selected method

Assessment of Status of Riboflavin Nutriture by Assay of Erythrocyte Glutathione Reductase Activity

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Introduction

The status of riboflavin nutriture in man has generally been estimated by using either fluorometric (*1–4*) or microbiological (*5–7*) assay methods to measure the amount of riboflavin present in urine (*8–20*), plasma or serum (*2, 15, 20–24*), erythrocytes (*2, 15, 18, 20, 22, 25, 26*), or whole blood (*7, 15, 24*). While these measurements correlate with the clinical signs associated with riboflavin deficiency, they are less than ideal as indicators of riboflavin nutriture.

A major objection is that the concentration of riboflavin in blood or urine largely reflects recent dietary intake, and has not been shown to be related to body stores or functional concentrations of riboflavin. Furthermore, sampling is difficult under many conditions because of the need either for 24-h urine specimens or for relatively large amounts of blood. Factors influencing the urinary excretion of riboflavin are the time of collection in the case of a random sample (*10, 16, 27*), physical activity (*20, 28*), urinary volume (*13*), dietary creatinine (*16*), nitrogen balance (*12, 14, 19, 20, 28*), stress (*14, 20, 24, 28*), and temperature (*9, 20, 28*).

What is actually needed is knowledge concerning the body reserves and metabolic state of riboflavin in an individual. In recent years, estimations of vitamin nutriture have been based on the participation of a vitamin in its coenzyme form in a specific enzymatic reaction. These approaches not only indicate whether adequate amounts of a vitamin are ingested but also give valuable information concerning the conversion of the vitamin precursor form to its biologically active coenzyme form. Examples of such functional tests are the transketolase (EC 2.2.1.1) assay for thiamine status (*29, 30*) and the aspartate aminotransferase (EC 2.6.1.1) assay for pyridoxine status (*31*). More recently, erythrocyte glutathione reductase[1] (EGR) has been proposed as an enzymatic index to the riboflavin nutriture of man (*32–35*). The enzyme catalyzes the reduction of oxidized glutathione (GSSG) in the following manner:

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

Glutathione reductase is one of at least two flavoproteins in the erythrocyte requiring, as a coenzyme, flavin-adenine dinucleotide (FAD), a phosphorylated form of riboflavin (*36–39*). Most of the riboflavin in the erythrocyte is present as FAD (*33, 34*). EGR activity is altered in vivo by dietary riboflavin (*32, 34, 40–43*) and in vitro by FAD (*32, 34, 40, 41, 44*), which is a tightly bound prosthetic group. The degree of in vitro stimulation of EGR activity depends on saturation of the apoenzyme with FAD, which in turn depends on the availability of riboflavin. The enzyme is usually not saturated with respect to FAD at any time (*45*), but a relatively constant percentage of FAD saturation of the EGR is found in humans receiving adequate amounts of riboflavin (*35*). Several reports have shown the EGR assay to be useful and sensitive for evaluating the riboflavin status of man (*32–35, 45–47*). The test requires only minute quantities of blood in which EGR is reasonably stable and the assay is highly reproducible. The method to be described is essentially that of Sauberlich et al. (*45*).

[1] NAD(P)H:oxidized-glutathione oxidoreductase (EC 1.6.4.2).

Principle

The intake of riboflavin considered to be the most nearly ideal is that at which the tissue stores are most nearly saturated. When erythrocyte stores of riboflavin are optimal, an additional supply of riboflavin, added in vitro as FAD, will have no effect on EGR activity. However, if erythrocyte stores of riboflavin are less than optimal, addition of FAD in vitro to the EGR assay system will cause a stimulated EGR activity. The extent of this stimulation is considered to indicate the status of riboflavin nutriture.

EGR activity is measured in hemolyzed erythrocytes in the presence and absence of in vitro FAD, by monitoring the oxidation of NADPH at 340 nm. This stimulatory effect is expressed as an activity coefficient, which is defined as the diminution of absorbance (oxidation) of NADPH in the presence of FAD (ΔA_2) divided by the diminution of absorbance of NADPH without added FAD (ΔA_1) during a given period of time.

$$\text{Activity coefficient} = \frac{\text{decrease in absorbance with added FAD/10 min}}{\text{decrease in absorbance without added FAD/10 min}} = \frac{\Delta A_2}{\Delta A_1}$$

Materials and Methods

Reagents

Potassium phosphate buffer (0.1 mol/liter, pH 7.4). Dissolve 16.41 g of K_2HPO_4 and 0.79 g of KH_2PO_4 in 800 ml of doubly distilled water. Adjust the pH, and dilute to 1000 ml.

Nicotinamide adenine dinucleotide phosphate, tetrasodium salt, 2.0 mmol/liter. Dissolve 16.6 mg of NADPH (Sigma Chemical Co., St. Louis, Mo. 63178; purity of 95% or better) in 10 ml of sodium bicarbonate solution (10 g/liter). Prepare freshly each day. Keep on ice after preparation.

Note: Evaluator H. E. S. mentions that NADPH tetrasodium salt is available as the trihydrate (Calbiochem, San Diego, Calif. 92112), which is satisfactory if the aqueous reagent is prepared in a molecularly equivalent concentration.

Flavin-adenine dinucleotide, monosodium salt, 0.25 mmol/liter. Dissolve 2.4 mg of FAD (Sigma; purity of 95% or better) in 10 ml of doubly distilled water. Prepare freshly each day. Keep on ice after preparation.

Dipotassium ethylenediaminetetraacetate, 80 mmol/liter. Dissolve 1.5 g in 50 ml of doubly distilled water.

Glutathione (oxidized), 7.5 mmol/liter. Dissolve 46 mg of oxidized glutathione (Sigma) in doubly distilled water. Add 0.1 ml of sodium hydroxide solution (1 mol/liter). Prepare freshly each day. Keep on ice after preparation.

Apparatus

A recording spectrophotometer with automatic cuvette changer, thermostatted at 37 °C (Model 2400; Gilford Instrument Labs., Inc.).

Circulating water bath (Haake; Polyscience Corp., Niles, Ill. 60648).

Collection and Handling of Specimens

Collect blood samples by venipuncture, with either sodium or potassium salts of either heparin or ethylenediaminetetraacetate as anticoagulant. Even though purified EGR can be heated 60 min at 60 °C without loss of enzymatic activity (*39*), the blood samples should either be placed on ice or refrigerated if there is any appreciable delay between collection and processing. (Uncentrifuged blood samples, refrigerated, lose no measurable activity in 24 h.)

To wash the erythrocytes, transfer a 0.2-ml aliquot of whole blood into a 10 × 75 mm culture tube and add 1.0 ml of cold isotonic saline. Mix the contents, then centrifuge at 3000 rpm for 5 to 10 min. Remove the supernatant fluid and repeat the washing procedure twice more. Freeze the packed cells and store them at −10 °C until assayed.

Note: Washed erythrocytes retain full glutathione reductase activity for several weeks when kept frozen. After four to six weeks an increasing amount of material appears that will not redissolve when water is added. This insoluble material may decrease the absolute enzyme activity, but has not been found to have any effect on the results of in vitro addition of FAD. The assays should be done within 10 to 14 days after collection. Evaluator J. D. L. found no change in the activity coefficient when the cells were stored at −10 °C for as long as 35 days. When the cells were stored at −70 °C instead of −10 °C, the activity coefficient decreased by about 10%.

On the day of assay, thaw the frozen cells by incubating them at 30 °C in a water bath. Remove the cells from the water bath and add 1.5 ml of ice-cold doubly distilled water.

Note: The exact dilution of the hemolyzed erythrocytes is not critical so long as the incubations with and without FAD contain identical amounts of hemolysate. Evaluator H. E. S. finds a 20-fold dilution to be generally more satisfactory than the 15-fold dilution suggested by the submitter, as it results in a slower reaction rate that is somewhat more reproducible.

In subsequent manipulations keep the samples ice cold. Mix the erythrocyte preparation well to ensure complete hemolysis. Centrifuge at 3000 rpm for 10 min and decant the hemolysate. Repeat the centrifugation if the hemolysate is not clear.

Assay Procedure

Keep all solutions and hemolysates on ice during preparation of the enzyme assay. For each sample to be assayed, prepare two 3-ml cuvettes according to the protocol in Table 1.

Note: Blank the spectrophotometer with cuvettes filled with doubly distilled water. This is done by using the

Table 1. Protocol for Assay of Erythrocyte Glutathione Reductase Activity

Addition	Cuvette 1 (ml)	Cuvette 2 (ml)
Phosphate buffer	2.0	2.0
NADPH	0.1	0.1
Hemolysate	0.1	0.1
FAD	0.0	0.1
Water	0.1	0.0
Ethylenediaminetetraacetate	0.05	0.05

Delivery of identical aliquots of hemolysates into the cuvettes with and without FAD is very important. Glass pipets such as the Levy-Lang type are quite satisfactory for this purpose. Likewise, automatic pipets with disposable plastic tips may be used if care is exercised (see *Clin. Chem.* March 1974).

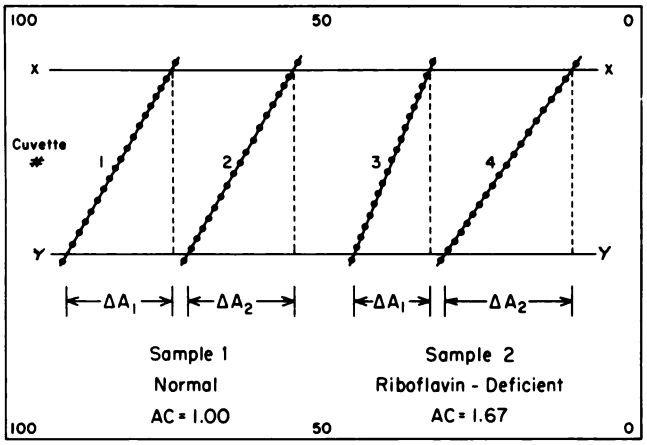

Fig. 1. Example of data calculations

slitwidth control knob to position cuvette No. 1 at a chart setting of 100. If cuvettes No. 2, 3, and 4 are properly matched, the chart setting will stay at 100. The daily variation in the slitwidth setting for blanking the spectrophotometer should be small.

Mix the contents of the cuvettes, place them in the thermostatted spectrophotometer and allow the temperature (37 °C) to equilibrate during an 8-min incubation.

Remove the cuvettes from the spectrophotometer and start the reaction by adding 0.1 ml of the solution of oxidized glutathione.

Mix the contents and place the cuvettes back into the spectrophotometer.

Use the absorbance control knob to position cuvette No. 1 at a chart setting of 90. Use the offset control knobs to position cuvettes 2, 3, and 4 at chart settings of 70, 50, and 30, respectively.

Monitor the change in absorbance at 340 nm for 10 min.

Note: A visible-light lamp used with a blue filter will give satisfactory results. The two cuvettes with and without FAD for a given sample should always be measured simultaneously on the same instrument. The readings are linearly related to time for at least 10 min. It may be necessary to dilute the hemolysate further if the degree of EGR activity is so great as to exhaust the supply of NADPH in the incubation mixture. If, on the other hand, too little EGR activity is present, the volume of hemolysate is doubled and the assay is repeated. With this assay, a blank without enzyme is not required. A dwell time of 5 s, a calibration setting of 0.5 (a full-scale deflection of the chart paper is equivalent to 0.50*A*) and a chart speed of 4 min per inch give satisfactory results. Use of the instrument suggested allows two complete samples to be assayed at the same time. "% Transmittance" chart paper should be used with the recorder.

Calculations

Calculate activity coefficients according to the equation given in the Introduction. The submitter has found it helpful to treat the curves in the following manner (see Figure 1). Draw the "best" straight line through the points, placing more emphasis on initial rates. Draw two parallel lines (*X* and *Y*) that delimit the 10-min incubation period. From the point where each curve intersects line *X*, construct a perpendicular to line *Y*. The distance from the point where the curve intercepts line *Y* to the point where the perpendicular from line *X* for that same curve intercepts line *Y* is a measure of the rate of EGR activity. In Figure 1 are examples of the effect of the in vitro addition of FAD on hemolysates obtained from normal (Sample 1) and riboflavin-deficient (Sample 2) persons.

Discussion

Measurements of the concentration of riboflavin in blood and urine do not reflect the severity of riboflavin deficiency. In contrast to free riboflavin in urine, serum, or erythrocytes, protein-bound riboflavin is not markedly affected by normal dietary riboflavin intakes, because tissue riboflavin, as flavin mononucleotide or flavin adenine dinucleotide (FAD), is not mobilized until the circulating riboflavin in the blood has been depleted. The sensitive response of EGR to FAD in vitro not only allows riboflavin nutriture to be assessed, but such an assessment is independent of the absolute rate of enzymatic activity. This minimizes or eliminates the effects of factors such as anemia. Surveys (*34, 42, 45–47*) and clinical trials (*33, 35*) indicate that activity coefficients will identify individuals receiving marginal dietary riboflavin over prolonged periods of time, as well as individuals suffering from severe ariboflavinosis. The in vitro response of EGR activity to FAD changes long before clinical symptoms of riboflavin deficiency manifest themselves, and the response can be reversed by riboflavin therapy.

Although the pH optimum for EGR is pH 6.4 to 6.9, EGR activity is routinely assayed at pH 7.4 without much apparent decrease in enzymatic activity. EGR has an absolute requirement for FAD; the K_m for FAD = 2×10^{-8} mol/liter for hemolysates from both normal and riboflavin-deficient subjects (*40*). FAD added in vitro to hemolysates from nor-

mal subjects stimulates NADH-methemoglobin reductase but not NADPH-methemoglobin reductase (*41*). As is the case with FAD, riboflavin deficiency has no effect on the K_m of EGR for NADPH (*48*). NADH can also serve as a substrate (*37, 39, 41, 49*) but does so only about 20% as effectively as NADPH.

It is somewhat surprising that concentrations of reduced glutathione are not affected by riboflavin deficiency (*48*). In a long-term experiment on rats, EGR was found to be more sensitive to ariboflavinosis than was liver glutathione reductase, even though the concentration of riboflavin in the liver diminished at a faster rate than that of the erythrocyte (*50*). EGR activity in rats is decreased by a deficiency of pyridoxine, but a deficiency of thiamine, folic acid, or ascorbic acid is without effect (*51*).

Occasional reports (*34, 35*) of activity coefficients of <1.0 remain unexplained at the moment. Because FAD from commercial sources is only about 90 to 95% pure, it has been suggested that the inhibition of EGR activity seen in some samples after addition of FAD in vitro is caused by some unknown contaminant in the FAD (*32, 34*).

Normal and Abnormal Values

It has been proposed that an activity coefficient of >1.30 suggests possible riboflavin deficiency (*32, 34*). Other reports (*33, 35, 45*) generally agree with this proposal. Correlation of activity coefficients with urinary excretion of riboflavin is improved when the activity coefficient is >1.30. The prediction of an upper limit of FAD unsaturation of EGR is not presently possible. Values as high as 2.34 have been reported for the activity coefficient (*35*). Its range for normal adolescents and adults is 0.90 to 1.15; newborns and pregnant women have similar normal values (*53*).

Measurements of the coefficient are not influenced by sex, age, or dietary protein (*34, 35*). The effect on it of diseases and other nutritional deficiencies are presently not known. It is widely recognized that a great number of clinical disorders are associated with altered EGR activity (*42*).

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In the editorial that introduced the first Selected Method [Clin. Chem. **19**, 1207 (1973)], *it was requested that readers carefully review the methods and offer suggestions for possible improvement, based on their experience with the method. We encourage comments on these previously published methods so that our ultimate goal for Selected Methods of Clinical Chemistry will be met.*

Authors	Method for	Clin. Chem.
Cali et al.	*Calcium*	**19**, *1208, Oct. 1973*
Fleisher et al.	*Carcinoembryonic Antigen*	**19**, *1214, Oct. 1973*
Dietz et al.	*Cholinesterase*	**19**, *1309, Nov. 1973*
Ratliff and Hall	*Cortisol*	**19**, *1400, Dec. 1973*
Cameron and Scarisbrick	*Progesterone*	**19**, *1403, Dec. 1973*
Dietz	*α_1-Antitrypsin*	**20**, *396, Mar. 1974*
Ambrose	*Tyrosine*	**20**, *505, Apr. 1974*
Lash and Fleischer	*Angiotensin*	**20**, *620, May 1974*