AN ABSTRACT OF THE THESIS OF

Shih-Ya Yeh for the degree of Master of Science

in Foods and Nutrition presented on October 17, 1985

Title: Riboflavin Status of Orientals in a U. S. Town

Abstract approved: _____
Lorraine T. Miller, Ph.D.

Riboflavin status was determined in 16 Oriental males and females who were residing off the Oregon State University campus. Before and after these subjects had received 10 mg of riboflavin daily for 7 days, we measured erythrocyte glutathione reductase (EGR) activity with and without FAD added in vitro and erythrocyte total riboflavin levels (RBC B-2). Riboflavin status was assessed by the EGR activity coefficient (EGRAC) (EGR activity with FAD added in vitro / EGR activity without FAD added in vitro). The subjects' dietary intake of riboflavin, protein and calories was estimated from their self chosen diets which were recorded for 3 days before the riboflavin supplementation. Both sex groups had adequate mean levels of riboflavin, protein and calories in their diets. Further, none of the subjects had an intake less than two-thirds of the recommended dietary allowances (FNB, 1980) for riboflavin. Before riboflavin

supplementation 5 of the 9 males and 2 of the 7 females had EGRAC greater than or equal to 1.2, suggesting that they were at high risk of marginal riboflavin deficiency, and to have a normal EGRAC they may need an intake of riboflavin greater than the present recommended allowance.

In all of the subjects, EGRAC decreased in response to the riboflavin supplements. Additionally, the subjects' RBC B-2, EGR basal activity (without FAD added in vitro) and FAD stimulated EGR activity increased significantly ($P < 0.01$) after 7 days of riboflavin supplementation. Although the RBC B-2 concentrations were lower than those reported by Bessey, Horwitt and Love (1956) and Bamji (1969), this measurement correlated significantly with EGRAC ($r = -0.64$, $P < 0.01$), EGR basal activity ($r = 0.74$, $P < 0.01$) and FAD stimulated EGR activity ($r = 0.57$, $P < 0.01$). Dietary riboflavin intake whether expressed as total riboflavin intake per day or riboflavin intake per 1000 Kcal was not correlated to any of these biochemical tests made before riboflavin supplementation. RBC B-2 values obtained from these 16 Orientals were similar to those obtained in 5 Caucasian subjects, indicating that these low values were not due to genetic differences.

Riboflavin Status of Orientals in a U. S. town

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed October 17, 1985

Commencement June 1986

APPROVED:

Professor of Foods and Nutrition in charge of major

Head of Department of Foods and Nutrition

Dean of Graduate School

Date thesis is presented October 17, 1985

Typed by Ching-kai Hsiao, Ming-Kuang Hsu,
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ACKNOWLEDGEMENTS

I wish to express my sincere thanks and appreciation to Dr. Lorraine T. Miller for her guidance, support and assistance throughout my graduate program.

I also wish to thank Dr. David R. Thomas of the Department of Statistics for his help with the statistical analysis.

Special thanks goto the volunteers for their participation and cooperation. Many thanks go to Lynda Barstow for her assistance with drawing of the blood samples and for her humor in times of need. I also wish to thank Ming-Kuang Hsu for his help to produce this document.

Deep appreciation go to my husband, Ching-Kai; my son, Gary; and my parents for their encouragement,comfort and support.

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INTRODUCTION

Riboflavin is a constituent of several oxidative enzyme systems which are involved in intermediary metabolism of carbohydrates, amino acids, lipids and nucleotides. This vitamin is also necessary for the conversion of vitamin B-6 to its active form (Anderson, 1980). Numerous investigations point towards a close metabolic link between thiamin and riboflavin (Tucker, Mickelsen and Keys, 1960; Sharada and Bamji, 1972). In addition, interactions between riboflavin and folacin exist (Bovina et al., 1969).

Because riboflavin is essential to numerous biochemical reactions in tissues, it is natural that deprivation of this vitamin can cause damage to many different tissues. The physiological significance of marginal riboflavin deficiency was reviewed recently by Thurnham (1981).

The purpose of this study was to examine the riboflavin status of a group of relatively healthy Orientals living off the Oregon State University campus. Since adult Chinese in general do not consume milk or milk products, the principal source of riboflavin in the American diet, we hypothesized that these people may have marginal riboflavin status. Accordingly, we estimated

their riboflavin intake from a 3-day dietary record, and measured erythrocyte glutathione reductase (EGR) activity with and without FAD added in vitro and erythrocyte total riboflavin level (RBC B-2). It is generally agreed that combining dietary records with biochemical assays helps in determining whether biochemical findings suggestive of a particular vitamin deficiency are primarily due to an inadequate dietary intake or to some other cause such as malabsorption or increased requirement (Garry, Goodwin and Hunt, 1982) The supplement of riboflavin to the subjects insured that abnormal biochemical status was related to riboflavin and not to other factors or disease.

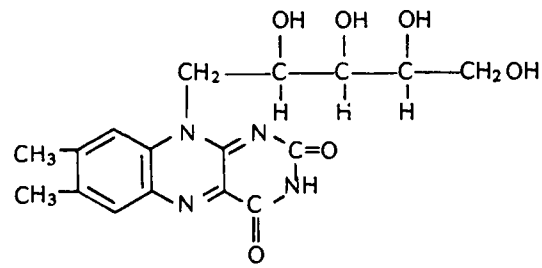
REVIEW OF LITERATURE

Chemical and Physical Properties

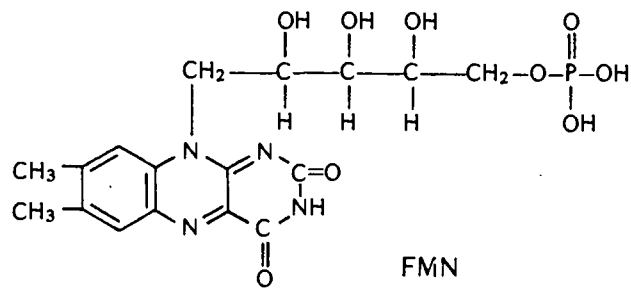
Riboflavin is an orange-yellow substance that imparts a greenish-yellow fluorescence to an aqueous medium. It is sparingly soluble in water and acidic solutions. One gram of riboflavin dissolves in from 3 to about 15 L of water, the variation in the solubility being due to differences in crystalline structures (Windholz et al., 1976). It is highly soluble in alkaline solutions. Riboflavin is stable to heat in neutral or acidic solutions, but it may be destroyed by heating in alkaline solutions or by exposure to light. The natural greenish-yellow fluorescence characteristic of riboflavin in solution is the basis for the fluorometric assay of this vitamin.

Physiological Fuction

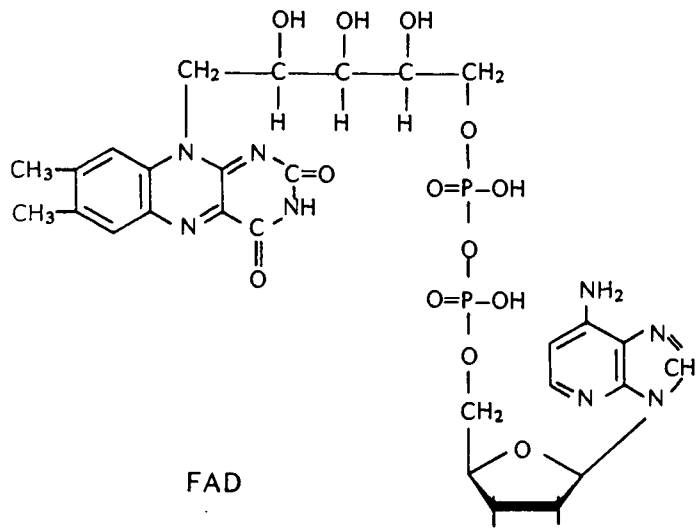
Riboflavin isimportant because of its coenzyme action in flavoproteins, which catalyze biological oxidation-reduction reactions. Two coenzyme forms of riboflavin, riboflavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), are known to exist in the body (Fig. 1). The active portion of the riboflavin moiety is the isoalloxazine ring, which is reversibly reduced (Fig. 2).



Riboflavin



FMN



FAD

Fig. 1. Riboflavin and its coenzyme forms, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD).

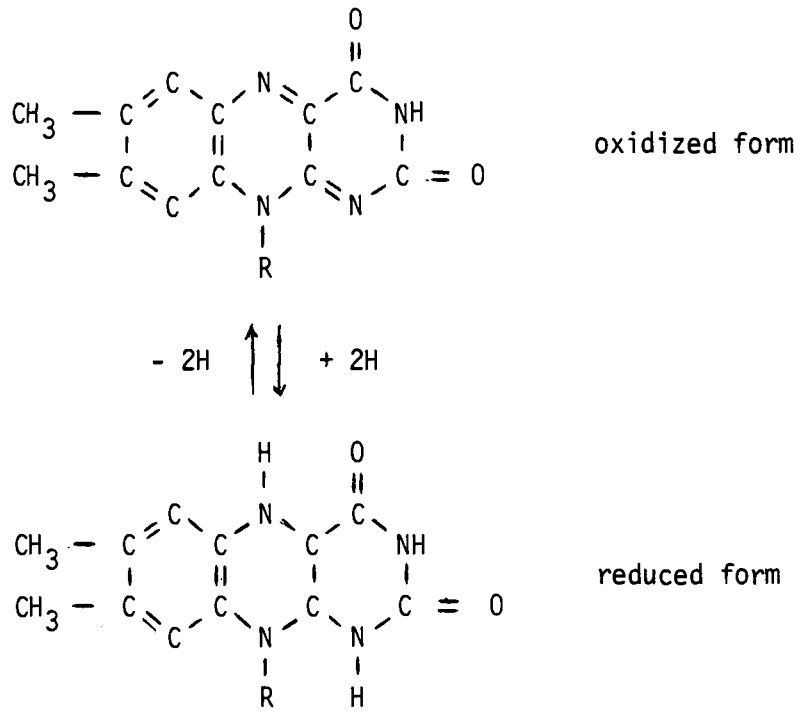


Fig. 2. The reversible reduction of flavin nucleotides by a shipting of bonds in the isoalloxazine ring. R represents the remainder of the flavin nucleotide molecule.

Riboflavin is important in energy metabolism. The flavoproteins act as hydrogen carriers in respiration and electron transport, leading to the formation of ATP, a higher energy compound. Enzymes requiring riboflavin include NADH dehydrogenase, succinate dehydrogenase, and dihydrolipoyl dehydrogenase. Flavoproteins participating in respiration and electron transport are presented in Table 1.

Flavoproteins are also involved in the specific dehydrogenation of adjacent carbon atoms, resulting in the introduction of double bonds into certain molecules. For example, in the first step of fatty acid oxidation acyl-CoA undergoes enzymatic dehydrogenation by acyl-CoA dehydrogenase, an FAD-containing enzyme, to form Δ-enoyl-CoA.

L-amino acid oxidase is an FMN-containing enzyme which catalyzes the oxidative deamination of L-amino acids to the corresponding α-keto acids. D-amino acid oxidase, an FAD-containing enzyme, catalyzes the oxidative deamination of D-amino acids.

Glutathione reductase contains FAD as its prosthetic group. It has been suggested that erythrocyteglutathione reductase (EGR) activity is a very sensitive test for measuring riboflavin deficiency in humans. This enzyme will be described in more detail later.

FMN and FAD are necessary to convert some vitamins

Table 1. Flavoproteins participating in respiration and electron transport.

Flavoprotein	Flavin nucleotide	Function
NADH dehydrogenase	FMN	catalyze transfer of electrons from NADH to the next number of the electron-transport chain
Dihydrolipoyl dehydrogenase	FAD	a component of the pyruvate and α-ketoglutarate dehydrogenase system
Succinate dehydrogenase	FAD	active in the tricarboxylic acid cycle succinate + E-FAD ---- fumarate + E-$FADH_2$
Acyl-CoA dehydrogenase	FAD	catalyze the first step dehydrogenase during fatty acid oxidation acyl CoA + E-FAD ---- Δ-enoyl-CoA + E-$FADH_2$
Electron-transferring flavoprotein	FAD	catalyze transfer of electrons from one number of the electron-transport chain to the next number

Adapted from Lehninger, 1982.

to their active forms. For example, pyridoxine (pyridoxamine) phosphate oxidase, an FMN-containing enzyme, catalyzes the conversion of both pyridoxine phosphate and pyridoxamine phosphate to pyridoxal phosphate, the active form of vitamin B-6 (Fig. 3). Anderson (1980) reported that in the red blood cell conversion of pyridoxine phosphate to pyridoxal phosphate is very sensitive to riboflavin status and is related to riboflavin metabolism.Further, riboflavin is important in tryptophan metabolism and the formation of niacin by its role in production of the pyridoxal phosphate. During riboflavin deficiency, this pathway is disrupted and urinary excretion of tryptophan metabolites is increased (Mason, 1953). Riboflavin also plays a role in the conversion of folic acid to its reduced enzyme form (Bovina et al., 1969). It also has a close link with thiamin. In thiamin deficiency, there is an increased urinary excretion of riboflavin (Tucker et al., 1960). The interrelationship of riboflavin with other nutrients is presented in Fig. 3 (Komindr and Nichoalds, 1980).

Metabolism

FMN and FAD are the major forms of riboflavin in the diet. They need to be hydrolyzed completely to free riboflavin before they are taken up by the epithelial cells of the mucosa (Daniel, Binninger and Rehner, 1983).

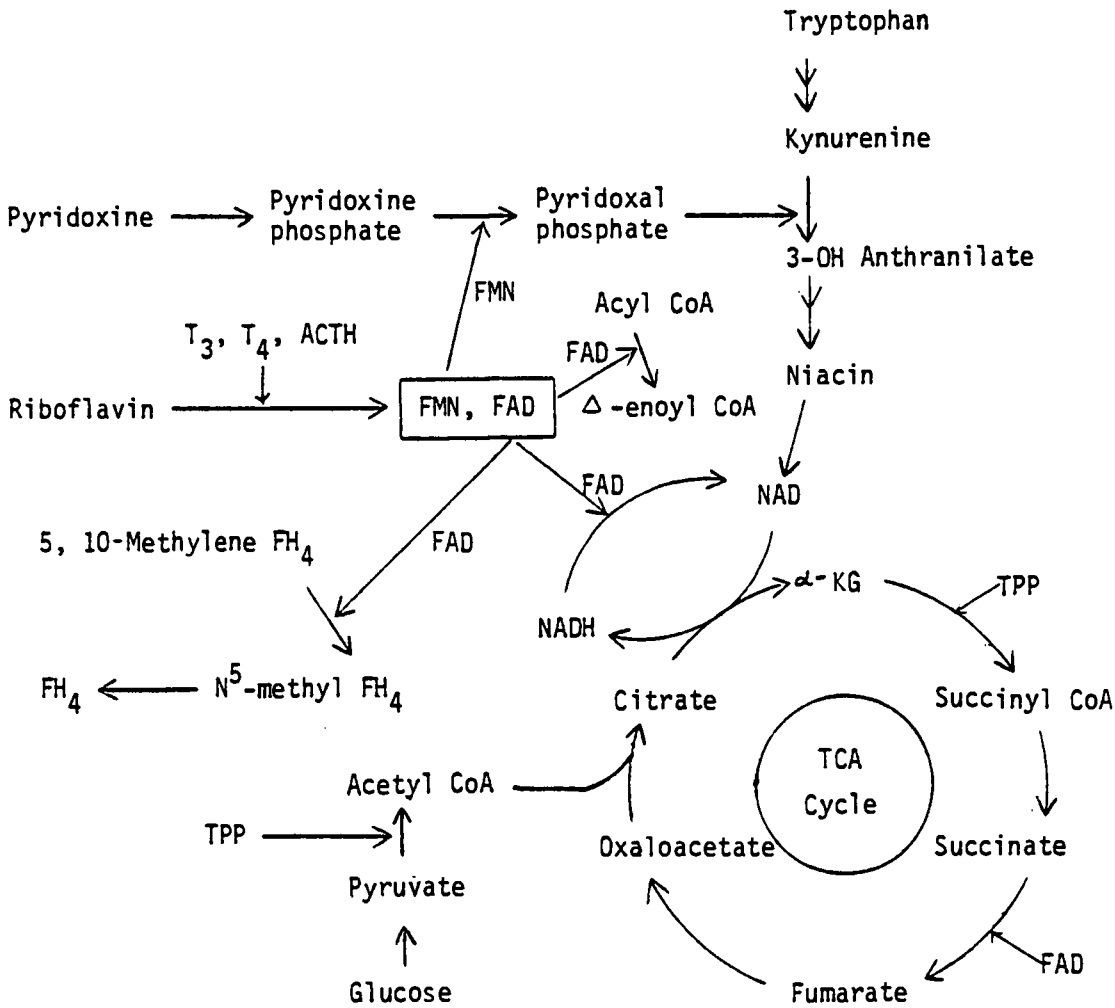
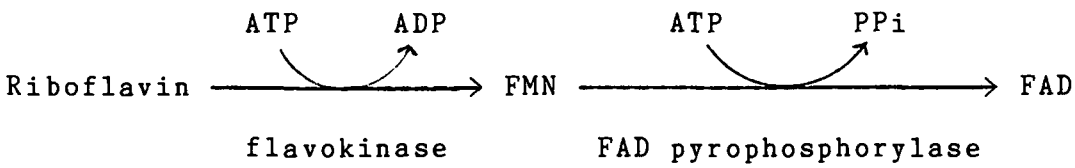


Fig. 3. Interrelationships of riboflavin with other nutrients and influence of hormones in riboflavin metabolism (adapted from Komindr and Nichoalds, 1980)

Key: TPP = thiamin pyrophosphate
FMN = flavin mononucleotide
FAD = flavin adenine dinucleotide
NAD = nicotinamide adenine dinucleotide (oxidized form)
NADH = nicotinamide adenine dinucleotide (reduced form)
FH_4 = tetrahydrofolic acid
KG = ketoglutarate
ACTH = adrenocorticotropic hormone
T_3 = triiodothyronine
T_4 = thyroxine

Free riboflavin is converted to FMN in the intestinal mucosa as a condition for its absorption. FMN then enters the portal vein and is transported to the liver where it is converted to FAD (see below).



Riboflavin can also be converted to FMN and FAD in the erythrocyte (Mandula and Beutler, 1970).

The site of riboflavin absorption has been localized to the proximal small intestine. It is absorbed by a specialized transport process involving a saturable mechanism, rather than by passive diffusion. (Campbell and Morrison, 1963; Levy and Jusko, 1966; Jusko and Levy 1975). When low levels of riboflavin are fed to normal persons, the amount recovered in urine is proportional to the amount ingested. When large oral doses of riboflavin as FMN are administered to normal adult subjects, however, the upper limit of intestinal absorption is about 25 mg under ordinary conditions, demonstrating that the intestinal absorptive capacity for the vitamin is limited (Jusko and Levy, 1967). When riboflavin or FMN is given orally with food, the absorption of the vitamin is increased. The effect of food probably involves a

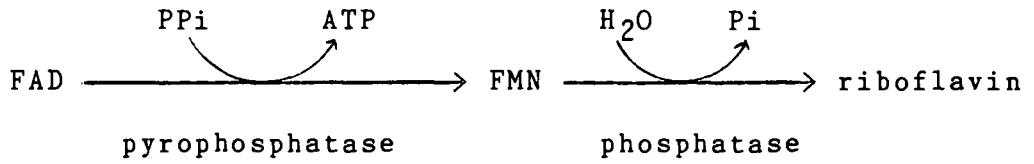
decreased rate of intestinal transit, which increases the degree and duration of vitamin exposure to the absorption site (Rivlin, 1984). Bile salts also increase the absorption of riboflavin (Mayersohn, Feldman and Gibaldi, 1969)

Once riboflavin has been absorbed, about one-half of the riboflavin and 80% of the FMN are bound to proteins, primarily to albumin (Jusko and Levy, 1975). The binding of FMN and riboflavin to albumin involves hydrogén bonding which is weak. In tissues, however, the coenzyme forms of riboflavin tend to be tightly bound to a large number of apoenzymes.

Riboflavin is found in almost all tissues, principally in the form of its coenzymes (McCormick, 1975). The liver, kidney, and heart have the highest concentration of this vitamin: 70 to 90 % is in the form of FAD. Free riboflavin constitutes less than 5 % of the stored flavins (Cerletti and Ipata, 1960). Riboflavin is not extensively decomposed in the body. There does not appear to be any specialized mechanism for storage of riboflavin. Excessive intake of riboflavin is excreted in the urine.

Riboflavin is excreted from the body in urine andin feces. The primary form of the vitamin in urine is almost exclusively in the form of riboflavin rather than as coenzyme derivative (Jusko and Levy, 1975). Yagi, Ohishi and

Ohkawa (1982) reported that other metabolites of riboflavin may be detected in human urine in small amounts, possibly arising from covalently bound flavins. The unbound FAD is subject to degradation (McCormick, 1975):



Rivlin (1984) proposed that the thyroid hormones exert a sensitive control over riboflavin utilization (Fig. 3). He observed that the activity of flavokinase, the rate of FMN and FAD formation, and the tissue concentration of these coenzymes and flavoproteins are reduced in the hypothyroid rat. He suggested that thyroid hormones can enhance the conversion of riboflavin to FMN and FAD by increasing the activities of flavokinase and FAD pyrophosphorylase, respectively. However, in animals that had been treated with excessive doses of thyroid hormones, the concentrations of FAD and FMN did not rise above normal levels. Adrenocorticotropic hormone (ACTH) and aldosterone also have shown to augment the formation of FMN and FAD from riboflavin in adrenal cortex, kidney and liver (Rivlin, 1984).

Requirements

Riboflavin plays a significant role in energy metabolism. For people of all ages, the daily riboflavin allowances recommended by the Food and Nutrition Board (FNB, 1980) have been based on a value of 0.6 mg of riboflavin for every 1000 kcal consumed. The RDA for men between the ages of 23 and 50 is 1.6 mg; and for women between 23 and 50 years of age, it is 1.2 mg. The need for riboflavin increases during pregnancy (+0.3 mg/day) and lactation (+0.5 mg/day). A minimum of about 1.2 mg of riboflavin per day in adults is necessary to maintain adequate body stores and urinary output, even if the caloric intake is below 2000 kcal. Recently research reported that oral contraceptive use and exercise may increase the requirement for riboflavin . This will be discussed later.

Food Sources

The best natural source of riboflavin is yeast, but it is not extensively used in the ordinary American diet. Milk and meat are the best contributors to dietary riboflavin intake in the United States. One cup of whole milk contributes approximately 0.4 mg of riboflavin, which is about 33 % and 25%, respectively, of the daily recommended allowance for riboflavin for women and men between the ages of 23 and 50 years. Rapidly growing green

vegetables (e.g. spinach, broccoli, and asparagus) are good sources; whole-grain or enriched cereal products also increase riboflavin intake. It has been estimated that milk and milk products contribute about 39 % of the riboflavin in the American diet, with meat, fish, poultry, eggs, and legumes contributing a total of about 29%. Fruits, vegetables and grain products together contribute about 31 % (Marston and Peterkin, 1980).

Riboflavin is thermostable. Ordinary cooking procedures do not affect the riboflavin content of foods. However, considerable loss may occur if foods are exposed to ultraviolet light or cooked in a large amount of water. The use of sodium bicarbonate in cooking vegetables can also destroy riboflavin.

Milling of rice and wheat results in considerable loss of riboflavin, since most of the riboflavin is in the germ and bran which are removed during this process. Polished rice contains approximately 59 % of the riboflavin originally present in brown rice (Dimler et al., 1960).

Effect of Deficiency

Since flavoproteins have a fundamental role in cellular metabolism, riboflavin deficiency is manifested in several ways. Angular stomatitis, cheilosis, glossitis, seborrheic dermatitis about the nose and scrotum are the

clinical signs associated with riboflavin deficiency (Horwitt, 1980). Additionally, certain functional and organic disorders of the eyes may result from riboflavin deficiency (Bunce, 1979). However, spotaneous deficiency is not due to riboflavin deficiency alone; it is usually accompanied by other nutrient deficiencies.

Anemia has been seen in riboflavin deficiency of man. Powers and Thurnham (1981) found that even a mild deficiency of riboflavin may interfere in iron metabolism, impair production of erythrocytes from bone marrow, depress the erythrocyte glutathione reductase activity, increase the instability of red blood cells and shorten the life span of red blood cells.

Riboflavin deficiency has been linked with esophageal cancer. Thurnham et al. (1982) reported that 97 % of the esophageal cancer patients in China had biochemical ariboflavinosis. They suggested that riboflavin deficiency may affect the integrity of esophageal epithelium, facilitating oxidative damage and /or making the tissue more vulnerable to environmental carcinogens.

Riboflavin deficiency also affects lipid metabolism. A deficiency of riboflavin prevents the beta-oxidation of fatty acids and inhibits their utilization. A recent review article (Anonymous, 1980) indicated that the decrease of fatty acid oxidation is related to the acyl-

CoA dehydrogenase activity, rather than other enzymes involved in beta-oxidation.

Assessment of Riboflavin Status

Several methods have been used to assess riboflavin status in man, including nutrition surveys, clinical signs, and biochemical methods. Nutrition surveys of riboflavin intake can provide general knowledge concerning riboflavin status, but surveys fail to provide direct information as to the body reserves and metabolic state of the vitamin in an individual. Clinical signs are difficult to assess and are often expressed only in severe deficiency. Biochemical methods are more quantitative and often can detect a deficiency before clinical signs appear. Appropriate biochemical tests for detecting deficiency of this vitamin include measuring the concentration of riboflavin in blood and urine, and the activity of erythrocyte glutathione reductase, an FAD-containing enzyme.

Urinary Riboflavin Excretion

Urinary riboflavin excretion has been commonly used as an index of the riboflavin nutritional status. The correlation between dietary riboflavin and urinary riboflavin excretion has been extensively studied in normal adults and in volunteers fed diets low in this

vitamin. Horwitt et al. (1950) reported that the urinary excretion of riboflavin dropped to approximately 50 ug/day within a few days of restriction to 0.55 mg riboflavin per day. As the riboflavin intake was increased, urinary riboflavin excretion also increased. He suggested that urinary excretions of less than 100 ug riboflavin in 24 hours reflected an inadequate recent intake of the vitamin, and urinary excretion below 50 ug riboflavin a day was a strong evidence that the individual had been on a diet deficient in riboflavin for some time.

The urinary excretion of riboflavin has limited use as an indicator of deficiency. It is affected by kidney function and tends to reflect current riboflavin intake rather than flavin stores. Further, it does not measure the severity of the vitamin deficiency in the absence of clinical symptoms. The amount of riboflavin excreted in the urine may be temporarily and misleadingly increased by the consumption of a small amount of a riboflavin-rich food. It may also vary with the time of sampling when random urine samples are used, nitrogen balance and dietary creatinine when expressing urinary data on the basis of creatinine excretion. Tucker et al. (1960) reported that sleep and short periods of heavy physical work decrease riboflavin excretion, while enforced bed rest and heat stress increase riboflavin excretion.

Erythrocyte Total Riboflavin Levels

Erythrocytes are presumably a storage site of riboflavin and, therefore, are not affected by current dietary intake (Cooperman and Lopez, 1984). The measurement of erythrocyte total riboflavin levels has been proposed as a method to evaluate riboflavin status. Bessey, Horwitt and Love (1956) reported that total riboflavin content of erythrocytes decreases significantly under conditions of restricted riboflavin intake. The normal value of erythrocyte total riboflavin is greater than or equal to 15 ug per 100 ml of packed erythrocytes. Concentrations of less than 10 ug total riboflavin per 100 ml packed erythrocytes are indicative of deficiency (Table 2). Bessey et al. (1956) also pointed out that erythrocyte total riboflavin levels do not change until after other riboflavin indices have been altered. In addition, the differences between levels of normal subjects and riboflavindeficient subjects were small (no greater than two-fold).

Neither plasma nor whole blood riboflavin levels can be used as a practical index for evaluating status of humans with regard to this nutrient. Plasma levels of riboflavin tend to be variable and reflect current intake. The riboflavin content of whole blood will vary with the proportion of erythrocytes to plasma, as well as with the nutritional status of the individual.

Table 2. Laboratory assessment of riboflavin (B-2) status*

	Measurement methods		
	Erythrocyte B-2 levels	Urinary B-2 levels	Erythrocyte glutathione reductase activity
Normal value	≥ 15 µg B-2/dl packed rbc	≥ 80 µg B-2 /g creatinine ≥ 120 µg B-2/ 24 hr	EGRAC < 1.2**
Deficient value	< 10 µg B-2/dl packed rbc	< 27 µg B-2/g creatinine < 40 µg B-2/ 24 hr	EGRAC ≥ 1.2
Comment	not very sensitive	subject to changes due to age, pregnancy, nitrogen balance, and exercise.	not subject to age or other related alteration

* Adapted from Selhub and Rosenberg (1984) and Sauberlich, Skala and Dowdy (1974).

** EGRAC represents erythrocyte glutathione reductase activity coefficient.

Erythrocyte Glutathione Reductase Activity Assay

Another biochemical method for evaluating riboflavin status in man is based on the change in the activity of erythrocyte glutathione reductase (GR; NADPH:glutathione oxidoreductase E.C.1.6.4.2.), one of two flavoproteins in the red cell requiring FAD. The other FAD-containing coenzyme in human red cells is NADH-dependent "diaphorase", but this system has not been applied for analytical work. A direct estimation of protein-bound riboflavin reflects the metabolic utilization of the vitamin and not dietary intake. Further, protein-bound riboflavin is not mobilized until the supply of riboflavin in the blood is depleted (Tillotson and Sauberlich, 1971).

Glutathione reductase (GR) is a key enzyme in the regulation of the hexose monophosphate pathway in erythrocytes. GR catalyzes the reduction of oxidized glutathione (GSSG) with the concomitent oxidation of NADPH to NADP, which provides stimulus for the pentose phosphate pathway (Fig. 4). The primary function of GR is to maintain a low level of GSSG and high level of reduced glutathione (GSH) in the cells. GSH protects the red cells from stress of oxidation by removing lipid peroxides and H_2O_2 through the mediation of glutathione peroxidase. Reduced glutathione is also required by erythrocytes for maintaining sulhydryl-containing enzymes in the active

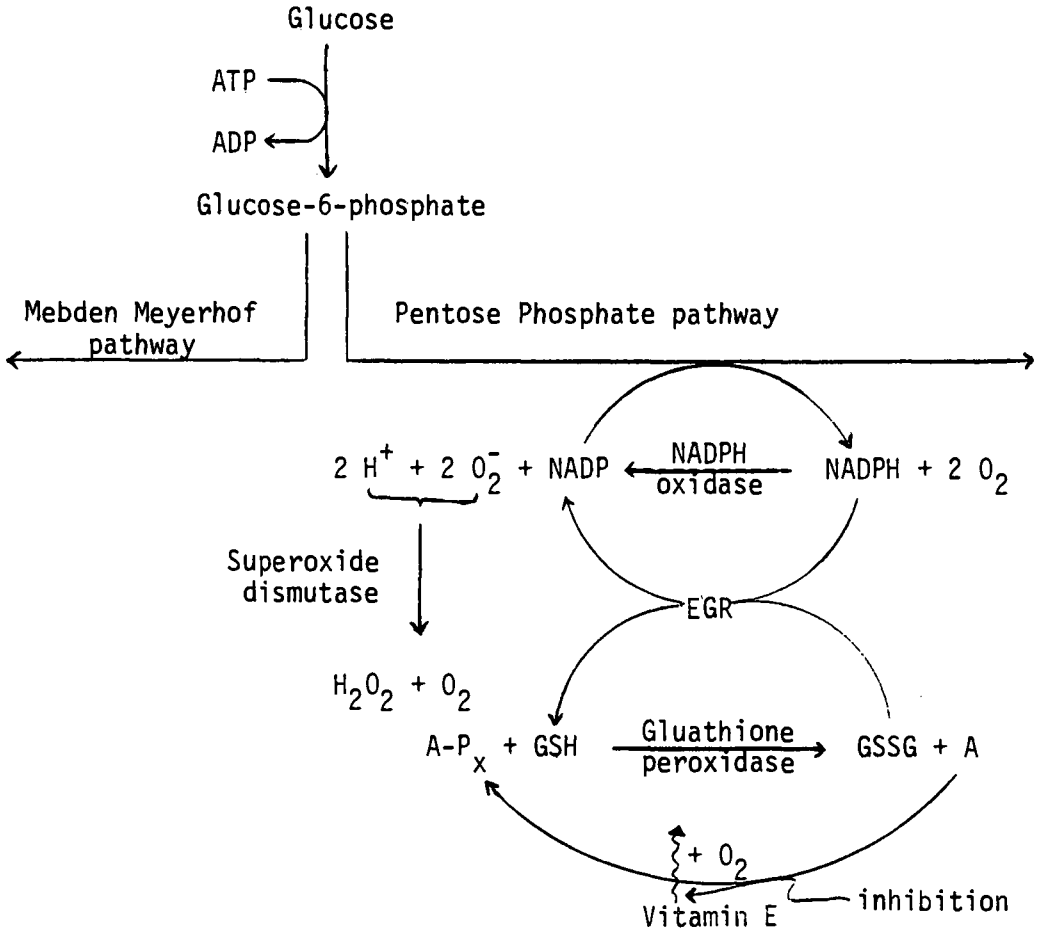


Fig. 4. Role of EGR in erythrocyte metabolism. A-P_x represents peroxide for A (e.g. fatty acid peroxide, hydrogen peroxide) (adapted from Varma et al., 1983).

state, and maintaining hemoglobin in its native form. A reduced efficiency of GR might lead to an accumlation of NADPH , which may be channeled into the superoxide pathway and increased H_2O_2 production. Further, a decrease in GR activity may impair the stability of red cells and shorten their life span (Fig. 4) (Varma et al., 1983).

The erythrocyte glutathione reductase (EGR) activity assay can be readily performed spectrophotometrically, requiring only a small quantity of blood. The activity of EGR is altered in vivo by riboflavin intake and in vitro by FAD (Beutler, 1969). The degree of in vitro stimulation by FAD is dependent on the FAD saturation of the enzyme, which in turn is dependent on the availability of riboflavin. The assay results are usually expressed in terms of "erythrocyte glutathione reductase activity coefficient" (EGRAC). It is calculated as follows:

$$\text{EGRAC} = \frac{\text{GR activity with FAD added in vitro}}{\text{GR activity without FAD added in vitro}}$$

Icen (1967) and Staal et al. (1969) demonstrated that EGR is not saturated in respect to FAD at anyone time. Tillotson and Baker (1972) reported that a relatively constant percentage of FAD stimulation of the EGR was found in humans receiving adequate amount of riboflavin.

An EGRAC greater than 1.2 may be suggestive of possible riboflavin deficiency based on controlled human riboflavin deficiency studies (Glatzle et al., 1970). However, an absolute cut-off level for evidence of a riboflavin deficiency has not been defined. The summary of laboratory assessments of riboflavin status is presented in Table 2.

The method described above has been shown to be a useful and sensitive method for evaluating the riboflavin status in man (Tillotson and Baker, 1972; Bamji, 1970). EGRAC can identify individuals receiving a marginal amount of riboflavin as well as individuals suffering from severe ariboflavinosis. Recently, Thurnham (1981) indicated that erythrocyte glutathione reductase is the first tissue enzyme to be affected by riboflavin depletion. Prentice and Bates (1981a,b) have used a chronically marginally riboflavin deficient rat as a model to evaluate the EGRAC as a measurement of riboflavin status. They found that EGRAC was correlated strongly with nearly all the riboflavin sensitive variables measured, including hepatic and renal riboflavin concentrations. These authors suggested the EGRAC test as a primary index of riboflavin status.

Measurements of the EGRAC are not influenced by sex, age, or dietary protein (Glatzle et al., 1970; Tillotson and Baker, 1972). At present the EGR activity is the most commonly used method to evaluate riboflavin status in

humans, including oral contraceptive users, (Roe et al., 1982; Carrigan, Machinist and Kershner, 1979; Newman et al., 1978), pregnant and lactating women (Bates et al., 1982), adolescents (Lopez, Schwartz and Cooperman, 1980; Ajayi and James, 1984; Sauberlich et al., 1972), during exercise and weight loss in women (Belko et al., 1983; Belko et al., 1984), and aged (Garry et al., 1982). However, EGR activity is affected by certain diseases, e.g. glucose-phosphate dehydrogenase deficiency (Schrier et al., 1958), and diabetes mellitus (Long and Carson, 1961), etc. Special precautions should be taken in interpreting the results.

Factors that Influence Riboflavin Status

Ariboflavinosis can be caused by insufficient intake or by certain metabolic disorders. Those metabolic disorders include 1) inhibition of riboflavin absorption; 2) inhibition of the conversion of riboflavin to the coenzymes; 3) inhibition of the formation of holoenzymes; 4) inhibition of enzyme reaction; and 5) increased catabolism of riboflavin (Yagi et al., 1982; Komindr and Nichoalds, 1980).

Low urinary riboflavin levels, low EGR basal activity and elevated EGRAC have been reported in women taking oral contraceptives (OC). This is especially true for those with longer duration of drug usage (Briggs and

Briggs, 1974; Newman et al., 1978; Rivlin, 1979). These findings suggest a possible increased requirement for riboflavin during OC usage. However, Carrigan et al. (1979) found no significant difference in riboflavin status between OC users and non-users. Further, Roe et al. (1982) using an experimentally controlled diet demonstrated that OC do not significantly influence riboflavin status. A definitive statement about OC cannot be made at the present time. The data are insufficient to suggest an increased dietary allowance (FNB, 1980).

Recently, Belko et al. (1983) reported that exercise increased the riboflavin requirement in young normal weight women, which was contrary to results obtained in many previous studies on riboflavin requirements. The authors used EGRAC test as the index of riboflavin status and the riboflavin requirements were defined as the intake of riboflavin needed to achieve any EGRAC within the normal range (< 1.2). Based on their study, young women who were not participating in regular exercise programs required a riboflavin intake of 0.96 mg / 1000 Kcal and those who were exercising regularly required a level of 1.1 mg / 1000 kcal. However, Horwitt (1984) thought that Belko and co-workers misinterpreted data from their experiment. He proposed that riboflavin requirement is not directly related to caloric consumption or exercise, but rather to growth and tissue maintenance.

The status of riboflavin nutriture in the elderly person appears to be highly variable (Alexander et al., 1984). In the study of the riboflavin status of institutionalised and non-institutionalised aged, Vir and Love (1977) found that the highest incidence of vitamin B-2 deficiency was noted in the subjects of sheltered dwelling and subjects living in their own homes but not taking multivitamin supplements. The authors suggested that regular vitamin B-2 supplementation may be of benefit to the aged. By contrast, riboflavin deficiency was only rarely observed by Rutishauser et al. (1979) and Garry et al. (1982) through dietary assessmesnt with EGRAC measurement. There is no evidence that aging affects riboflavin metabolism. That the riboflavin deficiency occurs among the aged is probably due to the inadequate riboflavin intake.

MATERIALS AND METHODS

This study was approved by the Human Subjects Committee at Oregon State University. An informed consent form approved by this committee was signed by each of the subjects before participating in this study.

Subjects

Sixteen Orientals (9 males and 7 females) and 5 Caucasians (2 males and 3 females) between the ages of 23 to 34 years, participated as subjects in this project. They are described in Table 3. The Oriental subjects, who came from Taiwan, R. O. C., were recruited from among the Chinese students and their spouses. They lived off the Oregon State University campus. The Caucasian subjects were also recruited from among the Oregon State University student population.

All subjects except subject 9 were OSU students. The descriptive data for all of the subjects are shown in Table 3. According to the values for hematocrit and hemoglobin, the general health status of the Oriental and Caucasian subjects was good. All subjects were within the acceptable limits (Sauberlich et al., 1974).

The subjects had no current liver, kidney, or any hormonal disease, and did not take any prescribed medications (except an oral contraceptive by no C2) as

Table 3. Description of subjects.

Group	Subject no	Age yr	Height cm	Weight kg	Hematocrit BS* %	Hematocrit AS* %	Hemoglobin BS g/dl	Hemoglobin AS g/dl
Oriental Male	2	27	170.0	70.0	52.0	51.3	17.7	17.3
	3	29	170.0	71.0	48.8	49.0	17.4	17.4
	6	33	170.0	65.0	44.0	47.0	15.0	16.0
	8	26	178.0	64.1	49.8	51.3	17.0	17.9
	10	30	163.0	70.0	47.5	47.4	15.7	15.8
	11	27	175.3	65.9	50.3	49.3	17.3	16.7
	12	24	168.0	65.0	48.2	46.3	16.3	15.8
	14	34	167.6	65.9	54.1	51.4	18.7	17.7
	19	32	172.7	68.6	46.9	47.0	16.6	16.3
Oriental Female	1	25	150.0	36.0	43.0	43.3	14.7	14.9
	4	24	157.0	50.9	42.5	43.5	15.2	15.7
	5	23	163.0	50.0	43.0	45.0	13.8	14.9
	7	30	155.0	49.0	39.3	39.9	13.0	13.4
	9	28	157.5	50.0	45.0	44.3	16.3	15.9
	13	26	160.0	46.4	41.0	43.0	13.9	15.0
	20	29	163.0	54.0	43.0	40.0	14.8	13.4
Caucasian Male	C1	23	180.3	64.9	46.4	47.0	16.2	16.5
	C4	32	182.9	81.8	46.5	50.0	16.3	17.8
Caucasian Female	C2	25	170.2	70.5	41.8	42.8	14.2	15.2
	C3	24	160.0	53.1	40.3	41.5	13.5	14.4
	C5	33	152.4	63.5	38.8	39.3	13.0	13.6

* Blood samples were collected before (BS) and after (AS) supplementation with 10 mg riboflavin daily for 7 days.

determined by a questionnaire. Subject C2 was using the oral contraceptive, Ortho-Novum 7/7/7/ (Ortho Pharmaceutical Corporation, Raritan, New Jersey). None of the woman subjects were pregnant. Out of 21 subjects, 7 reported having used a vitamin supplement occasionally before this study. All subjects except subjects nos. 3 and C4 discontinued use of their vitamin supplement at least 2 weeks before this investigation. No. C4 discontinued use of the vitamin supplement 3 days before the 7-day riboflavin supplementation period and no. 3 discontinued at the first day. All subjects took only the 10 mg riboflavin supplement daily provided by the investigator during the supplementation period.

Experimental Protocol

This study included a 3-day dietary record kept by the subjects (days 1-3, from Sunday through Tuesday), and a 7-day riboflavin supplementation period (days 4-10, from the following Wednesday through the next Tuesday) during which each subject received 10 mg of riboflavin (10 mg riboflavin per tablet, Eli Lilly and Company, Indianapolis, IN) orally each morning with breakfast. The subjects were reminded by telephone to take their supplements. All subjects reported that they followed directions. Blood (10 ml) was drawn from fasting subjects between 8:00 and 8:30 AM before (day 4) and after (day 11)

riboflavin supplementation. Erythrocyte total riboflavin, erythrocyte glutathione reductase activity and erythrocyte glutathione reductase activity coefficient, as well as hemoglobin and hematocrit were measured in the blood drawn before and after riboflavin supplementation.

The subjects recorded their dietary intake so that their daily dietary nutrient intake could be estimated. All subjects were informed of the importance of accurate dietary histories. They were given written and verbal instructions on how to record their diets. No effort was made to regulate the dietary intake of the subjects and each was instructed to continue his/her normal diet during this period of investigation. Subject no. C5 recorded her dietary history 4 days after the end of riboflavin supplementation period.

Blood Collection

Blood was drawn from the antecubital vein into heparinized evacuated tubes by a registered medical technologist. After centrifugation at 4°C, plasma and the buffy coat were removed from the erythrocytes. The erythrocytes subsequently were washed three times with ice-cold saline (0.85% NaCl). The packed erythrocytes were dispensed into 0.3 ml aliquots and stored at -15°C until assayed for total erythrocyte riboflavin and erythrocyte glutathione reductase activity. Hematocrit

and hemoglobin were determined in whole blood before centrifugation. Hemoglobin was also measured in red cell hemolysates.

Hemoglobin and Hematocrit

Hemoglobin was determined by the cyanomethemoglobin method (Richard, 1965). Hematocrit was determined by the procedure described by Richterich (1968).

Total Erythrocyte Riboflavin

Total erythrocyte riboflavin content was determined by a fluorometric method which was adapted from the procedures by Beutler (1969) and Burch, Bessey and Lowry (1948) (Appendix A). The assay was based on several factors: 1) the fluorescence of riboflavin in solution is proportional to concentration; 2) both coenzymatic forms of riboflavin, FMN and FAD, are bound to protein and are liberated by treatment with dilute trichloroacetic acid; 3) FAD is completely hydrolyzed to FMN in trichloroacetic acid after 20 hours at 37°C; and 4) at neutrality, riboflavin and FMN have the same fluorescence per mole. Measurements of total erythrocyte riboflavin content were made within two weeks following collection of blood. Data were expressed as ug total riboflavin per 100 ml red blood cells.

Erythrocyte Glutathione Reductase (EGR) Activity and Erythrocyte Glutathione Reductase Activity Coefficient (EGRAC)

EGR activity in red cell hemolysates was measured by a modification of the method of Bayoumi and Rosalki (1976) (Appendix B). This enzyme, which has an FAD prosthetic group, catalyzes the reduction of oxidized glutathione (GSSG) with the simultaneous oxidation of nicotinamide-adenine dinucleotide phosphate (NADPH):

$$GSSG + NADPH + H^+ \longrightarrow 2\ GSH + NADP^+$$

EGR activity is determined by the rate of NADPH oxidation, which is measured at 340 nm. EGR activity was expressed as International Units per g of hemoglobin in the hemolysate. EGRAC represents the degree of stimulation resulting from the in vitro addition of FAD. It is calculated from the ratio of the enzyme activity measured with FAD added in vitro to the enzyme activity measured without added FAD.

Dietary Analysis

The estimated daily nutrient intake was calculated by computer using the 1981 Ohio State University Nutrient Database (Schaum, Mason and Sharp, 1973) and other sources of nutrient information described by Sanders (1985). Nutrient values which were missing from the database were

also obtained from Leung, Butrum and Chang (1972).

Statistical Analyses

The data were statistically analyzed by using Statistical Interactive Programming System (SIPS) (Nie et al., 1975). The relationship between subjects' daily riboflavin intake and biochemical riboflavin status was determined by using correlation coefficients. The effects of the riboflavin supplement on biochemical riboflavin status, the difference in biochemical riboflavin status between Orientals and Caucasians, as well as difference between male and female subjects were also tested by paired and unpaired t-tests (Snedecor and Cochran, 1980). It was assumed that the variances of the Orientals vs. the Caucasians and the males vs. the females are equal. A probability (P) value of 0.05 or less was considered statistically significant.

RESULTS

Nutrient Intakes Based on 3-day Dietary Records

The dietary intake and riboflavin supplement of each subject at the beginning of the study are represented in Table 4. Based on body weight, the average caloric intakes of the four groups of subjects were adequate or slightly higher than the RDA for their ages (FNB, 1980). All of the subjects received adequate protein intakes; their protein intakes were above the RDA for the mixed proteins of the United States diet (0.8 g/kg of body weight per day). The protein intakes of all subjects ranged from 0.83 g/kg of body weight to 2.42 g/kg of body weight. The contribution of the major food groups to the total protein intake by the subjects is given in Table 5.

In this study all of the Caucasian subjects, 5 of the 9 Oriental males and 5 of the 7 Oriental females consumed an amount of riboflavin from food sources which was equal to or greater than the RDA for the ages of 23 to 50 (male 1.6 mg/day, female 1.2 mg/day). Riboflavin intake of the subjects who consumed less than the RDA ranged from 1.21 to 1.55 mg/day for Oriental males, and ranged from 0.95 to 0.98 mg/day for Oriental females. No one had less than two-thirds of the RDA.

The Food and Nutrition Board (1980) suggests that 0.6 mg of riboflavin be consumed for every 1000 kcal.

Table 4. Mean daily dietary intakes of energy, protein and riboflavin (B-2)* based on a 3-day dietary record, and personal B-2 supplement before the riboflavin supplementation period.

Subject		Energy	Energy / body wt	Protein	Protein / body wt	B-2	B-2 / energy	B-2 supplement**
sex	no	kcal	kcal / kg	g	g / kg	mg	mg / 1000 kcal	
Oriental								
male	2	2168	31.0	58	0.83	1.21	0.56	N
	3	2420	34.1	90	1.27	1.30	0.54	X
	6	2209	34.0	94	1.45	1.98	0.90	O
	8	2578	40.3	104	1.63	3.45	1.34	N
	10	2683	38.3	96	1.37	1.55	0.59	N
	11	3096	46.9	148	2.24	3.33	1.09	N
	12	1930	29.7	64	0.98	1.50	0.78	N
	14	2303	34.9	94	1.42	1.95	0.85	N
	19	2758	40.6	94	1.38	2.82	1.02	O
Average		2461	36.6	94	1.40	2.12	0.85	
Oriental								
female	1	1722	47.8	87	2.42	1.49	0.87	N
	4	2012	39.5	79	1.55	1.53	0.76	N
	5	1535	30.7	59	1.18	0.95	0.62	N
	7	1820	37.1	73	1.49	1.60	0.88	O
	9	1833	36.7	85	1.70	1.54	0.84	N
	13	1396	30.4	71	1.54	0.98	0.70	O
	20	2324	43.0	78	1.44	1.77	0.76	O
Average		1806	37.9	76	1.62	1.41	0.78	
Caucasian								
male	C1	3379	52.0	143	2.20	2.69	0.80	N
	C4	2895	35.3	122	1.49	3.23	1.12	Y
Average		3137	43.7	133	1.85	2.96	0.96	
Caucasian								
female	C2	1242	17.7	90	1.29	1.43	1.15	N
	C3	1826	34.5	76	1.43	1.96	1.07	N
	C5	2386	37.9	96	1.52	2.40	1.01	N
Average		1818	30.0	87	1.41	1.93	1.08	

* Amonut of riboflavin in supplement was not included in dietary intake.

** N: No
O: Occasionally. Discontinued vitamin supplement at least 2 weeks before the supplementation period.
X: Occasionally. Discontinued vitamin supplement at the first day of the supplementation period.
Y: Occasionally. Discontinued vitamin supplement 3 days before the supplementation period.

Table 5. Contribution of major food groups to the protein and riboflavin intakes of Oriental and Caucasian subjects.

	Oriental	Caucasian
Protein		
milk and milk products	11%	17%
meat, fish, poultry, eggs, and legumes	60%	57%
grain products	20%	14%
fruits and vegetables	8%	11%
miscellaneous items	1%	1%
Riboflavin		
milk and milk products	24%	32%
meat, fish, poultry, eggs, and legumes	31%	21%
grain products	22%	23%
fruits and vegetables	20%	21%
miscellaneous items	3%	3%

Only 3 of the 9 Oriental males consumed slightly less 0.6 mg of riboflavin per 1000 kcal with range from 0.54 to 0.59 mg/1000 kcal. All of the Caucasian subjects received more than the RDA for riboflavin. The contribution of major food groups to riboflavin intake for the two races is also presented in Table 5.

Biochemical Riboflavin Status and the Effect of the Riboflavin Supplement

Erythrocyte glutathione reductase (EGR) activity and erythrocyte total riboflavin (RBC B-2) levels in the Oriental subjects before and after the treatment with 10 mg riboflavin daily for 7 days are shown in Tables 6 and 7, respectively, and are summarized in Table 8. Since basal EGR activity, FAD stimulated EGR activity, EGRAC, and RBC B-2 levels were not significantly different between male and female subjects, all of the Oriental subjects are considered together unless it was necessary to distinguish between the data from the males and females.

After only one week of supplementation with 10 mg riboflavin daily, the mean values of basal EGR activity, FAD stimulated EGR activity and RBC B-2 levels increased significantly (all $P < 0.01$). Concomitantly, the EGRAC values decreased significantly after the treatment with riboflavin ($P < 0.01$). It should be noted that 5 of the 9 Oriental males and 2 of the 7 Oriental females, who had

Table 6. Erythrocyte glutathione reductase (EGR) activity and erythrocyte glutathione reductase activity coefficient (EGRAC) in Oriental subjects before (BS) and after (AS) the supplementation with 10 mg riboflavin daily for 7 days.

Subject		EGR basal activity *,**			FAD stimulated EGR activity ***			EGRAC ****	
Sex	no	(BS)	(AS)	$\frac{(AS)}{(BS)}$ #	(BS)	(AS)	$\frac{(AS)}{(BS)}$	(BS)	(AS)
Male	2	4.15	7.28	1.75	5.24	7.50	1.43	1.26	1.03
	3	7.50	7.78	1.04	7.60	8.01	1.05	1.01	1.03
	6	4.53	6.65	1.47	5.17	6.98	1.35	1.14	1.05
	8	5.15	5.65	1.10	5.88	5.78	0.98	1.14	1.02
	10	5.35	7.75	1.45	7.18	8.25	1.15	1.34	1.06
	11	4.71	6.02	1.28	5.89	6.26	1.06	1.25	1.04
	12	5.10	6.82	1.34	6.64	7.10	1.07	1.30	1.04
	14	6.51	7.62	1.17	7.79	7.66	0.98	1.20	1.00
	19	4.86	5.98	1.23	5.34	6.15	1.15	1.10	1.03
X ± SD		5.32 ±1.05	6.84 ±0.82	1.31 ±0.22	6.30 ±1.03	7.08 ±0.86	1.14 ±0.16	1.19 ±0.11	1.03 ±0.02
Female									
	1	3.86	6.00	1.55	4.59	6.18	1.35	1.19	1.03
	4	4.98	6.97	1.40	5.24	6.95	1.33	1.05	1.00
	5	5.96	7.46	1.25	7.11	7.79	1.10	1.19	1.04
	7	4.89	6.80	1.39	5.92	6.70	1.13	1.21	0.98
	9	5.95	6.62	1.11	6.64	6.62	1.00	1.12	1.00
	13	6.47	7.78	1.20	6.97	7.97	1.14	1.08	1.02
	20	4.81	6.57	1.37	6.33	7.23	1.14	1.31	1.10
X ± SD		5.27 ±0.90	6.89 ±0.59	1.32 ±0.15	6.11 ±0.93	7.06 ±0.65	1.17 ±0.13	1.16 ±0.09	1.02 ±0.04

* EGR activity was expressed as International Units per g of hemoglobin.

** EGR basal activity represented the EGR activity without FAD added in vitro.

*** FAD stimulated EGR activity represented the EGR activity with FAD added in vitro.

**** EGRAC represented the degree of stimulation resulting from the in vitro addition of FAD.

\# in vivo stimulation due to riboflavin supplement.

Table 7. Erythrocyte total riboflavin in Oriental subjects before (BS) and after (AS) the supplementation with 10 mg riboflavin daily for 7 days.

Group	Subject	RBC total riboflavin (BS)	(AS)	(AS)/(BS) *
	no	µg/dl rbc	µg/dl rbc	
Male	2	7.96	9.00	1.13
	3	9.26	10.50	1.13
	6	7.86	9.91	1.26
	8	9.13	9.93	1.09
	10	8.26	12.05	1.46
	11	7.91	9.32	1.18
	12	8.30	11.02	1.33
	14	8.44	9.40	1.11
	19	8.43	9.13	1.08
X ± SD		8.39±0.05	10.03±1.00	1.20±0.13
Female	1	7.77	9.98	1.28
	4	8.11	9.56	1.18
	5	8.23	10.40	1.26
	7	9.03	12.18	1.35
	9	8.03	9.66	1.20
	13	9.48	11.35	1.20
	20	8.27	10.57	1.28
X ± SD		8.40±0.61	10.53±0.95	1.25±0.06

* degree of increase due to riboflavin supplementation.

Table 8. A summary of biochemical riboflavin status in male and female Oriental subjects before (BS) and after (AS) the supplementation with 10 mg of riboflavin daily for 7 days

	Male (n=9)	Female (n=7)	All (n=16)
EGR basal activity *,** U/g Hb			
(BS)	5.32±1.05 #	5.27±0.93	5.30±0.95
(AS)	6.84±0.82	6.89±0.59	6.86±0.71
P value	< 0.01	< 0.01	< 0.01
FAD stimulated EGR activity *** U/g Hb			
(BS)	6.30±1.03	6.11±0.09	6.22±0.96
(AS)	7.08±0.86	7.06±0.65	7.07±0.75
P value	< 0.05	< 0.01	< 0.01
EGRAC ****			
(BS)	1.19±0.11	1.16±0.09	1.18±0.10
(AS)	1.03±0.02	1.02±0.04	1.03±0.03
P value	< 0.01	< 0.01	< 0.01
Total riboflavin in rbc µg/dl rbc			
(BS)	8.39±0.05	8.42±0.61	8.40±0.53
(AS)	10.03±1.00	10.53±0.95	10.25±0.98
P value	< 0.01	< 0.01	< 0.01

* EGR: erythrocyte glutathione reductase, expressed as International Units per g of hemoglobin.

** EGR basal activity represented the EGR activity without FAD added in vitro.

*** FAD stimulated FAD activity represented the EGR activity with FAD added in vitro.

**** EGRAC: erythrocyte glutathione reductase activity coefficient, represented the degree of stimulation resulting from the in vitro addition of FAD.

\# Mean ± SD

inital (before supplementation) EGRAC value greater than or equal to 1.20 (range 1.20 - 1.34), had EGRAC value less than or equal to 1.10 (range 1.00 - 1.10) after the administration of riboflavin. Subject no.3, who had still taken a vitamin supplement during the period of recording diets, had a very low initial EGRAC value (1.01) and supplemented EGRAC value (1.03).

The relationships of the initial individual EGRAC values and the effect of the riboflavin supplementation on the degree of increase of RBC B-2 levels and in vivo stimulation of EGR basal activity (value before supplementation / value after supplementation) in the individual subjects were also studied. It is of interest that a positive correlation ($r = 0.60$) was found between the magnitude of increase in RBC B-2 levels and the initial EGRAC value in the individuals ($P < 0.05$). This finding indicates that the degree of increase of RBC B-2 levels in response to riboflavin supplementation was greater in the subjects who had a higher initial EGRAC value. Further, a similar situation was found between the in vivo stimulation of basal EGR activity and the initial EGRAC values ($r = 0.50$, $P < 0.05$). However, no significant correlation was observed between the percentage of FAD stimulated EGR activity and the initial EGRAC value ($r = 0.03$).

Relationship Between RBC B-2 Levels and EGR Activity

The regression of basal EGR activity, FAD stimulated activity and EGRAC on RBC B-2 levels has been calculated from data obtained before and after riboflavin supplementation ($n = 32$). In this case of the 16 Oriental subjects, a significant, positive correlation with a coefficient of 0.74 was found between basal EGR activity and RBC B-2 levels ($P < 0.01$). A similar result was found between FAD stimulated EGR activity, with a correlation coefficient of 0.58 ($P < 0.01$), while a negative correlation was obtained between EGRAC and RBC B-2 levels ($r = -0.64$, $P < 0.01$).

Relationship Between Basal EGR Activity and EGRAC

The relationship between basal EGR activity and $EGRAC_S$ in the Oriental subjects was investigated and a significant negative correlation with coefficient of -0.69 was found ($P < 0.01$) (Fig. 5). It is apparent that higher EGRAC were associated with lower basic enzyme activity both before and after riboflavin supplementation.

Relationship Between Dietary Intake of Riboflavin and Biochemical Riboflavin Status

Since the energy intake varied with the sex of the subjects, the relationship between dietary riboflavin intake and biochemical riboflavin status was considered seperately in male and female Orientals and in all

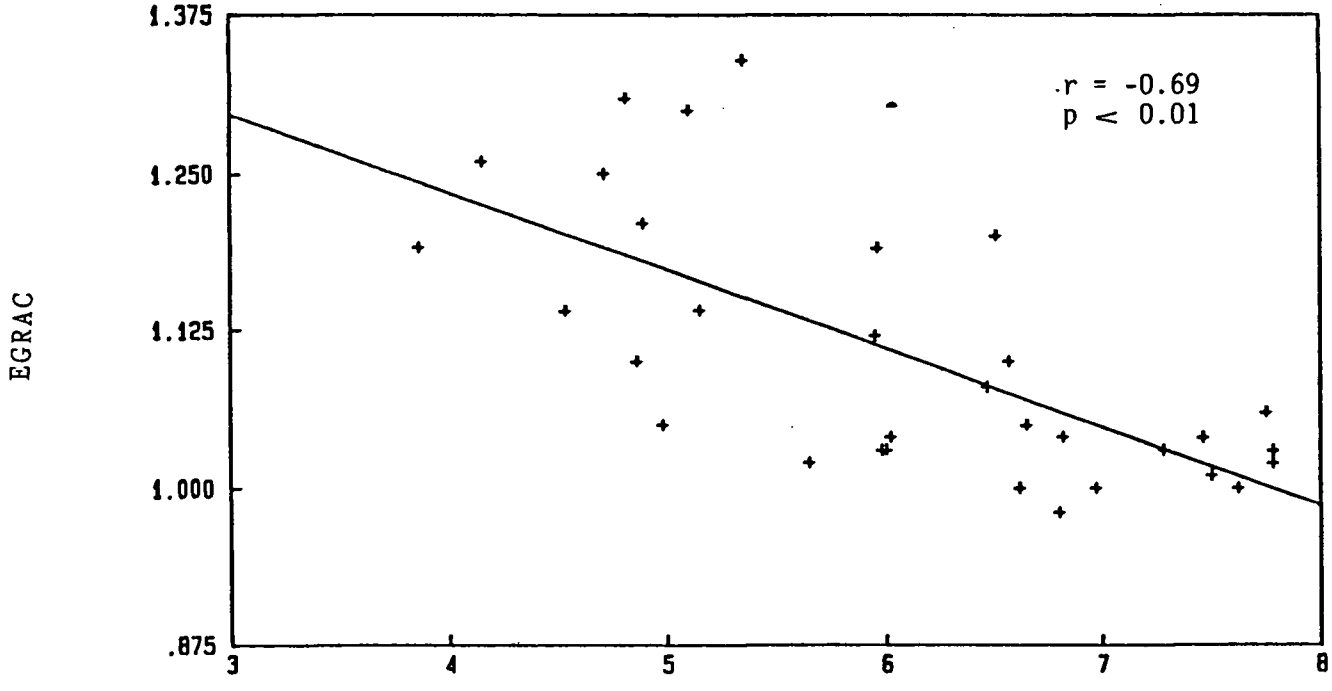


Fig. 5. Scatter diagram representing the correlection between erythrocyte glutathione reductase (EGR) basal activity and erythrocyte glutathione reductase activity coefficient (EGRAC).

Orientals. No significant correlation were found between dietary riboflavin intake, whether expressed as mg/day or mg/1000kcal, and any of the four biochemical measurements of riboflavin status.

Riboflavin Status in Caucasian vs. Oriental Subjects

The comparison of biochemical indices of riboflavin status between Oriental and Caucasian subjects before and after the supplementation with 10 mg of riboflavin daily for 7 days is shown in Table 9. There was no significant difference between these two groups in the initial state. The only exception was the RBC B-2 levels after the supplementation ($P < 0.05$) which may be due to the slightly heigher initial concentration of riboflavin in erythrocytes of the Caucasians.

Table 9. Biochemical riboflavin status before (BS) and after (AS) the supplementation with 10 mg of riboflavin daily for 7 days in Oriental vs. Caucasian subjects.

	Oriental (n=16)	Caucasian (n=5)	Statistical significance
EGR basal activity *,** U/g HB			
(BS)	5.30±0.95#	5.80±1.67	NS
(AS)	6.86±0.71	6.86±1.30	NS
FAD stimulated EGR activity *** U/g Hb			
(BS)	6.22±0.96	6.70±1.36	NS
(AS)	7.07±0.75	7.22±1.14	NS
EGRAC ****			
(BS)	1.18±0.10	1.26±0.13	NS
(AS)	1.03±0.03	1.06±0.07	NS
Total riboflavin in rbc µg/dl rbc			
(BS)	8.40±0.53	9.29±2.12	NS
(AS)	10.25±0.98	11.78±2.04	$P < 0.05$

* EGR: erythrocyte glutathione reductase, expressed as International Units per g of hemoglobin.

** EGR basal activity represented the EGR activity without FAD added in vitro.

*** FAD stimulated EGR activity represented the EGR activity with FAD added in vitro.

**** EGRAC: erythrocyte glutathione reductase activity coefficient, represented the degree of stimulation resulting from in vitro addition of FAD.

\# Mean ± SD

DISCUSSION

Nutrient Intakes

According to the recommended dietary allowances (FNB, 1980), all race and sex groups had adequate mean levels of energy, protein and riboflavin in their diets. Although most of the dietary riboflavin in United States is supplied by dairy products, meat, fish, eggs and legumes (Marston and Peterkin, 1980), the present study shows that grain products, fruits and vegetables also contribute significant amount of riboflavin to the diets of the subjects in the present study (Table 5). In this study the five Caucasians received a third of their total riboflavin from milk and milk products, while the Orientals obtained a third of the total riboflavin from meat, fish, poultry, eggs and legumes. The Caucasians received less total protein from grains than the Orientals. However, due to the enriched ready-to-eat breakfast cereals consumed by the Caucasian but not by the Orientals, the contribution of grains and cereals to the total riboflavin intake of the two racial groups was similar.

Erythrocyte Glutathione Reductase Activity Coefficient

EGRAC, a measure of the apoenzyme by in vitro stimulation of EGR by FAD, is a useful and sensitive

method for identifying individuals receiving marginal amounts of riboflavin over prolonged peroids of time as well as individuals suffering from severe ariboflavinosis (Tillotson et al., 1972; Glatzle et al., 1970; Bamji, 1969). However, the EGRAC value used as a guide for detecting inadequate riboflavin nutriture is not completely settled at this time. The concentration of FAD added in vitro to the EGR assay affects the degree of EGR activity stimulated by the coenzyme. According to Garry et al. (1982), concentrations of FAD greater than 5 μM added in vitro to the EGR assay result in lower EGRAC than when FAD concentrations from 1 to 3 μM are used . Since in the present study, the concentration of 10 μM of FAD was used to stimulate the enzyme, (Appendix B), EGRAC values of 1.2 or above as suggested by Glatzle and co-workers (1970) were used to indicate riboflavin inadequacy. Further, EGRAC values below 1.0 are occasionally observed (Garry et al., 1982; Nichoalds, Lawrence and Sauberlich, 1974; Glatzle et al., 1970). Glatzle and co-workers (1970) chromatographed crystalline FAD and obtained at least one accompanying non-FAD unidentified compound. They suggested that this unknown compound may inhibit EGR activity. For practical purposes, Glatzle proposed that activation coefficients below 1.0 be accepted for determining riboflavin status.

Overall, 5 of the 9 Oriental males and 2 of the 7

Oriental females in the present study had initial (before supplementation) EGRAC values greater than or equal to 1.2 (range 1.20 - 1.34). Only one male Oriental and one female Oriental subjects had EGRAC values greater than 1.30. Following the supplement of 10 mg of riboflavin daily for one week, EGRAC values were reduced to 1.10, indicating high risk of marginal riboflavin deficiency in these subjects. However, 4 of the 7 subjects who had inadequate initial EGRAC values ($\geq$ 1.20) consumed diets containing more than the RDA for riboflavin (FNB 1980). It is likely that an intake of riboflavin greater than the current RDA may be needed by these subjects if their 3-day dietary records are indication of their used diets. Recently Belko et al. (1983, 1984) reported that young healthy women may require more riboflavin (0.8-1.0 mg/1000 kcal) than the current RDA to achieve an EGRAC of 1.2. Other similar statements were made by Bates et al. (1981) and Beutler (1969). Additionally, exercise may also increase the requirement for riboflavin. The subjects' physical activity was not measured in present study.

Erythrocyte Glutathione Reductase Activity, Basal and Stimulated

It is of interest that not only the EGR basal activity, but also the activity of FAD stimulated EGR was significantly ($P < 0.01$) increased after a short term of riboflavin supplementation (Table 9). A similar result

was also observed by Beutler (1969) who suggested that EGR exists in at least three forms: (a) as an active enzyme; (b) as an enzyme which can readily be activated in vitro by FAD; (c) as an enzyme which can be activated in vivo by riboflavin but which resists in vitro activation. Beutler also pointed out that the administration of riboflavin may influence the synthesis of EGR apoenzyme in the developing erythroblasts. A similar state of affairs has been shown to exist with pyridoxine intake and red cell transaminase activity (Rose et al., 1973; Brown et al., 1975). Rose et al. and Brown et al. suggested that the coenzyme stabilizes the enzyme so that there is a reduced rate of degradation.

Erythrocyte Riboflavin Levels

The values for erythrocyte riboflavin (RBC B-2) levels observed in the Orientals of this study (Table 8) are lower than the values reported by other workers. Bamji (1970) reported that the mean value of RBC B-2 was 31.63 ug/100 ml rbc for apparently healthy people, 22.82 ug/100 ml rbc for the subjects with clinical evidence of riboflavin deficiency, and 34.10 ug/100 ml rbc for these deficient subjects after treatment with 10 mg of riboflavin daily for 7 days. In the study by Bessey et al. (1956), RBC B-2 levels were 20.2 to 27.6 ug/100 ml rbc in riboflavin supplemented subjects (riboflavin intake 2.55-

3.55 mg/day) and 10.0 to 13.1 ug/100 ml rbc in the deficient subjects (riboflavin intake 0.55 mg/day). The variation in RBC B-2 values obtained by these investigators and those found in the Oriental subjects in the present study can not be solely related to the race of the subjects. According to our data, the RBC B-2 levels of Orientals and Caucasians are not significantly different initially (before supplementation), although, based on a limited number of subjects, the RBC B-2 levels are slightly higher in Caucasians than in Orientals after the riboflavin administration (Table 10). The reasons for the difference in the RBC B-2 levels reported by other investigators and those obtained in the present study are not readily apparent. The marked increase in RBC B-2 levels after the treatment with riboflavin in this study is in keeping with the findings of other workers (Bamji, 1970; Bessey et al., 1956).

Correlations Within Biochemical Riboflavin Parameters

RBC B-2 level was significantly correlated with EGR basal activity ($r = 0.74$, $P < 0.01$) as well as with FAD stimulated EGR activity ($r = 0.58$, $P < 0.01$). These observations can be easily understood because 1) most of the riboflavin in erythrocytes is present as FAD (Beutler, 1969); 2) EGR basal activity depends on the FAD content of erythrocyte; 3) the coenzyme stablizes the enzyme so that

there is a reduced rate of degradation.

A significant negative correlation was obtained between EGRAC values and RBC B-2 levels ($r = -0.64$, $P < 0.01$) and between EGRAC values and EGR basal activity ($r = -0.69$, $P < 0.01$) (Fig. 5), showing that $EGRAC_S$ are inversely related to RBC B-2 levels and to EGR basal activity. Further, there were significant positive correlations between the initial EGRAC values and the effect of riboflavin supplement on the increased RBC B-2 levels ($r = 0.60$, $P < 0.05$) and in vivo stimulation of EGR basal activity ($r = 0.50$, $P < 0.05$). All of these findings indicate that the EGRAC value reflects riboflavin status of the body. These results suggest that EGRAC is sensitive and useful for evaluating riboflavin status in man. Other workers (Tillotson and Baker, 1972; Glatzle et al., 1970; Bamji, 1969) have also stated that EGRAC is a sensitive indicator of riboflavin status.

Correlation Between Riboflavin Intake and Biochemical Riboflavin Status

Dietary riboflavin intake per day or riboflavin intake in terms of energy intake did not significantly correlate with RBC B-2 levels, EGR basal activity, FAD stimulated EGR activity or EGRAC. Such results have been observed in several other studies (Rutishauser et al., 1979; Vir and Love, 1977; Buzina et al., 1971). This lack of correlation may be due to several factors. First, RBC

B-2 levels and EGR activity reflect the metabolic ultilization of riboflavin over a period of time. Absorption, renal excretion and metabolism of riboflavin (Komindr and Nichoalds, 1980; Yagi et al., 1982), and physical activity (Belko et al., 1983; Belko et al., 1984; Tucker et al., 1960) may influence the availability of and/or requirement for riboflavin, and therefore the correlation. Besides, in studies where riboflavin intake was experimentally controlled, EGRAC values differed significantly in subjects consuming the same diet (Bamji, 1969; Tillotson and Baker, 1972). Secondarily, a small number of subjects participated in the present study and a narrow range of riboflavin intake was consumed by the subjects (0.95-3.45 mg/day). Finally, limitations of food composition tables, inaccurate food records, errors in interpretation of the records, limitation of 3-day dietary records and bioavailability of riboflavin in foods may also cause unreliable data on riboflavin intake. In one study (Garry et al. 1982) reporting a highly significant correlation between the EGRAC and the total riboflavin intake ($p < 0.0001$), the intakes of riboflavin were in the range of 0.65 to 165 mg per day and the number of subjects was 270.

Although the expected relationship between biochemical riboflavin status and dietary riboflavin intake was not present in this study, the mean values of

RBC B-2 concentration, EGR basal activity, FAD stimulated EGR activity and EGRAC were significantly increased after the 7-day treatment with riboflavin. Such observations are consistent with those of several other studies where the riboflavin intake was experimentally controlled and with a step-wise increase in the subjects' riboflavin intake (Tillotson and Baker, 1972; Beutler, 1969; Bessey et al. 1956). These imply that there is significant inter-subject variability between biochemical riboflavin status and total riboflavin intake.

SUMMARY AND CONCLUSION

A combined dietary and biochemical study was carried out on 16 Oriental subjects residing off the Oregon State University campus. To evaluate the status of riboflavin nutrition and to assess the effect of riboflavin supplementation, we measured EGR activity with and without FAD added in vitro and RBC B-2 level. Riboflavin status was assessed by EGRAC. The subjects' dietary intake of riboflavin, protein and calories was estimated from their self chosen diets which were recorded for 3 days before the subjects received a supplement of 10 mg of riboflavin for 7 days. Both males and females had adequate mean levels of riboflavin, protein and calories in their diets, although 4 of the 9 males and 2 of the 7 females had a total dietary riboflavin intake slightly lower than the RDA averaged over the 3-day period. None of the subjects had intakes lower than two-thirds of the RDA. High risk of marginal biochemical riboflavin deficiency was noted in 5 of the 9 males and 2 of the 7 females by a EGRAC value greater than or equal to 1.2, suggesting that they may need intakes of riboflavin greater than the RDA to have a normal EGRAC .

After the supplement, the mean values of EGR basal activity, FAD stimulated EGR activity, RBC B-2 levels were significantly increased (all $P < 0.01$), while the mean

value of EGRAC was significantly decreased ($P < 0.01$). These observations indicate there are significant intra-relationships between the total riboflavin intake and all the four biochemical parameters measured. However, the inter-subject relationships between the dietary riboflavin intake and the biochemical riboflavin parameters were not present in this study.

Although the RBC B-2 concentrations obtained from the Oriental subjects were lower than those reported by other workers, this measurement correlated significantly with EGRAC ($r = -0.64$, $P < 0.01$), EGR basal activity $r = 0.74$, $P < 0.01$) and FAD stimulated EGR activity. The low RBC B-2 values obtained from these Oriental subjects cannot be solely related to the race of the subjects because similar results were obtained in 5 Caucasian subjects.

EGRAC was not only significantly negatively correlated with RBC B-2 levels and EGR basal activity, but was also positively correlated with the effect of riboflavin supplement on the degree of increase of RBC B-2 levels and in vivo stimulation of EGR basal activity. These indicate that any of these three biochemical measurements can be used to evaluate riboflavin adequacy in humans. However, measurement of EGRAC, which is expressed in relation to in vitro FAD stimulatory effect instead of enzyme activity, appears to be a simple,

sensitive and functional test of riboflavin status in humans. THe EGRAC test requires only a small quantity of blood and is highly reproducible.

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APPENDIX

Appendix A: Fluorometric Measurement of Total Riboflavin Content of Erythrocytes

This method is an adaptation of the procedures by Beutler (1969) and Burch et al. (1948).

Equipment:

Aminoco-Bowman spectrophotofluorometer.

Ultra-UV cuvettes, disposable, polystyrene, Elkay Product, Inc., Shrewsbury, MA.

Reagents:

Trichloroacetic acid (TCA) solution, 20%. Dissolve 20 g TCA in redistilled water; bring volume to 100 ml.

Dipotassium acid phosphate (k_2HPO_4) solution, 0.25M. Dissolve 4.356 g (as anhydrous K_2HPO_4) in redistilled water; bring volume to 100 ml.

Sodium hydrosulfite ($Na_2S_2O_4$) solution, 10%. Within 30 min. of use, dissolve 0.5 g $Na_2S_2O_4$ in 5 ml of 5% $NaHCO_3$. This reagent is kept in an ice bath to delay oxidation.

Riboflavin standard. Stock solution, 20 ug/ml. Dissolve 20 mg riboflavin (Sigma Chemical Co., St. Louis, MO, anhydrous) in 100 ml 0.01N HCl. Working solution, 20ug/100 ml. Dilute 1 ml of stock solution to 100 ml with 0.01N HCl. Prepare freshly on day of use in amber glass.

Procedure:

Use redistilled water throughout. Soak all glassware in one-half concentrated nitric acid for more than one day. Use glass graduated pipettes to measure packed erythrocytes. Work in subdued light. All measurements are run in duplicate.

1. To 1.4 ml of redistilled water, add 100 ul of packed erythrocytes. Rinse pipette several times with this solution. To precipitate protein, add 1.5 ml of 20% TCA solution and mix immediately.

2. Allow mixture to stand for 45 min at room temperature, mix again, and centrifuge for 10 min at3000 rpm.

3. Transfer 0.6 ml of TCA extract supernatant into a 3.0 ml photofluorometer cuvette and incubate at 37°C in the dark for 20 hours to hydrolyze FAD.

4. Neutralize sample with 2.4 ml of 0.25M K_2HPO_4. Cover with aluminum foil and mix by inversion. Read at 450 nm (activation) and 535 nm (fluorescence) after the lapse of one hour (R_1). Add 25 ul $Na_2S_2O_4$ to cuvette, mix by inversion and read (R_2).

R_1 = initial reading after neutralization with K_2HPO_4

R_2 = reading after addition of 25 ul 10% $Na_2S_2O_4$

Calculation:

The amount of riboflavin in erythrocytes is determined from a standard riboflavin solution (20 ug riboflavin / 100 ml 0.01N HCl) which is carried through the entire procedure.

$$\text{ug total riboflavin / 100 ml rbc} = \frac{R_{1(\text{sample})} - R_{2(\text{sample})}}{R_{1(\text{standard})} - R_{2(\text{standard})}} \times 20$$

The volume change caused by the addition of $Na_2S_2O_4$ reagent may be ignored because the volume only increases 0.8 % after the adding of the reducing agent.

Appendix B: Measurement of Erythrocyte Glutathione Reductase (EGR) Activity

This method is a slight modification of the procedure of Bayoumi et al. (1976).

Equipment:

Refrigerated centrifuge, at 4°C.

Beckman DU spectrophotomer with Gilford update thermostated at 37°C, using a circulating water bath.

Ultra-UV cuvettes, disposable, polystyrene, Elkay Product, Inc., Shrewsbury, MA.

Reagents:

Potassium phosphate buffer, 0.1M, pH 7.4 at 37°C. Dissolve 21.50 g of K_2HPO_4 $3H_2O$ and 0.79 g of KH_2PO_4 in 800 ml of redistilled water. When solution is at 37°C, adjust pH to 7.4 and dilute to 1000 ml.

Disodium ethylenediaminetetraacetate (EDTA), 80 mM. Dissolve 1.5 g of EDTA in 50 ml of redistilled water.

Flavin adenine dinucleotide, disodium salt, (FAD), 0.25 mM. Dissolve 1.1 mg of FAD disodium, $3H_2O$, 97% (Sigma Chemical Co., St. Louis, MO) in 5 ml of redistilled water. Prepare freshly on day of use. Keep prepared solution on ice.

Oxidized glutathione (GSSG), disodium salt, 50 mM. Dissolve 32.8 mg of oxidized disodium glutathione (Sigma)

in 1 ml of sodium hydroxide solution (2 g/l). Prepare on day of use. Keep prepared solution on ice.

Nicotinamide adenine dinucleotide phosphate, tetrasodium salt, reduced form, (NADPH), 4.0 mM. Dissolve 3.62 mg of NADPH tetrasodium salt, $3H_2O$, 98% (Sigma) in 1.0 ml sodium bicarbonate solution (10 g/l). Prepare on day of use. Keep prepared solution on ice.

Procedure:

Use redistilled water throughout. Keep all preparations on ice unless stated otherwise. Use glass graduated pipettes for measuring packed erythrocytes and automatic pipettes for the remaining reactants. Work in subdued light. Always measure activities with and without FAD added in vitro simultaneously. All measurements are run in duplicate.

1. To 1.9 ml of redistilled water add gradually 0.1 ml of washed erythrocytes. Rinse pipette several times with this solution. Cover with Parafilm and invert several times to completely hemolyze the erythrocytes.

2. Centrifuge at 4°C at 3000 rpm for 10 minutes. Decant supernatant, which should be clear.

3. To three 3-ml optical cuvettes, add reagents in order given

Addition	Cuvette 1 Blank	Cuvette 2 Sample	Cuvette 3 + FAD
Phosphate buffer	2.0 ml	2.0 ml	2.0 ml
EDTA	0.05 ml	0.05 ml	0.05 ml
Hemolysate	0.1 ml	0.1 ml	0.1 ml
Redistilled H_2O	0.1 ml	0.1 ml	---
FAD	---	---	0.1 ml
GSSG	---	0.1 ml	0.1 ml
NaOH	0.1 ml	---	---

4. Mix and preincubate at 37°C for 30 minutes

$NaHCO_3$	0.1 ml	---	---
NADPH	---	0.1 ml	0.1 ml

5. Immediately after mixing place in spectrophotometer set at 340 nm and at 37°C. Read absorbance of each cuvette exactly one minute apart for 10 minutes. Use stop watch for reading time.

6. Final concentration of reactants: Glutathione (oxidized), 2 mM; NADPH, 162 uM; FAD, 10 uM; and EDTA, 16 uM.

7. Hemoglobin content of hemolysates is determined by adding 200 ul hemolysate to 5 ml of potassium ferricyanide solution (diluent used for hemoglobin determination).

Calculation:

EGR activity is expressed as International Units (U)

per g of hemoglobin which is micromole of NADPH oxidized per g of hemoglobin per minute.

$$\text{EGR activity (U/g Hb)} = A \times \frac{1}{6.22 \times 10^{-3}} \times \frac{2.45}{0.1} \times \frac{1}{Hb}$$

Where A = decrease in absorbance per minute

6.22×10^{-3} = absorbance of 1 uM solutation of NADPH at 340 nm and 10 mm light path

2.45 = final volume in cuvette

0.1 = volume of hemolysate used

Hb = hemoglobin concentration in g/l

$$\text{EGR activity coefficient (EGRAC)} = \frac{\text{decrease in absorbance with FAD added / min}}{\text{decrease in absorbance without FAD added / min}}$$

Appendix C. Erythrocyte glutathione reductase (EGR) activity and erythrocyte glutathione reductase activity coefficient (EGRAC) in Caucasian subjects before (BS) and after (AS) the supplementation with 10 mg of riboflavin daily for 7 days.

Subject		EGR basal activity *,**			FAD stimulated EGR activity ***			EGRAC ****	
Sex	no	(BS)	(AS)	$\frac{(AS)}{(BS)}$ #	(BS)	(AS)	$\frac{(AS)}{(BS)}$	(BS)	(AS)
M	C1	4.94	6.30	1.28	6.00	6.54	1.09	1.21	1.04
F	C2	4.78	5.92	1.24	6.20	6.24	1.01	1.30	1.05
F	C3	4.14	5.92	1.43	6.05	6.99	1.16	1.46	1.18
M	C4	7.96	9.00	1.13	9.13	9.16	1.00	1.15	1.02
F	C5	7.18	7.18	1.00	6.14	7.17	1.17	1.17	1.00
X ± SD		5.80 ±1.67	6.86 ±1.30	1.22 ±0.16	6.70 ±1.36	7.22 ±1.14	1.09 ±0.08	1.26 ±0.13	1.06 ±0.07

* EGR activity was expressed as International Units per g of hemoglobin.

** EGR basal activity represented the EGR activity without FAD added in vitro.

*** FAD stimulated EGR activity represented the EGR activity with FAD added in vitro.

**** EGRAC represented the degree of stimulation resulting from the in vitro addition of FAD.

\# invivo stimulationdueto riboflavin supplementation.

Appendix D. Erythrocyte total riboflavin in Caucasian subjects before (BS) and after (AS) the supplementation with 10 mg riboflavin daily for 7 days.

Subject	Sex	RBC total riboflavin (BS)	(AS)	(AS)/(BS) *
no		µg/dl rbc	µg/dl rbc	
C1	M	8.21	10.80	1.32
C2	F	8.04	10.85	1.35
C3	F	7.10	10.15	1.43
C4	M	11.27	11.83	1.05
C5	F	11.83	15.26	1.29
X ± SD		9.29±2.12	11.78±2.04	1.29±0.14

* degree of increase due to riboflavin supplement.