Effect of Flavin Compounds on Glutathione Reductase Activity: In Vivo and In Vitro Studies

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ABSTRACT Increases or decreases of red cell glutathione reductase (GR) have been described in connection with many clinical abnormalities. We find that GR activity as measured in hemolysates represents only a portion of the available GR activity. The addition of small amounts of flavin adenine dinucleotide (FAD), but not of flavin mononucleotide or riboflavin, activates the GR of hemolysates. 1 μM FAD results in a maximal activation within 10 min; gradually increasing activation occurs at much lower, for example, 20 mμM FAD concentrations. Once FAD has activated GR, dilution or dialysis does not reverse activation of the enzyme. Activation of GR by FAD can be inhibited by adenosine triphosphate (ATP), and to a lesser extent by adenosine diphosphate (ADP) and adenosine monophosphate (AMP), if these adenine nucleotides are added before the addition of FAD, but only to a slight extent if FAD is added before the adenine nucleotides. The addition of FAD to GR does not alter its electrophoretic mobility but produces intensification of the bands.

The administration of 5 mg of riboflavin daily produces marked stimulation of red cell GR activity within only 2 days. After cessation of riboflavin administration, the GR activity again begins to fall. The degree of stimulation of GR activity by riboflavin is inversely correlated with the level of dietary riboflavin intake. The base line GR activity of normal individuals is directly correlated with the level of dietary riboflavin intake. The previously unexplained variations of glutathione reductase in health and disease must be reevaluated in light of the state of riboflavin nutrition and metabolism of the subject.

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INTRODUCTION

Glutathione reductase (GR) is a key enzyme in the regulation of metabolism along the hexose monophosphate pathway in erythrocytes. The activity of GR has been reported to be altered under a great many circumstances. Increases in activity have been reported to occur in glucose-6-phosphate dehydrogenase (G-6-PD) deficiency (1), diabetes mellitus (2), gout (3), after administration of pharmacologic doses of nicotinic acid (4), or of unspecified amounts of flavin mononucleotide (FMN) (5), after induction of methemoglobinemia in vitro (6), and after incubation of hemolysates with stroma (7). Deficiency of GR activity has been associated with many different clinical states, including drug-induced hemolytic anemia, hypoplastic anemia, thrombocytopenia, oligophrenia (8), homozygous hemoglobin C disease (9, 10), Gaucher's disease (11), and alpha thalassemia (5). A dominant pattern of inheritance has been reported to occur with GR deficiency (12). Because of the great variety of apparently unrelated clinical disorders which have been associated with GR deficiency, however, we have previously suggested that "it is possible that this lack represents a secondary manifestation of a poorly understood basic disorder" (13).

Purified GR has been shown to be a flavin enzyme, presumably with a flavin adenine dinucleotide (FAD) prosthetic group (14–16). We now report evidence that GR in red cells of normal individuals is not saturated with its coenzyme, flavin adenine dinucleotide (FAD). The activity of the enzyme as measured in hemolysates therefore depends to a great extent on the level of riboflavin intake, and it is possible to more than double the activity of the enzyme of some individuals by the administration of physiologic amounts of this vitamin. These findings suggest that the various apparently unrelated alterations in GR activity must be reevaluated

in the light of the state of riboflavin nutrition and metabolism of the individuals studied.

METHODS

Nonfasting venous blood samples were drawn from normal hospital employees and from their families and from inpatients and outpatients at the City of Hope Medical Center. GR deficient subjects were selected by screening a large number of blood samples, by use of previously described fluorescent screening technique (17). ATP, ADP, AMP, glutathione, oxidized form, (GSSG) (type II), triphosphopyridine nucleotide, reduced form, (TPNH), TPN, diphosphopyridine nucleotide (DPN), FMN, riboflavin, and FAD were all obtained from Sigma Chemical Co., St. Louis, Mo.

GR assays were carried out on samples of blood collected in 1–1.3 mg of neutralized ethylenediamine tetraacetate (EDTA) per ml of blood. The enzyme is extraordinarily stable in blood samples kept even for 3–4 wk at 4°C, but most assays were carried out within 1 or 2 days. An aliquot of red cells was washed three times in isotonic sodium chloride solution, lysed in 19 volumes of distilled water, frozen in a dry ice-acetone mixture, thawed, and centrifuged at 5000 *g* for 10 min. Unless otherwise indicated, the enzyme assay was carried out in a 1 ml system containing 50 μl of 1 M Tris-hydrochloride buffer, pH 8.0 (25°C), 10 μl of 0.2 M neutralized EDTA, 10 μl of hemolysate, 100 μl of FAD solution at the indicated concentration, 100 μl of 0.033 M neutralized GSSG, 100 μl of 1 mM TPNH, and 630 μl of water. The order in which FAD, GSSG, and TPNH were added influenced the rate of reaction. Maximal rates were found when the order of addition was: *a* FAD, *b* GSSG, and *c* TPNH. A partial reaction mixture was therefore preincubated at 37°C, and the sequential addition of FAD (or water), GSSG, and TPNH were carried out in that order at 10-min intervals. The optical density was then followed for 20–30 min at 340 mμ in a Gilford model 2000 or model 2400 recording spectrophotometer thermostated at 37°C. The maximum linear rate was used in computing enzyme activity, which was expressed as micromoles of TPNH oxidized per gram of hemoglobin per minute.

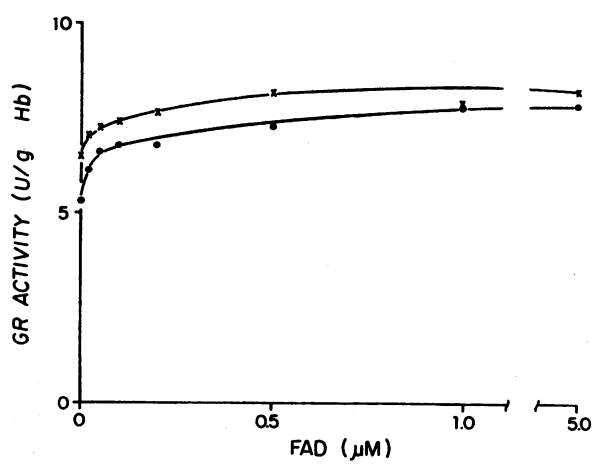

FIGURE 1 The effect of various concentrations of flavin adenine dinucleotide (FAD) on glutathione reductase (GR) activity of two normal hemolysates. In each case, the final concentration of FAD in the assay system is given.

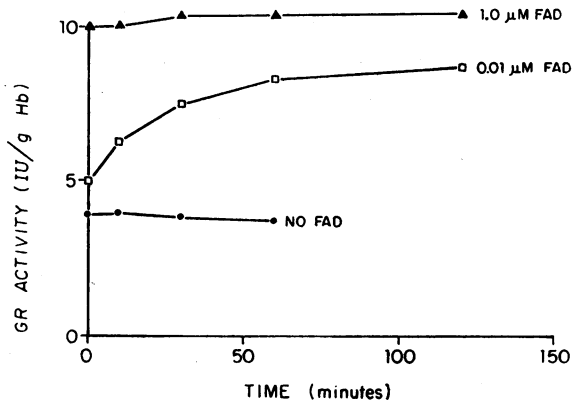

FIGURE 2 Activation of GR by 0.010 μmole/liter and 1.0 μmoles/liter (final concentration) FAD. The time indicated represents the moment at which glutathione, oxidized form (GSSG) was added. This was followed 10 min later by the addition of triphosphopyridine nucleotide, reduced form (TPNH) and measurement of the reaction velocity.

Red cell FAD, FMN, and riboflavin concentrations of packed red cells were measured by a modification of the methods of Burch, Bessey, Lowry, and Love (18, 19). An aliquot of blood was centrifuged, the plasma and buffy coat were removed, and the packed cells were frozen at −20°C. The hematocrit of the packed cells was estimated by carrying out a hemoglobin determination and assuming that the hemoglobin content of red cells was 330 mg/ml.

All glassware used in flavin determinations was specially cleaned in one-half concentrated nitric acid. 200 μl of packed cells were rinsed into 2.8 ml of distilled water, and proteins were precipitated by the addition of 3.0 ml of 20% trichloroacetic acid solution. The mixture was permitted to stand in the cold for 15 min, centrifuged in the cold, and 0.6 ml of the supernatant transferred into 3.0 ml of 0.2 M K_2HPO_4 in a photofluorometer cuvette. Fluorescence measurements were made in a Turner 110 or Turner 111 filter fluorometer using a Corning 5-58 primary and a 3-70 secondary filter. Readings were taken before and after the addition of a pinch of sodium dithionite. The remainder of the supernatant trichloroacetic acid extract was incubated at 37°C in the dark for 20 hr to hydrolyze FAD, and fluorometric readings were made as before. $\frac{1}{10}$ ml of a standard containing 1 μM riboflavin in 0.01 N HCl and a water blank were carried through the entire procedure, and riboflavin plus FMN and FAD levels were calculated (15, 16). Using this technique, good recoveries of FAD added to hemolysates was achieved.

The effect of the administration of 5 mg of riboflavin given daily was investigated in 13 subjects. The estimated daily dietary riboflavin intake was based on a 5–7 day dietary record kept by the subject and was calculated from standard food tables (20–23). One subject (L.B.-9) was found to have been taking a multivitamin supplement containing 2.5 mg of riboflavin, and this has been included in the calculation of dietary intake. No effort was made to regulate the dietary intake of the subjects, and each was instructed to continue their normal diet during the period of investigation.

Electrophoresis of GR was carried out on Cellogel strips according to the method of Blume, Rüdiger, and Löhr (24) and on starch gel using a previously described technique (25).

Hemolysates were "stripped" of FAD using a modification of Icen's (16) adaptation of Warburg's classical technique for the removal of the prosthetic group from flavin enzymes. 6 ml of saturated ammonium sulfate solution was added to a destromatized 1:20 hemolysate. The pH was adjusted to 3.0 using 0.1 N HCl. After 1–2 hr at 4°C the precipitated enzyme was collected by centrifugation and was redissolved by the addition of 0.3 ml of 1 M Tris-hydrochloride buffer, pH 8, and 1.0 ml of water. In some instances the entire procedure was then repeated once to yield an enzyme preparation with little or no activity in the absence of FAD.

RESULTS

Effect of 1 μM FAD on GR activity of hemolysates. The addition of FAD was found to exert a consistent activating effect on the GR activity of hemolysates. Fig. 1 presents the effect of the addition of various concentrations of FAD on the activity of two normal hemolysates. It is apparent that maximum activation is achieved at a 1 μM concentration of FAD, and that much lower concentrations of FAD also result in substantial activation of the enzyme. Although the addition of FAD in a concentration of 1 μmole/liter immediately resulted in maximal activation of hemolysates, the activation at low FAD concentrations proceeded for several hours, as shown in Fig. 2.

The GR activity of 40 hemolysates with and without addition of FAD to give a final concentration of 1 μmole/liter is shown in Fig. 3. It is apparent that all hemolysates studied were activated by the addition of FAD. The slope of the regression line calculated from unselected normal samples was 1.0, a finding indicating that the average absolute increment in the activity of the enzyme after addition of FAD was independent of the activity without added FAD. As a consequence, the proportional increase of activity of samples with relatively low initial activity was greater than the proportional increase of activity of samples with high initial activity. Samples which were selected on the basis of having low activity, however, showed greater stimulation by FAD both on an absolute and proportional basis. A blood

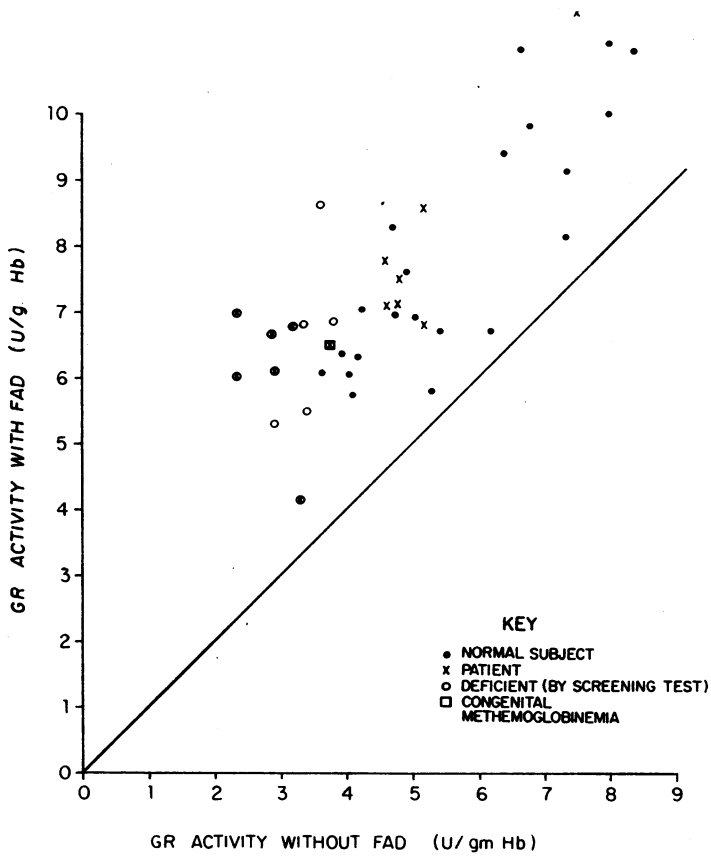

FIGURE 3 The GR activity of hemolysates from 26 clinically normal subjects and 14 patients with a variety of clinical disorders. Each hemolysate was assayed with and without the addition of FAD at a final concentration of 1 μmoles/liter.

sample from a patient with hereditary methemoglobinemia due to DPNH diaphorase deficiency, a disorder which is consistently associated with GR deficiency (*26, 27*), was also investigated. FAD caused considerable stimulation of GR activity of the hemolysate prepared from this patient. 1 mM riboflavin and FMN had no effect on red cell GR activity.

Relationship between FAD and GR activity. To determine whether FAD exerted a stimulating effect on the GR activity in the free form or whether it became tightly bound to the enzyme, FAD was added to hemolysates or "stripped enzyme," and the reversibility of activation was studied by dialysis or by dilution.

A mixture comprised of 1.0 ml of 1 μM FAD, 0.5 ml of 1 M Tris buffer, pH 8, 0.1 ml of 2 M EDTA, 0.1 ml of hemolysate, and 6.3 ml of water was divided into two dialysis bags. One aliquot was dialyzed against a mixture containing 100 ml of 1 μM FAD, 50 ml of 1 M Tris buffer, pH 8, 10 ml of 0.2 M EDTA, and 640 ml of water. The other aliquot was dialyzed against the same solution except that water was substituted for FAD. After 20 hr of dialysis against two changes of 80 volumes of dialyzing fluid, contents of the bags were removed, and the extent of dilution was estimated by measuring the optical density at 406 mμ. The GR activity was measured by adding 0.1 ml of GSSG and 0.1 ml of TPNH solution, in the usual manner, to 0.8 ml of the dialyzed partial reaction mixture. The activity of the two samples was identical: dialysis against medium without FAD had not resulted in reversal of the activation of the enzyme.

TABLE I
Effect of Dilution on FAD Activation of GR

FAD concentration during preincubation 10^{-9} moles/liter	FAD concentration in assay system, 10^{-9} moles/liter	GR activity *IU/g Hb*
0	0	4.44
2	0.04	5.08
0	2	4.43
20	0.40	5.64
0	20	5.90
100	2.00	7.86
0	100	7.48
1000	20	11.04
0	1000	10.39

FAD, flavin adenine dinucleotide; GR, glutathione reductase. Normal hemolysate was preincubated with various concentrations of FAD or with Tris buffer. GR assays were then carried out. When hemolysate had been preincubated with FAD, no FAD was added to the assay system. This resulted in a 50-fold dilution of FAD in the assay system. When no FAD was present in the preincubation system, FAD was added to the assay system. The activity of the enzyme was the same regardless of whether FAD had been present in the preincubation system, and therefore was diluted, or whether FAD wad added in the assay system.

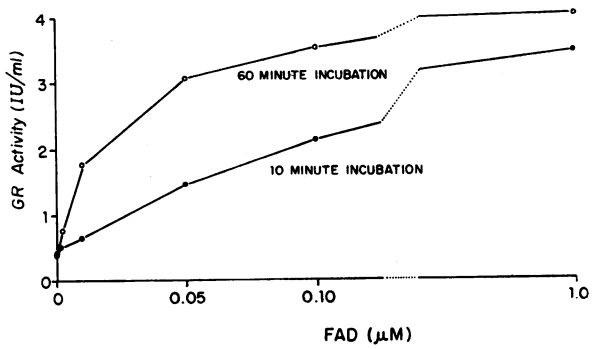

FIGURE 4 The effect of incubation of "stripped" GR with the various concentrations of FAD for 10 min or 60 min before the addition of GSSG and TPNH. The concentrations of FAD given are the final cuvette concentrations.

The relationship between FAD and enzyme was also studied by dilution. Various dilutions of FAD were prepared in 0.01 M Tris-HCl, pH 7.9 (25°C). 100 μl of 1:20 hemolysate were incubated with 100 μl of FAD solution or with 0.01 M Tris buffer for 1 hr. 20 μl of the preincubated hemolysate was then added to a cuvette containing 0.05 ml of Tris buffer, pH 8, 0.01 ml of 0.2 M EDTA, and 0.62 ml of water. This represents an immediate 35-fold and a final 50-fold dilution of the FAD-enzyme mixture. The cuvette with enzyme which had been preincubated only with buffer then received 0.1 ml of FAD in a concentration calculated to give the same final dilution as was present initially in the hemolysate which had been preincubated with FAD. After 10 min of further incubation the reaction was started in the usual manner by the sequential addition of GSSG and TPNH. The rate of reaction was followed spectrophotometrically and measured after approximately 40 min, so that the time of contact of each group of samples with the concentrated FAD solution was approximately the same. The results of this study are summarized in Table I. As shown in the Table, 50-fold dilution of the FAD-containing hemolysate resulted in no diminution of GR activity.

In order to quantitate further the relationship between GR activity and FAD concentration, the activity of "stripped" GR in the presence of various concentrations of FAD was also investigated. Fig. 4 shows the results of studies carried out when the usual assay procedure, involving 10 min of incubation with FAD before addition of GSSG and TPNH, was followed. Also shown is the result of lengthening the preincubation

period to 60 min. In this and in other studies (e.g. Fig. 2), it was apparent that at low concentrations of FAD there was progressive activation of enzyme with time. The rate of activation appeared to be relatively constant and showed no sign of reaching a stable level even after 2 hr when an FAD concentration of 10 or 20 mμmoles/liter was employed. In contrast, in a concentration of 1 μmole/liter, FAD activation of GR activity of hemolysates was essentially complete even after only 10 min of preincubation, and increased only very slightly between 10 and 60 min of incubation when "stripped" enzyme was studied.

Effect of ATP, ADP, AMP, DPN, and TPN on the activation of GR by FAD. The prior addition of ATP to give a final concentration of 1 mmole/liter was found to inhibit completely the activation of GR in hemolysates by 1 μM FAD. To study this effect in a more quantitative fashion various concentrations of ATP were added 10 min before or 10 min after the addition of FAD to "stripped" enzyme. The results of these studies are summarized in Table II A and II B. It is apparent that ATP strongly inhibits the activation of GR by FAD when it is added before FAD. The effect is much less pronounced when addition of FAD precedes addition of ATP. Although the inhibition of FAD activation resembled competitive inhibition in that the effect of low concentrations of ATP could be overcome by high concentrations of FAD, no valid kinetic analysis could be made because of the time dependency of FAD activation at low FAD concentrations, and because of the relative irreversibility of the GR-FAD reaction. The effect of ADP and AMP was found to be similar in all respects of ATP but was considerably less pronounced (Table II C).

TABLE II

Effect of Adenine and Pyridine Nucleotides on the Activity of "Stripped" GR in the Presence of Various Concentrations of FAD

A. Effect of ATP on GR activity when FAD added first

ATP concentration	FAD concentration, μmoles/liter				
	0	0.010	0.020	0.100	1.00
μmoles/liter	*IU/ml*				
0	1.08	4.66	5.23	6.75	7.32
100	1.01	3.18	4.42	6.27	6.51
1000	0.96	2.92	4.02	6.19	6.71

B. Effect of ATP on GR activity when ATP added first

ATP concentration	FAD concentration, μmoles/liter				
	0	0.010	0.020	0.100	1.00
μmoles/liter	*IU/ml*				
0	1.08	3.14	4.82	6.03	7.33
100	1.01	1.48	1.93	2.91	4.50
1000	0.96	1.00	1.32	1.13	3.02

C. Effect of AMP, ADP, and ATP on GR activity

Adenine nucleotide	Concentration	Activity with 0.02 μM FAD: Adenine nucleotide added before FAD	Activity with 0.02 μM FAD: Adenine nucleotide added after FAD	No added FAD
	μmoles/liter	*IU/ml*		
O		3.26		
AMP	100	2.48	3.63	0.85
AMP	1000	1.76		
ADP	100	1.62	3.45	0.88
ADP	1000	1.12		
ATP	100	1.41	3.31	0.74

TABLE II (*Continued*)

D. Effect of DPN and TPN on GR activity

Pyridine nucleotide	Concentration	Activity with 0.02 μM FAD: Pyridine nucleotide added before FAD	Activity with 0.02 μM FAD: Pyridine nucleotide added after FAD	No added FAD
	μmoles/liter	*IU/ml*		
O		2.79		0.49
DPN	1000	1.32	2.28	0.41
DPN	100	1.94		0.50
DPN	50	2.27		
TPN	1000	1.38	1.54	
TPN	100	2.07		
TPN	50	2.51		0.49

A mixture comprised of 0.05 ml of 1 M Tris-HCl, pH 8, 0.01 ml of 0.2 M ethylenediamine tetraacetate (EDTA), 0.01 ml of stripped enzyme, and 0.53 ml of H_2O was warmed to 37°C. In experiment A, 0.1 ml of a solution containing 0, 0.100, 0.200, 1.00, or 10.0 μmoles FAD/liter was added, and incubation continued for 10 min. Then 0.1 ml of a solution containing 0, 1, or 10 mM neutralized adenosine triphosphate (ATP), 0.1 ml of 0.033 M glutathione, oxidized form (GSSG), and 0.1 ml of 1 mM triphosphopyridine nucleotide (TPNH) were added at 10-min intervals, and optical density was measured at 340 mμ. In the experiment labeled B, the order of addition of ATP and FAD were reversed. The somewhat higher activities at low FAD concentration in the absence of ATP in series A is due to the fact that FAD activation of GR at low FAD concentration is strongly time dependent; the FAD was in contact with enzyme 10 min longer in series A than in series B. The same experimental design was used in carrying out experiment C and D, but two different batches of "stripped" enzyme were used

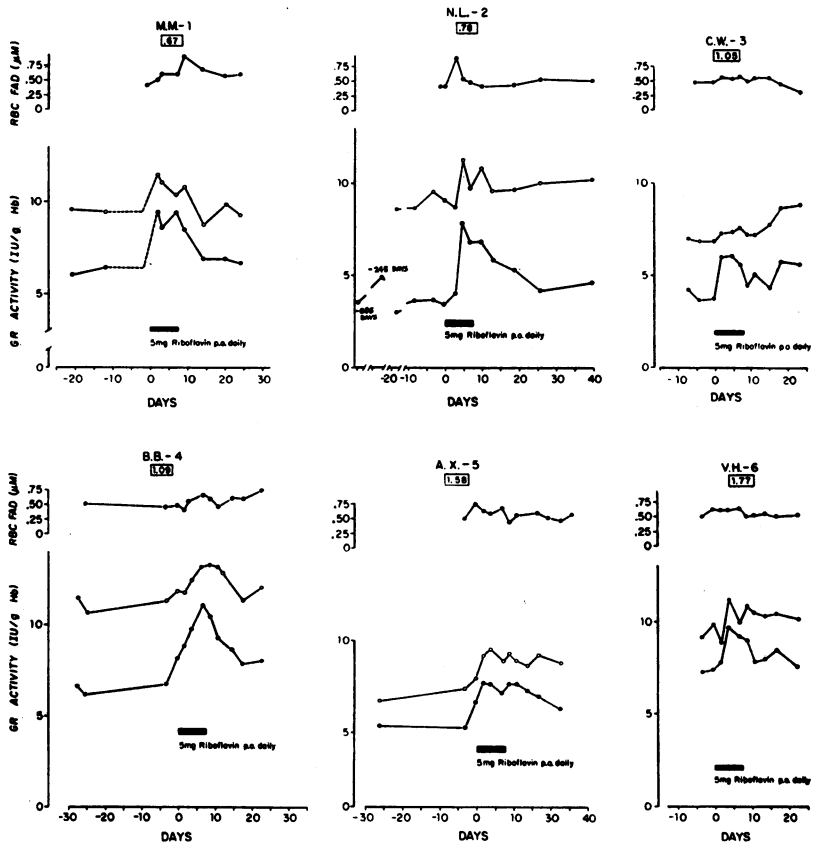

FIGURE 5 The effect of riboflavin administration on red cell GR activity and FAD levels. In each case GR activity measurements were made without the addition of FAD (●——●) and with the addition of FAD in a final concentration of 1 μmole/liter (○——○). Where available the average dietary riboflavin intake in milligrams of riboflavin per day is given in the rectangle below the subject designation. Further details regarding each subject are given in Table III.

In contrast to the situation with the FAD-GR reaction, the ATP effect could readily be abolished by dilution. When "stripped" enzyme was preincubated with ATP for 10 min and then diluted 35-fold by being added to a partial reaction system, FAD activation of the stripped enzyme was not inhibited.

Both TPN and DPN in a concentration of 1 mmole/liter were found to inhibit GR activity of hemolysates. The effect of TPN was somewhat greater than that of DPN, and it was evident even in the absence of added FAD. The effect of the pyridine nucleotides was studied also with "stripped" enzyme. The activation by FAD of "stripped" enzyme was inhibited by both TPN and DPN. The extent of inhibition was, in both cases, greater when the nucleotide was added before the addition of FAD than when FAD was added first (Table II D).

Electrophoretic studies. High-voltage electrophoresis of GR on Cellogel confirmed that the enzyme of normal hemolysates could be resolved into two bands. Addition of FAD in a final concentration of 1 μmole/liter to the hemolysate resulted in intensification of both bands but no change in their relative intensity or of their position. Similar results were obtained when, in addition, 1 μM FAD was added to the buffer system in which electrophoresis was carried out. Neither was any change in the position of GR bands noted on starch-gel electrophoresis when any hemolysate had been preincubated with 1 μM FAD, although the expected intensification of the GR band was readily observed.

Effect of riboflavin administration on GR activity. The administration of 5 mg of riboflavin daily for 8 days was investigated in 10 normal subjects and in three patients. The patients were selected on the basis of low GR activity discovered on screening (17). Pertinent clinical data and data regarding average daily riboflavin intake as estimated from food tables is presented in Table III. In each case assays for GR activity

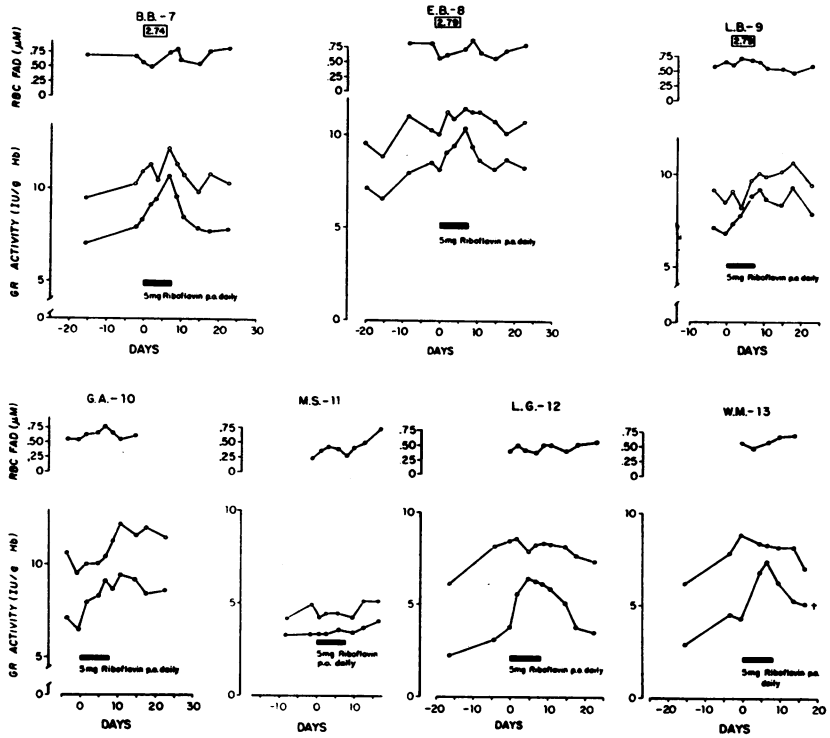

FIGURE 5 (*concluded*)

were carried out with and without the addition of 1 μM FAD, and measurements of red cell riboflavin, FMN, and FAD levels were made. The results of these studies are shown in Fig. 5. It is apparent that all but one prompt substantial increase of red cell GR activity, as measured without the addition of FAD. It is of interest that even the activity after in vitro activation with FAD increased during riboflavin administration. The subject who failed to respond, (M.S.-11), was suffering from ovarian carcinoma with intestinal obstruction. Because of frequent vomiting the actual ingested dose of riboflavin was uncertain. The mean values obtained on the nine normal subjects on whom reliable dietary histories were obtained are shown in Fig. 6. It is apparent that the magnitude of increase of GR activity was considerably greater in the individuals whose dietary intake was less than 1.5 mg of riboflavin daily than in the group with more than 1.5 mg of daily riboflavin intake. It is apparent, also, that a substantial increase in red cell FAD levels was observed in the group with the lower dietary riboflavin intake. The levels of riboflavin plus FMN were consistently very low, generally representing less than 10% of the total amount of flavin in the red cells. At these levels the results are quite imprecise, and no conclusions could be drawn regarding the levels of these FAD precursors.

Relationship between dietary intake of riboflavin and GR levels. The regression of GR activity on estimated daily riboflavin intake has been calculated. In the case of the 10 subjects for whom dietary intake estimates were made, a positive correlation with a correlation coefficient of 0.68 was found. This was significant at the 0.05 level. A much weaker correlation was found between dietary intake of riboflavin and GR reductase activity after FAD stimulation. The correlation of coefficient was only 0.35 and was not significant at the 0.2 level.

DISCUSSION

The addition of minute quantities of FAD has been shown to activate GR of hemolysates prepared from all of 26 clinically normal subjects and 14 patients with a variety of diseases. Staal et al. (5) have reported that partially purified enzyme from an individual believed to have hereditary GR deficiency could be activated by FAD, and that administration of flavin mononucleotide to this individual for several weeks resulted in a rise of red cell GR activity. However, Glatzle, Weber, and Wiss (28) found that hemolysates of normal individuals were not stimulated by FAD addition. They did not give the details of their experimental procedure, and it is likely the differences in technique are responsible for

TABLE III

Data Regarding Subjects Investigated before, during, and after Administration of 5 mg Riboflavin Daily for 8 Days

Subject	Sex	Race	Age	Weight	Diagnosis	Dietary riboflavin intake	GR activity: Average before riboflavin administration, No FAD	GR activity: Average before riboflavin administration, 1 μM FAD	GR activity: Average during riboflavin administration, No FAD	GR activity: Average during riboflavin administration, 1 μM FAD
				kg		*mg/day*	*IU/g Hb*			
M. M.- 1	F	Cau	25	69	Normal	0.67	6.22	9.54	9.12	10.95
N. L.- 2	F	Neg	35	68	Normal	0.78	3.50	8.99	5.80	9.46
C. W.- 3	F	Cau	31	61	Normal	1.05	3.75	6.91	5.92	7.31
B. B.- 4	F	Cau	40	48	Normal	1.09	6.88	11.59	9.90	12.46
A. X.- 5	F	Cau	27	66	Normal	1.58	5.80	7.37	7.51	9.20
V. H.- 6	F	Cau	43	52	Normal	1.77	7.34	9.50	8.85	9.97
B. B.- 7	M	Cau	11	41	Normal	2.74	7.78	10.25	9.75	11.31
E. B.- 8	M	Cau	14	50	Normal	2.79	7.65	9.92	9.62	11.16
L. B.- 9	F	Cau	22	59	Normal	3.70	6.99	8.86	8.01	8.66
G. A.- 10	F	Cau	40	76	Normal	—	7.03	10.23	8.51	10.20
M. S.- 11	F	Cau	60	55	Ovarian carcinoma	—	3.30	4.58	3.62	4.42
L. G.- 12	F	Cau	22	45	Rheumatoid arthritis	0.67	3.02	7.57	6.07	8.23
W. M.-13	M	Cau	28	40	Testicular carcinoma	—	3.86	7.61	7.07	8.31

the differences observed. We have found, for example, that the addition of GSSG or TPNH to the assay system before FAD addition markedly decreases the stimulating effect of FAD.

Although the quantity of FAD found in erythrocytes seemed ample to saturate GR, it must be recalled that FAD may often be tightly bound to enzymes. While treatment of hemolysates with trichloroacetic acid releases this FAD and makes it available for fluorometric assay, it may not readily be available to combine with the GR molecule. Furthermore, the presence of high concentrations of ATP within the red cell may, to some degree, limit the binding of FAD to GR. Thus, the administration of riboflavin was found to produce large increases in the GR activity of red cells.

It is of interest that not only the GR level measured in the ordinary way, but also the activity of the enzyme after FAD stimulation was increased by riboflavin administration. We interpret this observation as suggesting that GR exists in red cells in at least three distinct forms: *a* active enzyme; *b* enzyme which can readily be activated in our in vitro system by FAD; *c* enzyme which can be activated under in vivo conditions by the administration of riboflavin but which resists in vitro activation.

The activation of red cell GR by riboflavin administration occurs so rapidly that it must be due, almost entirely, to activation of preformed apoenzyme in circulating red blood cells. This does not rule out, however, the possibility that long-term administration of riboflavin may influence the synthesis of GR apoenzyme by developing erythroblasts. The fact that the GR activity of the red cells of most of our subjects had not returned to the base line value even 2 wk after the cessation of riboflavin administration could be interpreted as representing the effect of increased apoenzyme synthesis. A similar state of affairs has been shown to exist with pyridoxine intake and red cell transaminase levels (29) and iron intake and tissue aconitase levels (30).

These studies raise the question of whether the recommended daily allowance for riboflavin is too low. The recommended daily allowance is obviously not enough to "saturate" the tissues with riboflavin and riboflavin coenzymes. However, the present study permits no conclusions as to whether increasing the intake of riboflavin produces a more optimal physiologic state.

The finding that conventional methods of GR assay measure only a portion, sometimes less than one-third, of the enzyme present in the red cell may eventually help to clarify the many confusing observations which have been made about this enzyme in health and disease. It is apparent that riboflavin intake and metabolism can profoundly influence the levels of the enzyme in erythrocytes. Thus, clinical states which are associated with a decrease in red cell GR levels may have, as a common denominator, abnormalities in riboflavin nutrition or in the sysnthesis of FAD from riboflavin. It is of interest in this respect that hypoplastic anemia, one of the clinical states which has been reported com-

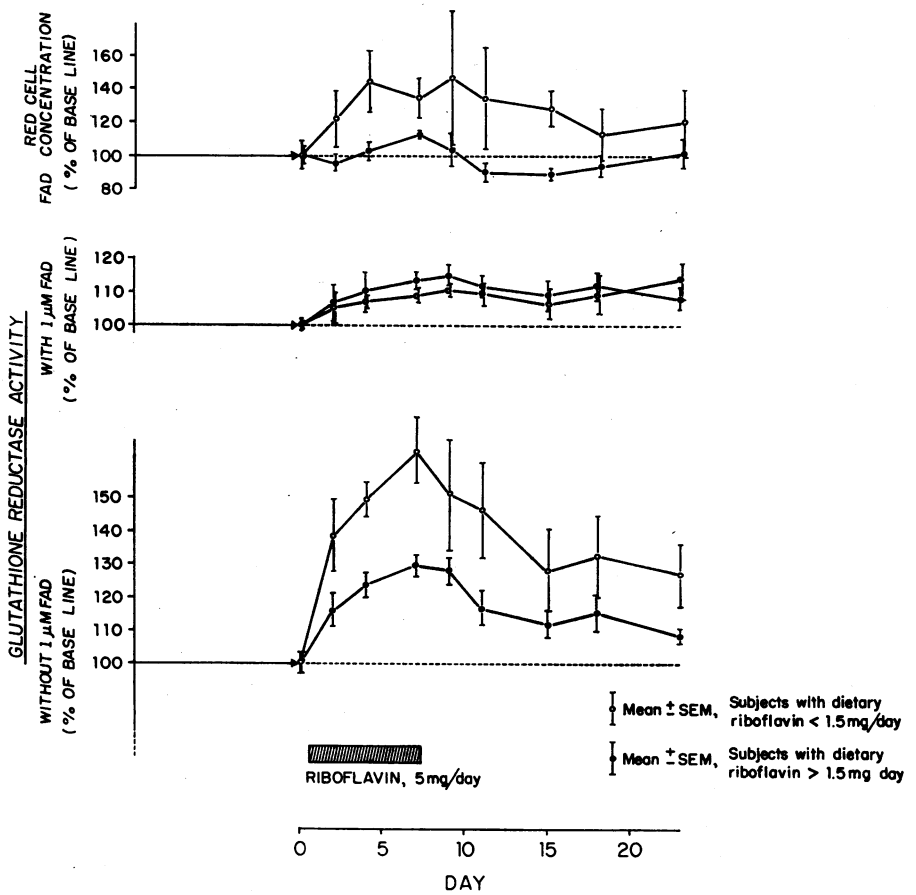

FIGURE 6 The average GR activities and red cell FAD levels of nine normal subjects before, during, and after administration of 5 mg of riboflavin daily for 8 days. The patients have been divided into two groups based on whether their daily dietary riboflavin intake exceeded the recommended daily allowance of 1.5 mg/day (five subjects), or whether it was below this level (four subjects). Activities are presented as per cent of base line activity.

monly to be associated with GR deficiency (8), has been found to occur after the administration of galactoflavin (31), a riboflavin antagonist, and possibly in riboflavin deficiency (32).

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