased significantly after 30 d of refeeding (1.9 nmol/L). The FT_3 concentration in this

TABLE 1
Anthropometric and biological data in anorexia nervosa patients and control subjects[1]

	Anorectic patients			
	Day 0 (n = 17)	Day 15 (n = 17)	Day 30 (n = 14)	Control subjects (n = 17)
Weight (kg)	38.1 ± 7.1[2]	41.5 ± 6.6[3]	44.6 ± 6.2[4,5]	52.9 ± 8.3
	(25.0–49.8)	(31.0–50.0)	(36.0–53.5)	(41.0–68.0)
BMI (kg/m^2)	14.8 ± 2.2[2]	16.5 ± 1.9[3]	17.5 ± 1.5[3,5]	20.5 ± 2.5
	(10.2–16.3)	(12.3–18.6)	(16.6–19.2)	(17.1–25.1)
Lean mass (kg)	29.1 ± 5.5	29.7 ± 4.9[5]	31.3 ± 4.9[6]	ND
Fat mass (kg)	8.2 ± 3.2	9.1 ± 3.5[5]	11.4 ± 2.5[6]	ND
Plasma				
Albumin (g/L)	43.5 ± 5.71	ND	43.2 ± 3.54	43.74 ± 3.6
Transthyretin (g/L)	0.28 ± 0.06	ND	0.32 ± 0.06[5]	0.26 ± 0.03
RBP (mg/L)	35.01 ± 10.9	ND	39.24 ± 7.9[5]	27.86 ± 4.19
NEAA:EAA	2.6 ± 0.73[3]	ND	1.74 ± 0.49[5]	1.5 ± 0.3
Urine				
Creatinine (mmol/L)	4.4 ± 2.5[3]	2.8 ± 1.3[3]	3.1 ± 0.2[3]	5.3 ± 1.5

[1] $\bar{x}$ ± SD; range in parentheses. Day 0, before refeeding; days 15 and 30, times of refeeding; RBP, retinol binding protein; NEAA, nonessential amino acids; EAA: essential amino acids; ND, not determined.

[2–4] Significantly different from control subjects (Bonferroni adjustment): [2]$P < 0.001$, [3]$P < 0.01$, [4]$P < 0.05$.

[5,6] Significantly different from day 0 (Bonferroni adjustment): [5]$P < 0.05$, [6]$P < 0.01$.

TABLE 2
Plasma thyroid hormones, erythrocyte and plasma riboflavin and riboflavin cofactors, and urinary organic acid concentrations in anorexia nervosa patients and control subjects[1]

Samples	Anorectic patients Day 0 (n = 17)	Anorectic patients Day 15 (n = 17)	Anorectic patients Day 30 (n = 14)	Control subjects (n = 17)
Plasma thyroid hormones				
T_3 (nmol/L)	1.4 ± 0.4[2] (0.7–2.1)	1.5 ± 0.4 (0.9–2.3)	1.5 ± 0.5 (0.9–2.5)	2.0 ± 0.6 (1.0–3.0)
T_4 (nmol/L)	117.3 ± 19.6[3] (81.3–137.7)	105.1 ± 6.1[2] (73.5–141.7)	92.3 ± 13.2[4,5] (71.1–112.9)	127.9 ± 20.0 (74.3–155.0)
FT_3 (pmol/L)	4.6 ± 1.2[2] (2.6–6.3)	4.1 ± 1.3[2] (2.4–6.7)	4.6 ± 0.9[2] (3.7–6.5)	6.0 ± 1.5 (3.7–7.9)
FT_4 (pmol/L)	11.0 ± 1.6[2] (8.6–13.7)	9.6 ± 1.7[4] (7.3–12.1)	10.0 ± 1.3[2] (8.1–12.1)	14.4 ± 3.6 (10.5–22.3)
Thyrotropin (mU/L)	1.7 ± 0.9 (0.4–3.3)	1.6 ± 0.9 (0.4–3.1)	1.7 ± 0.3 (0.7–3.3)	1.9 ± 1.3 (0.7–3.5)
Riboflavin and cofactors				
Erythrocyte				
FAD (nmol/mol Hb)	57.8 ± 18.5 (21.0–83.0)	55.7 ± 16.5 (28.0–89.0)	52.7 ± 9.9 (35.0–69.0)	55.1 ± 24.9 (30.0–120.0)
FMN (nmol/mol Hb)	16.6 ± 10.8 (3.0–48.0)	13 ± 7.1 (3.0–27.0)	15.0 ± 6.0 (6.0–36.0)	11.8 ± 3.8 (4.0–20.0)
Riboflavin (nmol/mol Hb)	3.5 ± 2.2[4] (1.0–7.0)	4.3 ± 2.7[4] (1.0–8.0)	3.6 ± 2.6[4] (1.0–9.0)	< 0.1 (trace)
Plasma				
FAD (nmol/L)	45.6 ± 23.9[3] (17.0–105.4)	48.1 ± 23.9[3] (26.0–90.9)	45.0 ± 18.9[3] (19.0–70.9)	78.5 ± 54.3 (30.0–206.0)
FMN (nmol/L)	11.8 ± 6.0 (3.8–23.0)	12.2 ± 5.2 (4.9–15.1)	13.4 ± 8.1 (4.0–31.0)	15.6 ± 9.3 (7.1–47.9)
Riboflavin (nmol/L)	14.8 ± 18.8 (1.0–69.0)	8.7 ± 5.1[4] (2.0–18.1)	14.9 ± 7.5 (4.0–31.0)	19.6 ± 15.2 (4.0–57.8)
Urinary organic acids				
Ethylmalonic acid (μmol/mmol creatinine)	7.1 ± 4.4[4] [14] (2.0–16.0)	10.8 ± 5.2[4] [14] (5.0–18.0)	9.4 ± 5.3[4] [11] (5.0–21.0)	1.3 ± 2.8 (0–5.0)
Isovalerylglycine (μmol/mmol creatinine)	7.7 ± 4.8[3] [14] (2.3–19.2)	10.7 ± 8.1[3] [14] (2.0–25.8)	12.5 ± 12.9[3] [11] (1.6–37.1)	3.8 ± 0.9 (2.6–5.2)
Glutaric acid (μmol/mmol creatinine)	3.4 ± 1.9 [14]	4.1 ± 2.6 [14]	2.41 ± 0.9 [11]	3.0 ± 1.2
Adipic acid (μmol/mmol creatinine)	8.6 ± 8.3 [14]	10.3 ± 6.1 [14]	5.5 ± 2.7 [11]	8.4 ± 5.3
Suberic acid (μmol/mmol creatinine)	9.5 ± 3.9 [14]	9.5 ± 4.0 [14]	10.6 ± 5.0 [11]	6.6 ± 5.5

[1] $\bar{x}$ ± SD; range in parentheses and n of anorectic patients in brackets. Day 0, before refeeding; days 15 and 30, times of refeeding; Hb, hemoglobin; T_3, triiodothyronine; T_4, thyroxine; FT_3, free T_3; FT_4, free T_4; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.
[2–4] Significantly different from control subjects (Bonferroni adjustment): [2] $P < 0.01$, [3] $P < 0.05$, [4] $P < 0.001$.
[5] Significantly different from day 0, $P < 0.05$ (Bonferroni adjustment).

patient also increased after 30 d of refeeding (from 3.7 to 5.4 pmol/L).

FAD, FMN, and riboflavin were assessed separately in erythrocytes and in plasma. HPLC retention times were 2.95 ± 0.03 min for FAD, 3.76 ± 0.08 min for FMN, and 8.40 ± 0.03 min for riboflavin. Extraction recovery was 91%, 95%, and 90% for FAD, FMN, and riboflavin, respectively. No erythrocyte riboflavin could be identified in samples from control subjects within the detection limit of the spectrofluorometer (0.5 nmol/L), in contrast with samples from anorectic patients (**Figure 1**), in whom significantly higher riboflavin concentrations were observed. Furthermore, no significant difference between control subjects and anorectic patients was observed for FAD and FMN concentrations in erythrocytes. In contrast, plasma FAD concentrations were lower in anorectic patients than in control subjects, but no significant difference was observed in plasma FMN and riboflavin concentrations between the 2 groups.

After refeeding, plasma riboflavin concentrations increased in 8 patients and plasma FAD concentrations increased in 3 patients, from 30 to 40 nmol/L, 49 to 68 nmol/L, and 34 to 64 nmol/L. Plasma FAD concentrations before refeeding did not change significantly after refeeding in 8 patients, but did decrease (NS) in 3 patients. The mean plasma FAD concentration in anorectic patients after refeeding was not significantly different from that before refeeding (Table 2). In the most emaciated patient, who had low T_3 and FT_3 concentrations on day 0, plasma FAD (47 nmol/L) and FMN (10 nmol/L) were lower than plasma riboflavin (69 nmol/L); after 30 d of refeeding, plasma FAD, FMN, and riboflavin decreased (33, 4, and 31 nmol/L, respectively). Erythrocyte riboflavin concentrations decreased in 3 patients after 30 d of

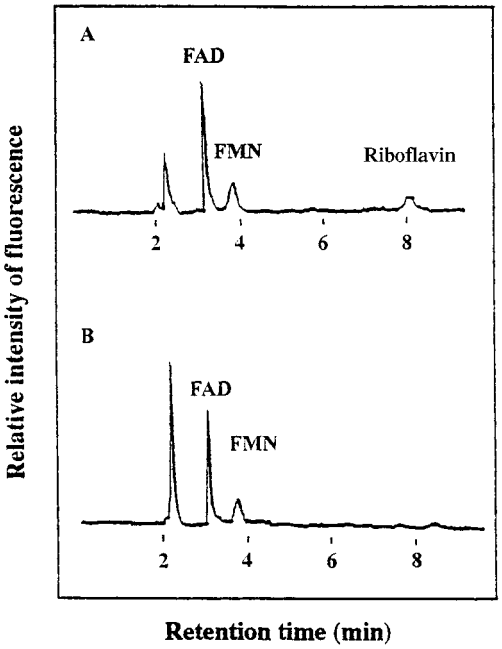

FIGURE 1. HPLC chromatogram of erythrocyte flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and riboflavin in anorexia nervosa patients (A) and control subjects (B). FAD, FMN, and riboflavin were detected by spectrofluorometry with an excitation wavelength of 445 nm and an emission wavelength of 530 nm. Riboflavin, FAD, and FMN were cleared with respective retention times of 8.40 ± 0.03, 2.95 ± 0.03, and 3.76 ± 0.08 min. The first peak of the chromatogram was an artifact.

refeeding. Two months after the patients had left the hospital, erythrocyte riboflavin concentrations had decreased in 4 of the anorectic patients (<0.1 nmol/mol hemoglobin; $P < 0.001$ for values after 2 mo compared with those on day 0).

Plasma riboflavin concentrations in anorectic patients were negatively correlated with T_3 concentrations on day 0 ($r = -0.69$, $P < 0.01$) and became positively correlated after refeeding ($r = 0.59$, $P < 0.05$). Plasma FMN was negatively correlated with T_3 on days 15 and 30 ($r = -0.90$ and $r = -0.74$, respectively; $P < 0.01$). The ratio of plasma FMN to riboflavin was positively correlated with T_3 before refeeding ($r = 0.73$, $P < 0.01$) and was negatively correlated after refeeding ($r = -0.81$, $P < 0.01$). Erythrocyte FMN was positively correlated with T_3 concentrations on day 30 ($r = 0.67$, $P < 0.01$), whereas the ratio of erythrocyte to riboflavin FMN was positively correlated with T_3 on day 15 ($r = 0.54$, $P < 0.05$). A summary of correlations between FT_3 and riboflavin, FAD, and FMN at different time points is given in **Table 3**.

FT_4 was negatively correlated with plasma FMN on day 30 ($r = -0.66$, $P < 0.01$), whereas T_4 was positively correlated with erythrocyte FAD on day 30 ($r = 0.63$, $P < 0.01$). On day 0, thyrotropin was negatively correlated with plasma FAD ($r = -0.55$, $P < 0.05$) and positively correlated with plasma FMN ($r = 0.67$, $P < 0.01$), but there was no significant difference between these indexes after refeeding. In the control subjects, there were no significant correlations between riboflavin, FAD, and FMN and T_3 or FT_3, but FT_4 was positively correlated with erythrocyte FAD and FMN ($r = 0.81$ and $r = 0.88$, respectively; $P < 0.01$).

TABLE 3
Correlation coefficients between free triiodothyronine (FT_3) and plasma riboflavin and riboflavin cofactors in anorexia nervosa patients and control subjects[1]

	FT_3 in anorectic patients			
	Day 0 (n = 17)	Day 15 (n = 17)	Day 30 (n = 14)	FT_3 in control subjects (n = 17)
Plasma				
FAD	0.16	0.50[2]	−0.16	−0.14
FMN	0.43	−0.67[3]	−0.48	0.26
Riboflavin	−0.19	−0.47	0.90[3]	−0.14
Erythrocytes				
FAD	0.70[3]	−0.51[2]	−0.08	0.11
FMN	0.39	−0.05	0.65[3]	−0.09
Riboflavin	−0.06	−0.06	0.39	ND

[1]Day 0, before refeeding; days 15 and 30, times of refeeding; ND, not determined; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide.
[2]$P < 0.05$.
[3]$P < 0.01$.

Furthermore, thyrotropin was positively correlated with plasma FMN ($r = 0.65$, $P < 0.01$).

Urinary concentrations of dicarboxylic acids (ethylmalonic, glutaric, adipic, and suberic acids) and isovalerylglycine in anorectic patients are presented in Table 2. Three patients were excluded from the statistical analysis of organic acid content in the urine (n = 14 on days 0 and 15 and n = 11 on day 30) because they had received enteral nutrition containing C_8–C_{12} medium-chain triacylglycerols, the metabolites (medium-chain dicarboxylic acids) of which could have interfered with the urinary excretion of adipic and suberic acids. Before and after refeeding, ethylmalonic acid and isovalerylglycine concentrations were significantly higher in anorectic patients than in control subjects. No significant difference between anorectic patients and control subjects was observed for glutaric, adipic, or suberic acid concentrations.

Plasma FMN was negatively correlated with ethylmalonic acid before ($r = -0.48$, $P < 0.05$) and 15 d after ($r = -0.61$, $P < 0.01$) refeeding; the correlation was not significant on day 30. The plasma FAD concentration in anorectic patients was not significantly correlated with urinary ethylmalonic acid before or after refeeding. FT_3 concentrations in anorectic patients were negatively correlated with ethylmalonic acid ($r = -0.30$, $P > 0.05$) and isovalerylglycine ($r = -0.55$, $P < 0.05$), on entry to the treatment program, whereas the correlation was not significant after refeeding. No significant correlations between these indexes were observed in control subjects. No significant correlation was observed between T_3, T_4, and FT_4 concentrations and ethylmalonic acid and isovalerylglycine in anorectic patients or control subjects.

DISCUSSION

Riboflavin deficiency in anorexia nervosa was determined previously in several studies either by analysis of the erythrocyte glutathione reductase activity coefficient (EGRAC) (6, 34, 35) or the plasma FAD concentration (6). With the EGRAC method, riboflavin deficiency was observed in 21% (35) and 50% (36) of anorectic patients. Van Binsbergen et al (6) reported a decrease

in whole-blood FAD concentrations in anorectic patients, none of whom had an abnormal EGRAC.

Our study was the first to simultaneously analyze FAD, FMN, and riboflavin in plasma and erythrocytes from anorectic patients. Results showed an accumulation of riboflavin in the erythrocytes and a lower plasma FAD concentration in anorectic patients than in control subjects. The sum of plasma and erythrocyte FAD concentrations in anorectic patients (254.3 ± 63.9, 256.3 ± 69.9, and 223.2 ± 35.5 nmol/L on days 0, 15, and 30, respectively) was similar to that observed by Van Binsbergen et al (6) in whole-blood samples (245.0 ± 45 nmol/L) from anorectic patients. Three anorectic patients (18%) in the present study had plasma total riboflavin concentrations below the lowest value of the control subjects and were considered to be riboflavin deficient, although none had total erythrocyte riboflavin deficiency. Under physiologic conditions, riboflavin is stored in cells as the flavocoenzymes (FAD and FMN) linked to flavoenzymes (37). Riboflavin conversion into FMN and FAD is catalyzed by riboflavin kinase and FMN adenyltransferase in the presence of ATP and Zn^{2+} (38). Both energy depletion and a decrease in riboflavin kinase activity might be responsible for the insufficient conversion of riboflavin into flavocoenzymes.

A decrease in T_3 and in the resting metabolic rate in anorectic patients could have been responsible for a decrease in riboflavin kinase biosynthesis. Consequently, new molecules of riboflavin entering erythrocytes would have had no enzymatic site available to be converted into flavocoenzymes and, as a result, would have been accumulated in the cells as free riboflavin. Because the anorectic patients in this study were not consuming a riboflavin-deficient diet, the fact that plasma riboflavin did not increase in 6 of 14 patients (43%) after refeeding could have been due to a pertubation in the capacity of the intestine to absorb riboflavin. T_3 increased slightly after refeeding and weight gain, but still remained at a low concentration, as reported previously by Kiyoara et al (11).

Our results also showed that T_4 decreased during the refeeding period, suggesting that the peripheral conversion of T_4 into T_3 was only partially restored. The low T_3 concentration, which was observed before refeeding, may have been responsible for deficient riboflavin kinase activity because the expression of this enzyme can be induced by T_3 (13). This might explain the negative correlation observed between plasma riboflavin and T_3 before refeeding and why this correlation became significantly positive after 30 d of refeeding. This hypothesis is strengthened by the positive correlation observed between the ratio of plasma FMN to riboflavin and T_3 before refeeding because FMN and riboflavin are the respective product and substrate of riboflavin kinase.

The perturbation in riboflavin metabolism observed in erythrocytes in the present study might also occur in other cells, particularly in hepatocytes. Considering that hepatocytes are the principal location of FAD-dependent acyl-CoA dehydrogenase activities, the increase in urinary ethylmalonic acid and isovalerylglycine concentrations before and after refeeding could reflect an intramitochondrial FAD deficiency resulting from the defective conversion of hepatocyte riboflavin into flavonucleotides. If hepatocyte riboflavin accumulates in the cytoplasm instead of entering into the mitochondria, flavocoenzymes could be unavailable to acyl-CoA dehydrogenases. This would lead to acyl-CoA accumulation in the mitochondria and short-chain acyl-CoA elimination after conversion into ethylmalonic acid or isovalerylglycine. The negative correlations observed between plasma T_3 and FT_3 status and urinary ethylmalonic acid and isovalerylglycine concentrations in anorectic patients entering the treatment program suggest that thyroid hormone status affects mitochondrial acyl-CoA metabolism via its role in riboflavin metabolism within the mitochondria.

In experimental riboflavin deficiency in rats, it was shown that hepatic flavocoenzymes decrease before plasma flavocoenzymes do (39), leading to a decrease in hepatic acyl-CoA dehydrogenase activity, which in turn is responsible for an increase in the urinary excretion of dicarboxylic acids and glycine conjugates (40). Our results suggest that butyryl-CoA and isovaleryl-CoA catabolism might be affected by FAD depletion, thereby producing a rise in the urinary excretion of ethylmalonic acid and isovalerylglycine. In the present study, the concentration of organic acids was referenced to urinary creatinine. As such, it was found that the rise in organic acids was not due to a decrease in creatinine excretion after refeeding (Table 1).

The excretion of ethylmalonic acid reflects an intramitochondrial accumulation of butyryl-CoA (41). Butyryl-CoA dehydrogenase has been reported to be the hepatic dehydrogenase most affected in cases of riboflavin deficiency (42). Isovalerylglycine synthesis occurs via isovaleryl-CoA from leucine catabolism (43). The fact that glutaric, adipic, and suberic aciduria in the anorectic patients before and after refeeding was not significantly different from that in the control subjects suggests that the urinary organic acid pattern of anorexia nervosa malnutrition was different from that of the genetic form of riboflavin-responsive ethylmalonic-adipic aciduria (43). The fact that concentrations of ethylmalonic, adipic, and suberic acids measured in our anorexia nervosa patients were significantly lower than concentrations we observed previously in malnourished Mauritanian children (44) suggests that FAD-dependent acyl-CoA dehydrogenase activities were less affected in anorectic patients than in malnourished children. We would have expected a reduction in ethylmalonic acid and isovalerylglycine concentrations after refeeding; however, our results showed that hepatic FAD-dependent dehydrogenases had not recovered their optimum activities over the refeeding period.

In conclusion, in anorectic patients we observed decreases in plasma T_3, T_4, and FAD concentrations and increases in erythrocyte riboflavin concentrations and urinary ethylmalonic acid and isovalerylglycine excretion. Our study indicates a possible relation between riboflavin and related organic acids, together with thyroid hormone status. It appears that thyroid dysfunction induced by malnutrition affects riboflavin and acyl-CoA metabolism.

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