

Riboflavin, Flavin Mononucleotide, and Flavin Adenine Dinucleotide in Human Plasma and Erythrocytes at Baseline and after Low-Dose Riboflavin Supplementation

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Background: Vitamin B₂ exists in blood as riboflavin and its cofactors, flavin mononucleotide (FMN) and FAD. The erythrocyte glutathione reductase activation coefficient (EGRAC) has traditionally been used to assess vitamin B₂ status in humans. We investigated the relationships of EGRAC and plasma and erythrocyte concentrations of riboflavin, FMN, and FAD in elderly volunteers and their responses to riboflavin administration.

Methods: EGRAC and plasma and erythrocyte concentrations of riboflavin, FMN, and FAD were determined in 124 healthy individuals with a mean age of 69 years. The same measurements were made in a subgroup of 46 individuals with EGRAC ≥ 1.20 who participated in a randomized double-blind 12-week intervention study and received riboflavin (1.6 mg/day; n = 23) or placebo (n = 23).

Results: Median plasma concentrations were 10.5 nmol/L for riboflavin, 6.6 nmol/L for FMN, and 74 nmol/L for FAD. In erythrocytes, there were only trace amounts of riboflavin, whereas median FMN and FAD concentrations were 44 and 469 nmol/L, respectively. Erythrocyte FMN and FAD correlated with each other and with EGRAC and plasma riboflavin ($P < 0.05$). All variables except plasma FAD responded significantly to riboflavin supplementation compared with placebo (P

≤ 0.04). The strongest increases were for riboflavin in plasma (83%) and for FMN in erythrocytes (87%).

Conclusions: Concentrations of all B₂ vitamers except plasma FAD are potential indicators of vitamin B₂ status, and plasma riboflavin and erythrocyte FMN may be useful for the assessment of vitamin B₂ status in population studies.

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Riboflavin⁴ is the precursor of flavin mononucleotide (FMN) and FAD (1). These compounds serve as cofactors for several reduction-oxidation enzymes, which play an important part in energy metabolism (1). They are also involved in the metabolism of folate, vitamin B₁₂, vitamin B₆, and other vitamins, and this probably explains why plasma riboflavin is a determinant of plasma homocysteine, which is associated with cardiovascular disease, pregnancy complications, and cognitive impairment (2).

Vitamin B₂ deficiency is common in many parts of the world, particularly in developing countries (3, 4). Several studies have indicated that vitamin B₂ deficiency may be widespread in industrialized countries as well, both in the elderly (5, 6) and in young adults (7).

Vitamin B₂ status in humans has usually been assessed by measuring the erythrocyte glutathione reductase activation coefficient (EGRAC), which is the ratio between enzyme activity determined with and without the addition of the cofactor, FAD (8–10). Apparently, glutathione reductase loses FAD at an early stage in vitamin B₂ deficiency, in contrast to key enzymes involved in energy metabolism (11), and this makes EGRAC a useful method

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⁴ Riboflavin is used specifically to name the precursor of FMN and FAD, whereas the term vitamin B₂ is used in the generic sense to denote riboflavin, FMN, and FAD.

for the diagnosis of vitamin B₂ deficiency (12, 13). The method is less reliable in populations with a high prevalence of glucose 6-phosphate dehydrogenase deficiency (8) or β -thalassemia (14), and some other conditions may also influence EGRAC values (8, 15).

The urinary excretion of vitamin B₂ (16) and blood vitamin concentrations (13, 17) have also been used as indicators of vitamin B₂ status in humans. Relationships between vitamin B₂ concentrations in erythrocytes and other indices have been investigated in cross-sectional studies (3, 9, 18–20), and in general, associations between vitamin B₂ and riboflavin intake (18–20) and between vitamin B₂ and EGRAC (3, 9) have been weak or absent. Riboflavin intervention studies have been carried out in humans and animals, and most studies demonstrate a decrease in vitamin B₂ in erythrocytes when an organism is depleted (21–23) and increased concentrations after riboflavin supplementation (3, 8, 9, 21, 24). Similarly, vitamin B₂ concentrations in plasma or serum have been investigated in cross-sectional (18, 20) and intervention studies (21, 22, 25, 26), but the results have been less consistent than for erythrocytes. Different study designs make the comparison of results difficult, particularly because only total B₂ or FAD was measured in most studies (3, 8, 9, 18–20, 23, 24). Additionally, different types of anticoagulants have been used for the preparation of plasma, and this may influence sample stability and relative vitamers concentrations (27).

The aim of the present study was to determine whether concentrations of riboflavin, FMN, and FAD in EDTA plasma and erythrocytes reflect vitamin B₂ status in humans. We investigated the relationship between these analytes and their correlation with EGRAC in healthy elderly individuals. In a group of individuals with EGRAC ≥ 1.20 , indicating suboptimal vitamin B₂ status (13), we performed a randomized placebo-controlled, low-dose riboflavin intervention and compared the responses of the separate vitamers.

Participants and Methods

STUDY DESIGN

Between January and April 1998, 124 elderly individuals were recruited through senior citizens groups and local folds in Northern Ireland. Their mean age was 69 years, and 69% were women (Table 1). Individuals with gastrointestinal, hematologic, vascular, renal, or hepatic disorders or with impaired cognitive function (score < 7 on Hodgkinson 10-Point Mental State Questionnaire) were not included, nor were individuals with serum B₁₂ < 111 pmol/L or individuals using B-vitamin supplementation. Ethical approval was granted by the Research Ethical Committee of the University of Ulster, and participants gave written, informed consent.

EGRAC was used to determine vitamin B₂ status in the study population, and 52 individuals with suboptimal vitamin B₂ status (EGRAC ≥ 1.20) were invited to participate in a 12-week randomized, double-blind, placebo-

Table 1. Characteristics of the study population.

	n	Mean ^a	Median (10th–90th percentiles)
Sex, % male	124	31	
Age, years	124	69	69 (61–79)
EGRAC	124	1.18	1.18 (1.07–1.29)
Erythrocyte FMN, nmol/L	54	50	44 (26–80)
Erythrocyte FAD, nmol/L	54	467	469 (351–558)
Plasma riboflavin, nmol/L	118	15.3	10.5 (5.4–28.4)
Plasma FMN, nmol/L	118	7.5	6.6 (4.0–11.7)
Plasma FAD, nmol/L	118	75	74 (56–97)
Creatinine, $\mu\text{mol/L}$	112	96	94 (77–117)
Body mass index, kg/m ²	124	26	26 (22–32)

^a When not otherwise indicated.

controlled, low-dose riboflavin intervention. Among these, 46 agreed and received riboflavin (1.6 mg/day; n = 23) or placebo (n = 23), which were taken in the morning. Participants were instructed to maintain their usual diets and to refrain from commencing any form of vitamin supplementation during the intervention.

BLOOD SAMPLING

Blood samples were collected from all participants at the time of screening, and before the start and after completion of the 12-week intervention for those who participated in the intervention study. All samples were collected after an overnight fast, which included the riboflavin and placebo tablets.

Tripotassium EDTA tubes (Vacuette; Greiner Labor-technik GmbH) were used for whole blood, which was placed on ice and centrifuged within 2 h to obtain plasma. The remaining erythrocytes were washed three times with phosphate-buffered saline, the saline and the buffy coat were removed after each centrifugation, and the resulting cells were stored. To obtain serum, blood was collected into Vacuette tubes with clot activator and gel (Greiner) and centrifuged within 2 h. Erythrocytes, EDTA plasma, and serum were stored at -70 °C until analysis.

BIOCHEMICAL ANALYSES

EGRAC (28) was measured by enzyme assay on the Cobas Fara centrifugal analyzer (Roche Diagnostics).

Plasma riboflavin, FMN, and FAD were measured by a modification of the method described by Hustad et al. (27). Briefly, a 40- μL plasma sample was mixed with 400 μL of trichloroacetic acid (100 g/L) containing 15 nmol/L isoriboflavin (internal standard), and 330 μL of the supernatant was neutralized by the addition of 108 μL of K₂HPO₄ (2 mol/L). The neutralized trichloroacetic acid-treated plasma was subjected to solid-phase extraction by use of C-18 columns as described in the original publication (27), except that the columns were eluted with doubly distilled water instead of phosphate buffer. The eluate was lyophilized overnight (Lyovac GT2; Leybold-Heraeus GmbH), and the analytes were then dissolved in

40 μ L of water. The vitamers were separated by capillary electrophoresis on a Beckman P/ACE MDQ (Beckman Coulter Inc.) and detected by laser-induced fluorescence.

Erythrocyte B₂ vitamers were analyzed in the same manner, except that samples were homogenized after the addition of trichloroacetic acid by use of a rotating polypropylene pestle.

Serum creatinine was determined by the Jaffe alkaline picrate method for the Hitachi 911 system (F. Hoffmann-La Roche Ltd.).

EGRAC was analyzed in triplicate, whereas riboflavin, FMN, and FAD were analyzed in duplicate. Analytical CVs were 7% for EGRAC and in the range 6–8% for plasma and erythrocyte B₂ vitamers. EGRAC and the B₂ vitamers were analyzed in separate laboratories, and results were blinded for those who performed the analyses. Quality control for all assays was provided by repeated analysis of stored batches of pooled erythrocytes, plasma, or serum.

STATISTICAL METHODS

Medians with 10th and 90th percentiles and means with SDs were used for descriptive statistics. The biological (intra- and interindividual) CV (CV_B) was calculated according to the formula:

$$CV_B = (CV_T^2 - CV_A^2)^{0.5}$$

where CV_T is the total CV and CV_A is the analytical CV. Correlation analyses were performed using Spearman coefficients.

The χ^2 test was used to compare categorical variables, and the Student *t*-test was used to compare means of continuous numeric variables. Responses to intervention were examined by univariate ANOVA with change (pretreatment – posttreatment values) as the dependent variable and treatment (riboflavin or placebo) as the factor variable. Because data on erythrocyte FMN and FAD were not available for all participants, r^2 was calculated to facilitate the comparison of these variables with other vitamin B₂ indices.

Tests were two-tailed, and $P < 0.05$ was considered statistically significant. SPSS Ver. 10.0 for Macintosh (SPSS Inc.) was used for all statistical analyses.

Results

POPULATION CHARACTERISTICS AND BLOOD INDICES

The study population consisted of 38 men and 86 women with a mean age of 69 years (Table 1). EGRAC and plasma and erythrocyte concentrations of B₂ vitamers showed no gender differences ($P > 0.3$). The median (10th–90th percentiles) EGRAC values were 1.18 (1.07–1.29; Table 1). There were only trace amounts of erythrocyte riboflavin (< 1 nmol/L), whereas erythrocyte FMN and FAD concentrations were 44 nmol/L (26–80 nmol/L) and 469 nmol/L (351–558 nmol/L), respectively (Table 1). Plasma concentrations were 10.5 nmol/L (5.4–28.4 nmol/L) for riboflavin, 6.6 nmol/L (4.0–11.7 nmol/L) for FMN, and 74 nmol/L (56–97 nmol/L) for FAD (Table 1).

In plasma, the biological (intra- and interindividual) variation of riboflavin concentrations was pronounced, and the CV_B was 153%, compared with 55% for FMN and 18% for FAD (data not shown). The variation was similar in plasma and erythrocytes, and the CV_B values for erythrocyte FMN and FAD were 45% and 16%, respectively.

BIVARIATE CORRELATIONS

Erythrocyte concentrations of FMN and FAD correlated significantly ($r = 0.57$), and both coenzyme forms were negatively associated with EGRAC ($r = -0.45$ and -0.30 , respectively) and positively associated with plasma riboflavin ($r = 0.39$ and 0.32 , respectively; Table 2). Plasma concentrations of riboflavin and FMN correlated strongly ($r = 0.58$), whereas the association between plasma FMN and FAD was less pronounced ($r = 0.30$; Table 2).

The inverse relationship between plasma riboflavin and EGRAC was not significant (Table 2). None of the variables was related to age, creatinine, or body mass index (Table 2).

Table 2. Spearman correlation coefficients for vitamin B₂ indices, age, creatinine, and body mass index.^a

	EGRAC	E-FMN ^b	E-FAD	P-Riboflavin	P-FMN	P-FAD	Age	Creatinine
E-FMN	-0.45 ^c (54)							
E-FAD	-0.30 ^d (54)	0.57 ^c (54)						
P-Riboflavin	-0.09 (118)	0.39 ^c (54)	0.32 ^d (54)					
P-FMN	0.08 (118)	0.04 (54)	0.05 (54)	0.58 ^c (118)				
P-FAD	0.18 (118)	-0.17 (54)	0.02 (54)	-0.08 (118)	0.30 ^c (118)			
Age	-0.02 (124)	0.14 (54)	0.06 (54)	0.07 (118)	-0.17 (118)	-0.15 (118)		
Creatinine	-0.02 (112)	-0.03 (54)	-0.22 (54)	0.22 (106)	-0.10 (106)	0.01 (106)	0.17 (112)	
BMI	0.01 (124)	-0.10 (54)	0.12 (54)	0.03 (118)	-0.05 (118)	-0.04 (118)	-0.09 (124)	0.05 (112)

^a The number of individuals is given in parentheses.

^b E, erythrocyte; P, plasma; BMI, body mass index.

^c $P < 0.01$.

^d $P < 0.05$.

Table 3. EGRAC and concentrations of B₂ vitamers before and after the intervention.^a

	Placebo			Riboflavin			<i>P</i> ^b	<i>r</i> ²
	<i>n</i>	Before	After	<i>n</i>	Before	After		
EGRAC	23	1.26 (0.06)	1.24 (0.06)	23	1.26 (0.05)	1.10 (0.07)	<0.001	0.45
Erythrocyte FMN, nmol/L	11	28 (9)	28 (7)	13	32 (19)	54 (24)	<0.001	0.63
Erythrocyte FAD, nmol/L	11	434 (43)	451 (45)	13	463 (69)	525 (74)	0.01	0.27
Plasma riboflavin, nmol/L	23	14.8 (11.4)	13.5 (8.1)	23	13.2 (14.9)	19.5 (16.9)	0.001	0.22
Plasma FMN, nmol/L	23	7.0 (2.6)	6.9 (2.0)	23	6.5 (2.3)	7.9 (2.5)	0.04	0.09
Plasma FAD, nmol/L	23	72 (11)	72 (14)	23	74 (13)	72 (15)	0.7	0.00

^a Data are mean (SD).

^b By univariate ANOVA, with change (postintervention – preintervention values) as the dependent variable and treatment (placebo vs riboflavin) as the factor variable.

RESPONSES TO LOW-DOSE RIBOFLAVIN INTERVENTION

Forty-six individuals with EGRAC ≥ 1.20 received either riboflavin (1.6 mg/day; $n = 23$) or placebo ($n = 23$) for 12 weeks. Mean dietary intakes of riboflavin (1.6 mg/day) and energy (9.4 MJ/day) were not significantly different between the riboflavin supplementation and the placebo groups ($P \geq 0.2$). EGRAC and concentrations of B₂ vitamers in plasma and erythrocytes (Table 3), as well as sex distribution, creatinine, and body mass index did not differ between the groups ($P \geq 0.3$), whereas the mean age was 70 years in the riboflavin group and 67 years in the placebo group ($P = 0.04$).

EGRAC and all B₂ vitamers except plasma FAD responded significantly to riboflavin supplementation compared with placebo (Table 3 and Fig. 1). In plasma, the mean riboflavin increase was 83%, whereas FMN increased by 27% and FAD did not change (data not shown). In erythrocytes, the riboflavin response could not be determined because concentrations were below the limit of quantification (<1 nmol/L), but FMN increased

by 87%, whereas FAD increased by only 14% (data not shown) in the riboflavin group.

The intervention (placebo or riboflavin) explained 45% of the variance of the EGRAC change (Table 3). Moreover, it accounted for 9–63% of the variance of B₂ vitamer responses, except for plasma FAD, which was independent of the intervention (Table 3).

The magnitude of riboflavin, FMN, and FAD responses differed, and relative vitamer concentrations changed in individuals who were supplemented, in both the plasma and erythrocytes. Thus, the riboflavin:FMN ratio increased significantly in plasma, indicating that riboflavin became more abundant relative to FMN, and the FMN:FAD ratio increased in both the plasma and erythrocytes (Table 4).

Discussion

The objective of the present study was to evaluate plasma and erythrocyte concentrations of riboflavin, FMN, and FAD as indicators of vitamin B₂ status. This was done by

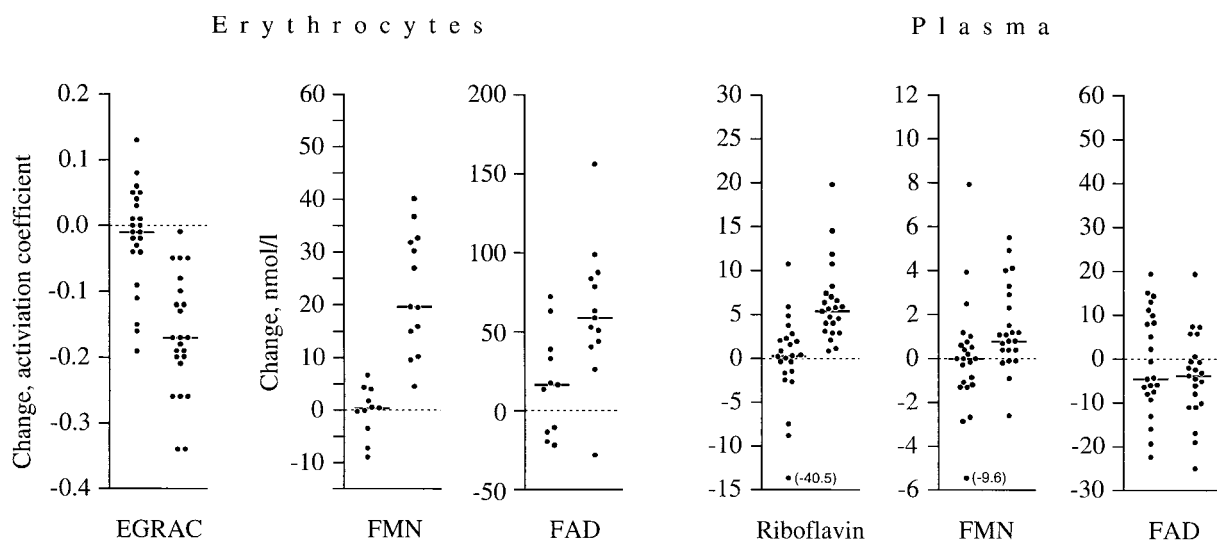


Fig. 1. Changes of EGRAC and B₂ vitamer concentrations in individuals receiving placebo or riboflavin.

EGRAC, riboflavin, FMN, and FAD responses (postintervention – preintervention values) are shown in the placebo (left-hand columns in panels) and riboflavin (right-hand columns in panels) groups. Medians are indicated by solid horizontal lines. For erythrocyte FMN and FAD, $n = 11$ and 13 in the placebo and riboflavin groups, respectively. For the other variables, $n = 23$ in both groups.

Table 4. Ratios between concentrations of B₂ vitamers before and after the intervention.^a

	Placebo			Riboflavin			P ^b
	n	Before	After	n	Before	After	
Plasma riboflavin:FMN ratio	23	2.12 (1.51)	1.92 (0.78)	23	1.93 (1.31)	2.43 (1.27)	0.02
Plasma FMN:FAD ratio	23	0.10 (0.03)	0.10 (0.03)	23	0.09 (0.04)	0.11 (0.04)	0.02
Erythrocyte FMN:FAD ratio	11	0.06 (0.02)	0.06 (0.01)	13	0.07 (0.03)	0.10 (0.04)	<0.001

^a Data are mean (SD).

^b By univariate ANOVA, with change (postintervention – preintervention values) as the dependent variable and treatment (placebo vs riboflavin) as the factor variable.

comparing relationships between potential indicators of vitamin B₂ status in 124 healthy elderly individuals and by assessing responses to low-dose riboflavin supplementation or placebo in 46 individuals with EGRAC ≥ 1.20 , indicating suboptimal vitamin B₂ status. We found that EGRAC and plasma riboflavin correlated with FMN and FAD in erythrocytes (Table 2). Riboflavin supplementation significantly reduced the EGRAC compared with placebo, and all B₂ vitamers except plasma FAD increased (Table 3 and Fig. 1).

STUDY DESIGN AND LIMITATIONS

The intervention study was randomized and placebo-controlled, and baseline measurements were repeated to partly correct for regression toward the mean. Because EGRAC was used both to select for the intervention and as a response variable, the design is not ideal for comparing responses of EGRAC to responses of other vitamin B₂ indices.

The intervention riboflavin dose of 1.6 mg/day was comparable to United Kingdom Reference Nutrient Intakes (29). By selecting a low dosage and withholding riboflavin on the days of blood sampling, we avoided interference from a transient increase in plasma riboflavin after vitamin intake. This increase may be substantial for high-dose riboflavin (30), but it is probably less important in connection with ordinary meals (21, 30).

Erythrocytes from the intervention period, sufficient to measure FMN and FAD in addition to EGRAC, were available for only 24 participants. These individuals did not differ from the rest of the individuals who took part in the intervention study with respect to sex distribution, age, dietary riboflavin, energy intake, EGRAC, and B₂ vitamer concentrations ($P \geq 0.1$).

CONCENTRATIONS OF B₂ VITAMERS IN PLASMA AND ERYTHROCYTES

Plasma and erythrocyte concentrations of riboflavin, FMN, and FAD (Table 1) were in the same range as reported in several other studies (17, 31). The biological variability of riboflavin was pronounced compared with FMN and FAD (Table 2). This is in agreement with previous reports (2, 27, 30) and may reflect that concentrations of flavin coenzymes, particularly FAD, are regulated within a relatively narrow range, as opposed to riboflavin.

In plasma, FMN was strongly associated with its precursor, riboflavin, whereas FAD correlated less strongly with its precursor, FMN (Table 2). The strength of these relationships was the same as reported in other studies (2, 27). Associations between concentrations of proximate metabolites within the same compartment and between EGRAC and concentrations of erythrocyte FAD (Table 2) are expected because of metabolic interdependence. Furthermore, covariations may also reflect that EGRAC and concentrations of riboflavin, FMN, and FAD are determined by vitamin B₂ status.

VITAMIN B₂ HOMEOSTASIS

Concentrations of individual B₂ vitamers in the blood have been examined in vitamin B₂ deficiency (22, 25). In men maintained on restricted riboflavin intake for several months, the sum of plasma riboflavin and plasma FMN was lower than in the control group, whereas plasma FAD was not significantly different (22). In Cebus monkeys who were severely vitamin B₂-deficient, both FAD and riboflavin decreased in serum, but riboflavin decreased before FAD during the development of deficiency (25). These vitamers have been investigated after riboflavin repletion as well, and in vitamin B₂-deficient rats, whose growth was improved by successive addition of dietary riboflavin, serum riboflavin increased relatively more than FAD (21).

Individual B₂ vitamers have also been investigated in tissues, and hepatic concentrations of riboflavin and FMN were lower than FAD in vitamin B₂-deficient rats, compared with controls (12, 32, 33). Moreover, FMN decreased more than FAD in rat liver during the development of vitamin B₂ deficiency, and FMN concentrations were subsequently restored more slowly on realimentation (34).

The differential responsiveness of riboflavin, FMN, and FAD during vitamin B₂ depletion and repletion probably reflects mechanisms involved in vitamin B₂ homeostasis (11, 33, 35). Such mechanisms may ensure adequate tissue concentrations of coenzymes, particularly of FAD (11, 33, 35). Preservation of FAD concentrations within a narrow range, at the expense of other B₂ vitamers, might explain why riboflavin and FMN are the most responsive vitamers in individuals with suboptimal vitamin B₂ status who receive low-dose riboflavin supplementation (Table 4).

RIBOFLAVIN, FMN, AND FAD AS INDICATORS OF VITAMIN B₂ STATUS

Riboflavin was the most responsive plasma index in individuals who were supplemented (Table 4), but the variability of the response was high, and the intervention explained only 22% of the variance (Table 3), which probably reflects that factors other than vitamin B₂ status determine plasma concentrations of riboflavin. Consequently, this index is probably useful for population studies, but it may be less suitable for the assessment of vitamin B₂ status in individuals.

Patients with hypothyroidism are prone to develop nonalimentary vitamin B₂ deficiency, which is associated with impaired activity of riboflavin kinase (36, 37). This enzyme converts riboflavin to FMN and is therefore important for flavin coenzyme synthesis (36, 37). Thus, riboflavin may not be useful for the assessment of vitamin B₂ deficiency in hypothyroidism. Moreover, vitamin B₂ metabolism may be influenced by respiratory infections, catabolic states, and certain other conditions (15, 38, 39), which may be associated with increased mobilization of the vitamin from the liver and other tissues into the blood (15, 39).

The increase in plasma FMN was just significant in the riboflavin-supplemented group compared with the placebo group (Table 3 and Fig. 1), but in erythrocytes, FMN increased 87%, and all individuals who were supplemented responded (Fig. 1). Moreover, the intervention accounted for more than one-half of the variance of the response (Table 3), suggesting that erythrocyte FMN may be more useful than plasma riboflavin for the determination of vitamin B₂ status for a particular individual.

Plasma FAD does not appear to reflect changes of vitamin B₂ status because concentrations did not increase on supplementation (Table 3 and Fig. 1). Erythrocyte FAD responded significantly, however, although not as strongly as erythrocyte FMN (Table 4). Moreover, erythrocyte FAD concentrations correlated significantly with several other vitamin B₂ indices, including plasma riboflavin, and this supports the idea that concentrations of erythrocyte FAD are determined by vitamin B₂ status.

In conclusion, concentrations of all B₂ vitamers except plasma FAD are potential indicators of vitamin B₂ status, and plasma riboflavin and erythrocyte FMN may be useful for the assessment of vitamin B₂ status in population studies. Riboflavin and FMN responded more strongly than FAD in individuals with suboptimal vitamin B₂ status who were riboflavin supplemented. This probably reflects homeostatic mechanisms maintaining adequate concentrations of flavin coenzymes, particularly FAD, under conditions of variable vitamin B₂ intake.

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