

**SYMPOSIUM ON  
'RECENT ADVANCES IN THE ASSESSMENT OF VITAMIN STATUS  
IN MAN'**

**The assessment of riboflavin status**

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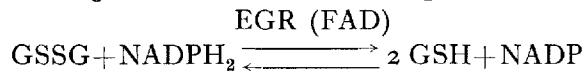
For the assessment of nutritional riboflavin status in man, several methods have been applied which are, in their basic principles, comparable with the methods for assessing the status of other vitamins. One approach involves the determination of the dietary riboflavin intake by means of food composition tables or by chemical analysis of food samples (Roine & Pekkarinen, 1968; van Schaik, 1968). These methods are, in general, laborious and expensive and are only available for use in connection with nutrition surveys or for studying the diet in certain institutions.

Other methods for determining the riboflavin status are based on the estimation of either urinary riboflavin excretion, with or without a preceding riboflavin load, or of the riboflavin or FAD levels or both in blood or erythrocytes (see Pearson, 1967; Glatzle, Körner, Christeller & Wiss, 1970). These procedures have certain disadvantages, since urinary riboflavin excretion may depend on the recent intake of the vitamin, on the nitrogen balance and on kidney function. Furthermore, riboflavin and FAD concentrations in blood or erythrocytes are difficult to measure very accurately, so that the results may be unreliable, especially when concentrations are very low, e.g. in people on a marginal vitamin intake.

To assess the riboflavin status as accurately as possible and by an easily applicable method, we became interested in looking for an enzymic assay which depends on the biochemical or physiological function of riboflavin or its active forms in the body. We therefore investigated the NADPH<sub>2</sub>-dependent glutathione reductase (*EC* 1.6.4.2), which requires FAD as coenzyme, in erythrocytes of rats and human subjects and proposed this enzymic approach in the detection of riboflavin deficiency (Glatzle, Weber & Wiss, 1968). Similar procedures have subsequently been reported by Bamji (1969) and Beutler (1969) using the same enzyme but different assay conditions.

The basis of the erythrocyte glutathione reductase (EGR) assay is similar to that of other enzymic functional tests, e.g. the transketolase activation test by Brin (1962) for evaluating the thiamin status and the glutamate-oxaloacetate aminotransferase activation assay by Raica & Sauberlich (1964) for the assessment of the pyridoxine status. During riboflavin deficiency, the activity of EGR is lowered, but it can be

returned to normal level by the *in vitro* addition of the coenzyme, FAD (Glatzle *et al.* 1968; Glatzle *et al.* 1970). The enzyme activity is estimated spectrophotometrically by measuring the oxidation of NADPH<sub>2</sub> in the following reaction:



where GSSG = oxidized glutathione, GSH = reduced glutathione.

The activation of the EGR by FAD is expressed as the activation coefficient  $\alpha$ , which is defined by the quotient:

$$\alpha = \frac{\text{activity after FAD addition}}{\text{activity without FAD addition}}$$

(Glatzle, 1970).

From the statistical evaluation of the activation coefficient  $\alpha$  for 185 apparently healthy blood donors, it was proposed that in our test system a value of  $\alpha > 1.20$  could be indicative of a marginal or inadequate riboflavin supply (Glatzle *et al.* 1970). While these activation coefficients showed a normal frequency distribution around 1.0, the distribution of the  $\alpha$  values for 124 geriatric patients was 'skew' and shifted to higher values (Fig. 1). These high activation coefficients could, however, be corrected by the administration of 10 mg riboflavin/d for 2 weeks (Fig. 1). A good negative correlation between the activation of EGR and the total riboflavin levels in red blood cells was found by Glatzle (1970).

On the other hand, Sharada & Bamji (1972) questioned the existence of a correlation between riboflavin levels in the red blood cell and the EGR activity in studies under field conditions. They believe that the two variables show parallel change to dietary riboflavin only in controlled studies. In experiments with rats, Sharada & Bamji (1972) observed that severe pyridoxine deficiency may markedly increase the riboflavin concentration of the red blood cell without any change in the FAD activation effect of EGR. These authors, therefore, concluded that the erythrocyte riboflavin levels may be a poor index of the riboflavin status. In a controlled study on human volunteers, Bamji (1969) found EGR activity and urinary riboflavin excretion more sensitive to dietary intake of the vitamin than the red blood cell riboflavin concentration.

In a study of the assessment of riboflavin intake in twenty-four Istrian farmers, Buzina, Jušić, Brodarec, Milanović, Brubacher, Vuilleumier, Wiss & Christeller (1971) reported a negative, statistically significant correlation between the EGR assay and urinary riboflavin excretion among those subjects with an activation coefficient  $\geq 1.10$ . In comparison with the ICNND guidelines (Interdepartmental Committee on Nutrition for National Defense, 1963) for the interpretation of results for urinary riboflavin excretion, EGR activation coefficients of  $\alpha \geq 1.28$  were classified as unacceptable. This should, however, be considered as tentative, since the results are only based on thirteen observations.

In American high school students with high EGR activation coefficients, Sauberlich, Judd, Nicholds, Broquist & Darby (1972) found, with one exception, urinary riboflavin levels considered as low or deficient according to the ICNND guidelines.

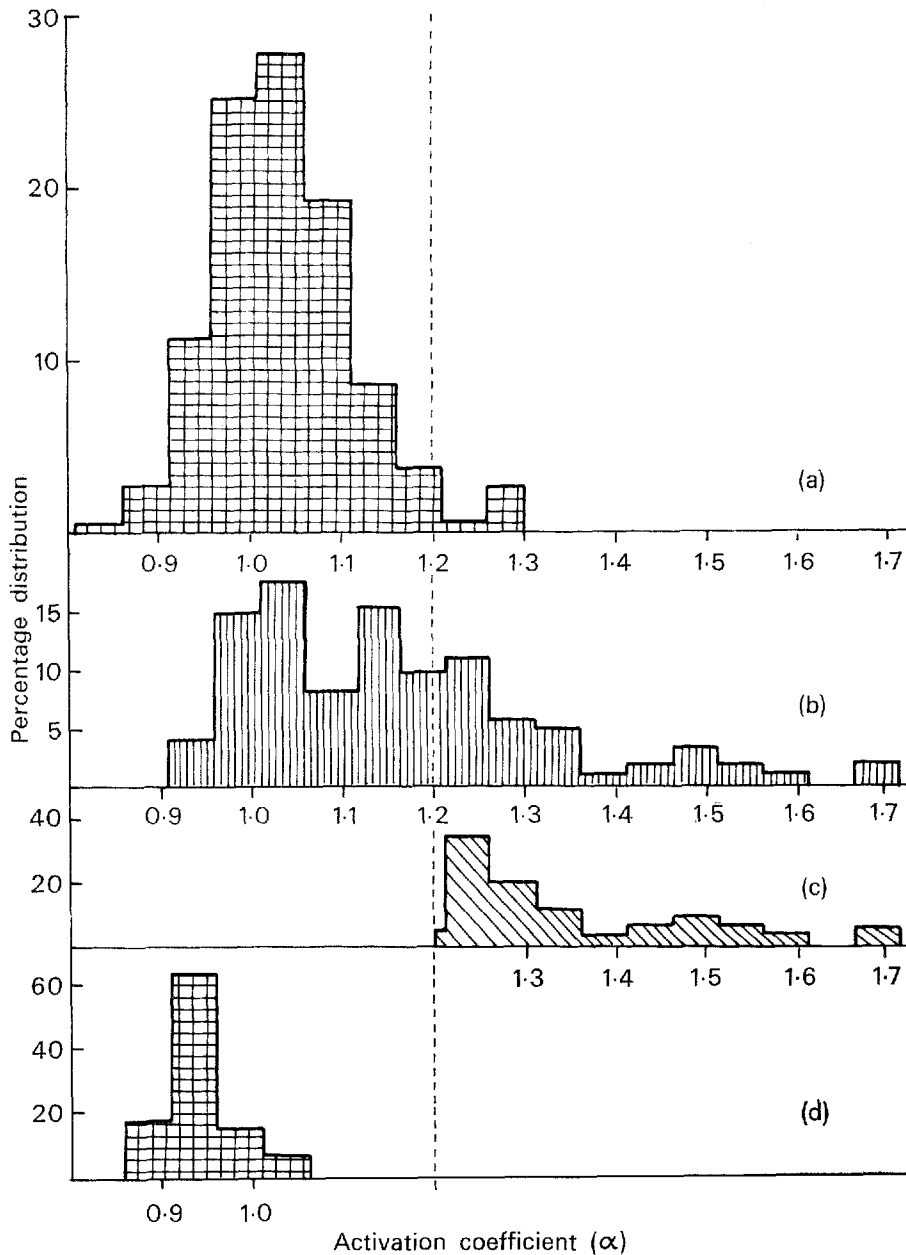


Fig. 1. Frequency distribution of the activation coefficients ( $\alpha$ ) for the erythrocyte glutathione reductase of: (a) 190 blood donors (b) 124 geriatric patients (c) thirty-five geriatric patients with  $\alpha$ -values  $> 1.20$  (selected from the group shown in (b)) before administration of riboflavin (d) the same group shown in (c) after the administration of 10 mg riboflavin/d for 2 weeks.

Tillotson & Baker (1972) carried out a 12-week study on six male volunteers with partial riboflavin depletion and subsequent repletion. The urinary riboflavin excretion decreased during the 1st week and did not significantly change during the

following 7 weeks of depletion. The urinary riboflavin level did not increase markedly until about the 10th day of the repletion period, but the EGR assay proved to be a sensitive measure of the riboflavin status throughout the whole study.

In all instances where a certain method is applied for the assessment of nutritional status, there is a question of whether the variable used does in fact represent the over-all situation in the body. We investigated this question in experiments on rats in which riboflavin intake was varied and found good correlations of the activation coefficients for glutathione reductase in erythrocytes, whole blood, liver and small intestine both between values and with the total riboflavin levels of liver and kidney (Glatzle, Weiser, Weber & Wiss, 1973). This study has shown that the glutathione reductase assay can be carried out with whole blood (BGR) instead of erythrocytes (EGR), thus offering analytical and technical advantages. The following correlation coefficients between BGR and EGR could be found: 0.86 for sixty-four rats (Glatzle *et al.* 1973) and 0.96 for 110 humans (Glatzle, unpublished results).

These experiments support the hypothesis that the EGR or BGR reflect fairly well the riboflavin body pool of an individual.

A difficult problem is the study of a correlation between the increase in EGR or BGR activation coefficients and the occurrence of clinical symptoms of ariboflavinosis. In the study of Tillotson & Baker (1972) on a partial riboflavin deprivation in human subjects, no clinical symptoms were observed. Bamji (1969) found significantly lower activities of glutathione reductase and increased activation coefficients in subjects with clinical evidence of riboflavin deficiency, such as angular stomatitis and glossitis. We investigated the EGR and BGR of twelve children from Thailand. Five children showed no clinical signs and the corresponding activation coefficients were found to be  $< 1.30$  for EGR and  $< 1.25$  for BGR. In five of the seven children with clinical signs of riboflavin deficiency, the activation coefficients were markedly increased and reached values between 1.30 and 1.80 for EGR, and between 1.25 and 1.50 for BGR. Thurnham, Migasena & Vudhivai (1971) studied the prevalence of angular stomatitis and 'biochemical' ariboflavinosis, as measured by the glutathione reductase assay of Glatzle *et al.* (1968), during a 2-year period in two villages of North-East Thailand. In one village the percentage prevalence of the two criteria appeared to follow a common pattern throughout the year, while in the other village the 'biochemical' ariboflavinosis seemed to be more prevalent and angular stomatitis occurred less often.

All these studies demonstrate that a certain correlation exists between the clinical symptoms and biochemical lesions of riboflavin deficiency, but the correlation does not appear to be very strong. There may be several reasons for this, e.g. a lack of specificity of the clinical symptoms or a contribution by nutritional factors other than riboflavin to the prevalence of stomatitis, time lags between biochemical changes and the appearance or disappearance of clinical signs.

We have already used the EGR or BGR assay in various studies of the assessment of the riboflavin status, e.g. in 651 pregnant women (S. Heller, R. M. Salkeld & W. F. Körner, unpublished results). In this study, 25% of pregnant women in the first trimester—increasing to 40% at term—appeared to be marginally supplied

with riboflavin according to our suggested classification of activation coefficient values. In another study with pregnant women, we observed a higher incidence of low haemoglobin levels in blood when the total blood riboflavin levels were decreased and the activation coefficients for the glutathione reductase were increased. The blood haemoglobin levels and values for packed cell volume correlated positively with the levels of total riboflavin in blood and negatively with the activation coefficients for EGR and BGR (Glatzle, Decker & Hinselmann, unpublished results).

From our present experience, we know of no instances where the glutathione reductase assay failed to work. Wrong interpretations may, however, occur in cases of  $\alpha$ -thalassaemia, where the protein moiety of the glutathione reductase in the erythrocytes is affected and the association constant of the apoenzyme with FAD is diminished (Staal, Helleman, de Wael & Veeger, 1969). This may lead to high activation coefficients at red blood cell flavin levels which are usually considered to be acceptable, as much more FAD is needed to reach full enzyme activity in this instance. Persons who are deficient in erythrocyte glucose-6-phosphate dehydrogenase often possess an increased glutathione reductase activity in erythrocytes which cannot be activated by FAD to the usual extent. This may falsely be interpreted as a good riboflavin status for the individual, but it is possibly true only for the status of the erythrocytes, since erythrocytes that are deficient in glucose-6-phosphate dehydrogenase have higher FAD concentrations than normal erythrocytes (Flatz & Simmersbach, 1970).

In conclusion, the assay of glutathione reductase activity is an attempt to evaluate the riboflavin status in a rather simple in vitro assay on the basis of the function of an FAD-dependent enzyme. It has proved to be a reliable and practicable method for the assessment of riboflavin status in human subjects, and appears to have some advantages over the variables previously used.

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