Erythrocyte Glutathione Reductase as a Measure of Riboflavin Nutritional Status of Pregnant Women and Newborns (37313)

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Until recently the methods used to assess riboflavin nutritional status of humans have not been satisfactory for specifically detecting the deficiency (1). However, the measurement of erythrocyte glutathione reductase (EGR), a flavin adenine dinucleotide (FAD) containing enzyme, has been shown to be an accurate index of riboflavin deficiency in adults (2–4).

The usefulness of this method in the pregnant woman and newborn has not been determined. It has been shown that FAD is not transferred placentally from mother to fetus (5). In addition the ability of the fetal erythrocyte to make FAD and the degree of saturation of EGR with FAD in both fetal and maternal erythrocyte were not known. In view of the foregoing, studies were carried out to determine the applicability of the EGR test to the pregnant woman and the newborn.

Material and Methods. Twenty normal, well-nourished pregnant women with no medical or obstetrical complications were studied. Seventeen took vitamin supplements containing riboflavin for various periods of time during pregnancy. Maternal venous blood was obtained at time of delivery and cord blood immediately after expulsion of the placenta. Five milliliters of blood were collected in test tubes containing 1.5 ml ACD solution (7.3 g anhydrous citric acid, 22 g sodium citrate $\cdot 2H_2O$, + 24.5 g glucose $\cdot H_2O$ per liter). The bloods were centrifuged at 1000 rpm in a desk top centrifuge for 10 min and the supernate discarded. The erythrocytes were washed 3 times with 5 volumes of cold 0.85% NaCl solution. The supernate and leukocytes were removed. To prepare the hemolyzate, 0.1 ml of erythrocytes was added to 1.9 ml cold distilled water, mixed, and allowed to sit for 30 min in a refrigerator. The mixture was then centrifuged for 15 min at 1500 rpm and the clear supernate collected.

The EGR activity of the hemolyzate was determined by a modification of the method of Glatzle *et al.* (4). The end point of this method is the decrease in absorbance in optical density units (OD) at 340 nm owing to the oxidation of NADPH both in the absence and presence of FAD. The result is expressed as the activity coefficient (AC) which is the ratio of the difference in OD with FAD to that without FAD. (AC = Δ OD with added FAD/Δ OD without added FAD). In normal adults AC values range from 0.9 to 1.2 (4).

NADPH, FAD and GSSG (oxidized glutathione) were obtained from the Boehringer Mannheim Corp, NY. NADPH (1.9 m*M*) was prepared by dissolving 16.6 mg of the tetrasodium salt in 10 ml 1% $NaHCO_3$. FAD (0.14 m*M*) was prepared by dissolving 1.2 mg of the monosodium salt in 10 ml distilled water and GSSG (3.7 m*M*) by dissolving 23 mg in 10 ml distilled water and 0.8 ml 1 *N* NaOH.

The assay is carried out in quartz cuvettes with a capacity of about 1.5 ml with a 1-cm light path. Each cuvette contains 0.6 ml of 0.1 *M* potassium phosphate buffer, pH 7.4, 0.1 ml 40 m*M* EDTA, 0.1 ml 3.7 m*M* GSSG, and 0.05 ml hemolyzate. Since it is essential that exactly the same amount of hemolyzate be added to each cuvette, sufficient amounts of the above ingredients for 4 cuvettes are added to a test tube, mixed and the proper amount doled out to each of 4 cuvettes (2 are controls and 2 are blanks). To two cuvettes 0.1 ml of 0.14 m*M* FAD is added. The 4 cuvettes are then incubated at 37°

TABLE I. Erythrocyte Glutathione Reductase Activity Coefficients (AC) in Paired Maternal and Cord Bloods.

	AC values	
	Mean ± SD (range)	
No.	Maternal bloods	Cord bloods
20	0.98 ± 0.05 (0.9–1.09)	1.00 ± 0.07 (0.9–1.20)

for 8 min. Then, 0.1 ml of 1.9 m*M* NADPH is added to the 2 controls (one with FAD) and sufficient distilled water to give a total volume of 1.1 ml in each cuvette. The contents are mixed thoroughly using a small plastic stick. Initial absorbance readings of the control cuvettes are made at 340 nm in a Beckman DU Spectrophotometer using the blank cuvettes to zero the instrument. The cuvettes are then incubated at 37° for 10 min at the end of which time readings are again obtained and from the differences in OD units the AC is calculated.

The erythrocytes of the infants were screened for glucose-6-phosphate dehydrogenase (G-6-PD) activity by the method of Motulsky and associates as described by Dacie and Lewis (6).

Results. The AC values for both maternal and cord bloods are listed in Table I. It can be seen that means of 0.98 ± 0.05 (SD) and 1.00 ± 0.07 (SD) for maternal and cord bloods, respectively, are not significantly different. These values are similar to those found in normal adults. The AC values, a measure of the degree of saturation of the apoenzyme, indicate that both normal maternal and fetal EGR are saturated with FAD.

The actual levels of EGR can be estimated from the decrease in absorbance owing to the oxidation of NADPH in the cuvettes containing no added FAD (Δ OD). These were determined and means of 0.100 ± 0.026 (SD) and 0.134 ± 0.035 (SD) were found for the 20 maternal and 20 cord blood hemolyzates, respectively. These differences are highly significant ($p < 0.005$) and indicate that the EGR content of the erythrocyte of the newborn is higher than that of the mother. This conclusion is based upon the fact that equal volumes of erythrocytes were used in assays of both maternal and cord blood. However, when one considers that the newborns' erythrocytes are larger than those of adults, it would appear the differences in EGR content may be even greater.

All of the newborns were normal by the erythrocyte G-6-PD test.

Discussion. After it had been shown that EGR was a FAD containing enzyme (7, 8), several methods to quantitate the determination of EGR were proposed in order that this parameter could be used as an index of riboflavin deficiency in man (2–4). In our experience, the method of Glatzle *et al.* (4) has proved to be the most sensitive and most suitable for routine use. We have modified this technique so that it can be easily performed utilizing a spectrophotometer ordinarily found in most laboratories.

The use of AC values simplifies the performance and interpretation of the test. Since the determining factor is the degree of saturation of the EGR apoenzyme with FAD, it is only necessary to measure the decrease in absorbance at 340 nm in the presence and absence of FAD. Thus, the level of hemoglobin or erythrocytes in the individual's blood does not affect the assay.

The decrease in saturation of EGR resulting in AC values above 1.2 was shown to be an early indicator of experimental riboflavin deficiency produced in both the rat (9) and man (1) maintained on a riboflavin low diet. The values returned to within normal limits after the rats and men were repleted with riboflavin.

This method has been found useful in surveys of population groups for riboflavin deficiency (10–12). However, this report is the first application of this method to the pregnant woman and the newborn. In the pregnant woman, the major portion of circulating riboflavin is in the form of FAD whereas that in the cord blood is free riboflavin owing to enzymatic degradation of FAD in the placenta (5). The ability and rate of fetal erythrocytes to make FAD had not been reported. In addition, the effect on the assay of the relatively high amounts of

reticulocytes in the newborn's blood was not known. The studies reported here show that the EGR levels in the cord blood erythrocytes are elevated. Others, studying enzymes in the erythrocyte, have reported high levels of the EGR holoenzyme in the newborn (13). Several factors may result in high EGR activity. It has been reported that such increased activity exists in those with erythrocyte G-6-PD deficiency (11, 14). In our study, all infants were screened for G-6-PD deficiency and all had apparently normal levels of this enzyme. In addition, reticulocytes have been shown to have higher levels of glutathione reductase than mature erythrocytes (15), and this may account for the high levels observed in cord blood. However, the AC method of expressing results depends upon the degree of saturation of EGR with FAD rather than the actual levels of EGR. In these studies, we have shown that the glutathione reductase of the mixture of reticulocytes and erythrocytes found in cord blood is saturated with FAD, and therefore the AC method is applicable under these conditions. Other methods which measure absolute levels of EGR may not be applicable to individuals whose blood contains higher than average levels of reticulocytes.

The usefulness of this method in the pregnant woman and newborn extends the range of this method to population groups which may be vulnerable to nutritional deficiencies.

Summary. The assay of erythrocyte glutathione reductase (EGR) used to assess the riboflavin nutritional status has been modified to increase its utility.

The levels of EGR in cord blood are significantly higher than those in the blood of well-nourished pregnant women at term.

In both maternal and cord blood the activity coefficients (AC) were near 1, indicating that the EGR in both was saturated with FAD. This method to determine riboflavin deficiency can be applied to both pregnant women and newborns, and is not affected by increased reticulocytes.

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