Elevated Glutathione Reductase Activity Coefficients in Erythrocytes after Treatment *In Vitro* with Inosine and Adenine (42882)

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Abstract. **Normal erythrocytes incubated with inosine and adenine in an acid citrate/dextrose medium underwent a marked, but reversible, loss of glutathione reductase activity reflected by erythrocyte glutathione reductase activity coefficients in the range 3.21 ± 0.39. The system therefore appears to simulate riboflavin deficiency in isolated red cells as assessed by this index. Deactivation was dependent on cell metabolism and was inhibited by treatment with methylene blue, ascorbate, and fluoride or by low temperature. The rate of deactivation was greatly increased by lowering the pH and by the presence of phosphate ions.** [P.S.E.B.M. 1989, Vol 191]

During a pilot study on the uptake of riboflavin by isolated red cells, attempts to increase the intracellular level of ATP by incubation of the cells with inosine and adenine in a standard acid citrate/dextrose (ACD) medium (1, 2) resulted in a marked decrease in glutathione reductase activity. This unusual deactivation was fully reversed by a short incubation of the hemolysate with flavin adenine dinucleotide (FAD), a cofactor normally firmly but noncovalently bound to the glutathione reductase apoenzyme. Glutathione reductase in red cells, even under normal conditions, is frequently undersaturated with FAD (3–6), but the degree of undersaturation is exacerbated by riboflavin deficiency. The essential role of FAD for enzymatic function is conveniently expressed by the erythrocyte glutathione reductase activity coefficient (EGRAC) defined as the ratio of erythrocyte glutathione reductase activity determined in the presence of added FAD to that in the absence of added FAD. Values for this coefficient above 1.3 reflect an increasing pathologic deficiency of the vitamin (7) and is a standard index for assessing the riboflavin status of patients. The present communication delineates some of the factors which influence the effect of adenine and inosine in an acid citrate medium (pH 4.0–6.0) on the EGRAC of isolated intact red cells. Although the conditions employed were not physiologic, they provide a novel method for facilitating flavin dissociation in glutathione reductase not previously described. This method of deactivation is noteworthy also in that it was applicable to intact cells. Hitherto, elevated EGRAC in such cells have only been produced by manipulation of the riboflavin content of the diet. Insofar as elevated EGRAC are a reflection of riboflavin status, the present procedure enables a pseudoriboflavin deficiency to be produced in isolated, riboflavin-replete red cells. Such a system may be of value in simulating riboflavin deficiency in selected model systems. At the same time it demonstrates that elevated EGRAC do not necessarily exclusively reflect riboflavin deficiency in a given subject.

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Materials and Methods

Blood was collected in EDTA and was largely donated by student volunteers. A number of samples were from pregnant subjects obtained in the course of routine obstetric hospital service and were generally from a socioeconomically disadvantaged population. The red cells were isolated by centrifugation, washed three times, the buffy coat removed by aspiration, and the cells resuspended in an equal volume of saline. Except where noted, deactivation was accomplished by a standard treatment in which four volumes of red cell suspension and one volume of ACD containing inosine and adenine were incubated with gentle rotation at 37°C for 1.5 hr. In these experiments the final concentrations were 25 m*M* inosine and 12.5 m*M* adenine. The standard ACD solution had a pH of about 5.2 and contained 44 g of citric acid, 13.2 g of trisodium citrate, and 14.7 g of glucose in 1 liter of water. For the study of the effect of pH on the deactivation, ACD solutions

in the pH range 4.0–6.0 were prepared at constant sodium concentration (8). Glutathione reductase activity coefficients were determined (7, 9) on stroma-free hemolysates prepared from the sedimented red cells from the reaction mixture (50 μl) centrifuged through dibutyl phthalate (150 μl) in a microfuge tube. The cells were hemolyzed by treatment with water (0.5 ml) and recentrifuged. Dibutyl phthalate was without effect on the enzyme assay. Adenosine phosphates (10, 11) and hemoglobin (12) were determined by standard methods.

Results

The basal EGRAC of student volunteers employed in the study were usually within the normal range (Table I) whereas erythrocytes from the second group of subjects frequently showed elevated values indicative of riboflavin deficiency. These findings were consistent with the general socioeconomic background of the two groups but was possibly exacerbated in the latter group by nutritional stress imposed by pregnancy (13). Irrespective of initial riboflavin status, all samples developed EGRAC values greatly above their initial level following a 1.5-hr incubation in a standard ACD/inosine/adenine mixture. In general, treatment resulted in EGRAC values (Table I) in the range 3.21 ± 0.39 (normal riboflavin status) or 3.91 ± 0.39 (riboflavin-deficient status) which, in both cases, were higher than those observed in even severe cases of riboflavin deficiency (7). Despite the somewhat higher value noted in the riboflavin-deficient population, both groups appeared to respond to deactivating conditions in essentially the same way and no special significance could be attributed to the preexisting riboflavin deficiency as a factor in the effect.

Table I. Inosine and Adenine in Acid Citrate/Dextrose as Factors which Promote Reversible Glutathione Reductase Deactivation in Red Cells[a]

	EGRAC	
	Time[b]	
	0 min	90 min
Effect of ACD/inosine/adenine[c]		
Treated:		
Poor riboflavin status	1.53 ± 0.08 (6)	3.91 ± 0.62 (6)
Normal riboflavin status	1.13 ± 0.04 (10)	3.21 ± 0.39 (10)
Control[d]	1.22 ± 0.05 (7)	1.43 ± 0.13 (7)
Role of adenine and inosine in ACD solution[c]		
ACD + adenine (12.5 m*M*)[e]	1.32 ± 0.09 (4)	3.17 ± 0.61 (4)
ACD + inosine (25 m*M*)	1.32 ± 0.09 (4)	2.57 ± 0.39 (4)
ACD + inosine (37.5 m*M*)	1.32 ± 0.09 (4)	2.73 ± 0.52 (4)
Role of intact cell		
Saline suspension of red cells in deactivation solution[c]	1.50 ± 0.27 (5)	4.29 ± 0.52 (5)
Lysate + ACD/inosine/adenine[f]	1.66 ± 0.33 (5)	1.95 ± 0.44 (5)

[a] Each value represents the mean ± SEM of (*n*) values.
[b] Time at which a sample was incubated before analysis.
[c] Washed erythrocytes in saline (approximately 50%) treated with ACD solution in ratio 4:1 at 37°C.
[d] ACD only.
[e] Final concentration.
[f] Sample prepared as described in footnote *c* but erythrocytes were hemolyzed by repeated freeze/thawing.

Deactivation was not immediate but increased with the period of the incubation and resulted, in some cases, in almost total deactivation when assayed in the absence of FAD. The data (Table I) were compiled using cells from both fresh and cold-stored blood (2–3 days) and all showed a similar deactivation. Although the glutathione reductase activity in terms of units per gram of hemoglobin was not routinely determined, a representative sample of such findings (Table II) indicated that the activity of deactivated enzyme was restored to the pretreatment level after incubation with FAD and irreversible deactivation was minimal over the short time periods employed. Attempts to promote elevated EGRAC in hemolysates by treatment with deactivating solutions were unsuccessful and resulted in only minor change (Table I). None of the components of the incubation mixture inhibited the reductase when tested in control experiments. Adenine (12.5 m*M*) in ACD solution tended to be somewhat more effective in deactivating glutathione reductase than inosine at a similar concentration, but solubility problems limited the range which could be tested. An adenine/inosine mixture appeared to be slightly more effective than an equimolecular amount of inosine (Table I). Lowering the level of both inosine and adenine in the deactivation mixture reduced the rate of deactivation. In a typical experiment, for example, in which the concentration of adenine and inosine had been reduced to one-tenth of the standard amount, a 4-hr incubation was required for the EGRAC to increase from 1.14 to 2.04. A control experiment using the standard amounts of inosine and adenine, by contrast, developed an EGRAC of 2.05 after only 90 min of incubation. It was apparent from this and similar experiments that the concentration of inosine and adenine influenced the rate of deactivation. Deactivation was further facilitated by the presence of inorganic phosphate (Table III), and this was consistent with the requirement of phosphate for the metabolism of inosine to hypoxanthine and ribose-1-phosphate (14). Thus, the inclusion of phosphate buffer (pH 5.2) in the mixture at a final concentration of 10 m*M* increased the EGRAC from 1.81 ± 0.20 in ACD/inosine/adenine alone to 2.48 ± 0.26. The mean control value in this series of experiments was 1.35 ± 0.08.

Table II. Reversibility of Glutathione Reductase Activity in Erythrocytes Deactivated with ACD/Inosine/Adenine

Time (min)	Glutathione reductase activity[a]					
	Control[b]			Treated[c]		
	Assay conditions +FAD	Assay conditions −FAD	EGRAC	Assay conditions +FAD	Assay conditions −FAD	EGRAC
0	7.8 ± 0.6[d]	6.6 ± 0.7[d]	1.17 ± 0.06	7.5 ± 0.5[d]	6.5 ± 0.6[d]	1.20 ± 0.08
90	7.7 ± 0.6	5.9 ± 0.5	1.32 ± 0.08	7.8 ± 0.4	3.5 ± 0.3	2.30 ± 0.17

[a] In micromoles NADPH oxidised/min/g hemoglobin.
[b] Four volumes of washed red cell suspension + 1 volume of ACD/10 m*M* phosphate.
[c] Four volumes of washed red cell suspension + 1 volume ACD/adenine (25 m*M*)/inosine (12.5 m*M*)/10 m*M* phosphate.
[d] Mean ± SEM ($n = 6$).

The rate of glutathione reductase deactivation, as outlined, was facilitated by low pH. The pH within the red cell was not determined but, after treatment with standard deactivation mixture, hemolysates in a number of experiments showed a spread of values between 5.8 and 6.3 depending on the sample of red cells used. The effect of pH was examined by using ACD-deactivating solutions in the range pH 4.0–6.0 (Table III). Treatment of a red cell suspension with a one-quarter volume of deactivating solution at pH 4.0 resulted in much greater rates of deactivation than observed with ACD/inosine/adenine mixtures of higher pH values. A small increase in the EGRAC from low pH alone in control experiments was minor. When the pH of the ACD/inosine/adenine deactivation mixture was increased to 6.0, the rate of deactivation of glutathione reductase became very slow and in the example quoted (Table IV), significant deactivation was not observed over several hours. Only after prolonged incubation of cells (overnight) did this treatment yield a high EGRAC. Several similar experiments confirmed this effect. Analogous results were obtained when the washed red cell suspension in the standard experiment was replaced by whole blood and this was attributed to the increased buffering capacity available from the plasma.

In these experiments elevated EGRAC were obtained only after prolonged (overnight) incubation. Experiments in which the inosine and adenine were either omitted from the ACD (pH 4.0–6.0) or were added directly to the enzyme assay showed no deactivation and suggested that deactivation arose predominantly through the metabolism of these compounds within the cell.

Methylene blue stimulates the oxidation of NADPH in erythrocytes both directly and indirectly (15, 16) and thereby increases the flux through the hexose monophosphate pathway (HMP). The inclusion of methylene blue (0.2 m*M*) in cell suspensions treated with ACD/inosine/adenine for 90 min was found to stabilize the system so that the EGRAC remained essentially normal (Table III). A similar finding was also observed when ascorbate (50 m*M*) was included in the mixture. This metabolite is also reported to stimulate NADP formation (15, 17), but probably by a different mechanism. Low temperature and fluoride also inhibited the deactivation process (Table III).

Individual adenosine phosphate levels within the cells were compared under two different conditions for which the effect of treatment with deactivation mixture on the EGRAC had been firmly established, namely, (i) at pH 4.0 and 5.2 and (ii) with methylene blue cotreatment (pH 5.2). In general, conditions in (i) facilitated greatly increased activity coefficients whereas those in (ii) stabilized the reductase against deactivation. It was shown that the low pH maintained in (i) was associated with a decline in intracellular ATP levels in combination with a large increase in both ADP and AMP (Table V). A smaller, but still increased level of ADP and AMP (compared with controls containing ACD/inosine/adenine alone) was also observed in erythrocytes treated with ACD/inosine/adenine supplemented with methylene blue. Thus, the adenosine phosphates tended to move in the same direction under both experimental conditions despite significant differences in the resulting EGRAC. Red cells treated with ACD/inosine/adenine at pH 6.0 (90 min), by contrast, showed lower than normal levels of ADP and AMP while the reductase remained fully active. In all cases, however, the total adenosine phosphate concentration of the cells remained approximately constant and there appeared to be little evidence for a viable correlation between adenosine phosphate levels and deactivation. Moreover, the increased ATP synthesis reported in ATP-depleted erythrocytes (1, 2) in whole blood treated with ACD/inosine/adenine was not observed under the conditions of the present study and appeared to require the higher intracellular pH resulting from the additional buffering capacity of the plasma proteins.

Discussion

The reversible deactivation of erythrocyte glutathione reductase by inosine and adenine in ACD buffer was shown to be a time-dependent phenomenon. Prolonged incubation under deactivation conditions invariably led to a very high loss of activity when assayed in the absence of FAD. Although the mechanism of the

Table III. Some Factors Shown to Influence Reversible Deactivation of Glutathione Reductase[a]

Treatment	EGRAC Time[b] 0 min	EGRAC Time[b] 90 min
Effect of the ACD solution pH[c]		
ACD (pH 4.0)/inosine/adenine	1.04 ± 0.10 (3)	2.01 ± 0.51 (5)
ACD (pH 5.0)/inosine/adenine	1.04 ± 0.10 (3)	1.86 ± 0.49 (3)
ACD (pH 6.0)/inosine/adenine	1.04 ± 0.10 (3)	1.28 ± 0.21 (3)
Effect of stimulation of HMP[c]		
ACD/inosine/adenine + methylene blue (0.2 m*M*)	1.31 ± 0.19 (4)	1.43 ± 0.12 (4)
ACD/inosine/adenine + ascorbate (50 m*M*)	1.32 ± 0.16 (4)	1.22 ± 0.16 (4)
Effect of phosphate ions		
ACD/phosphate buffer (10 m*M*)	1.21 ± 0.06 (5)	1.35 ± 0.07 (5)
ACD/inosine/adenine[c]	1.19 ± 0.07 (5)	1.81 ± 0.20 (5)
ACD/inosine/adenine/phosphate	1.19 ± 0.05 (5)	2.48 ± 0.26 (5)
Effect of fluoride		
2-hr preincubation with fluoride[d] (10 m*M*) then ACD/inosine/adenine[c]	1.58 ± 0.09 (2)	1.66 ± 0.16 (2)
Effect of low temperature[c]		
ACD/inosine/adenine at 37°C	1.68 ± 0.18 (2)	3.96 ± 0.24 (2)
ACD/inosine/adenine at 0°C	1.72 ± 0.24 (2)	1.70 ± 0.23 (2)

[a] Each value represents the mean ± SEM of (*n*) determinations.
[b] Time of incubation before analysis.
[c] Four volumes of washed erythrocytes in saline (approximately 50%) treated with 1 volume of ACD/inosine (25 m*M*)/adenine (12.5 m*M*).
[d] Ten millimolar (final) in saline.

deactivation remains unclear, it appeared that flavin became effectively unavailable to the enzyme in a time-dependent manner. As none of the components of the deactivation mixture affected the reductase when tested in control experiments, the deactivation inhibition apparently arose from metabolic changes within the cell. The inclusion in the incubation of a low level of inorganic phosphate resulted in a significant stimulation of the deactivation. As phosphate plays a known role in inosine metabolism (14), it further supported the concept of a metabolic change as a factor in the deactivation. Deactivation by inosine/adenine appeared to be restricted to the intact cell and conditions were not found to extend the effect to hemolysates. However, the deactivation of glutathione reductase in hemolysates by incubation with 6-phosphogluconate (18, 19) was confirmed.

This deactivation bore considerable similarity to the present deactivation and indicated *inter alia* that the reversible deactivation of glutathione reductase by normal metabolites of the red cell was not inherently dependent on the intact cell. The failure to deactivate the reductase in hemolysates with inosine/adenine was therefore attributed to the loss of a suitable milieu for the metabolism required. The inability to initiate deactivation when treatment was carried out either (i) at the temperature of melting ice, (ii) after a 2-hr preincubation with fluoride in a saline/glucose medium, or (iii) by the inclusion of methylene blue or ascorbate in the ACD/inosine/adenine solution supported the conclusion that deactivation resulted from metabolic change. Fluoride, by its inhibition of enolase, depletes the intracellular ATP and prevents *inter alia* metabolism of glucose. Methylene blue and ascorbate have a number of potential metabolic sequelae when added to blood which include an improved maintenance of the 2.3-diphosphoglycerate content of erythrocytes during storage (20) and an increase in the size of the NADP pool (17). The latter is an important regulator of the HMP. Consequently, the relative proportion of the HMP and Embden-Meyerhof pathways utilized, directly or indirectly, in treated cells may be important in the deactivation mechanism.

It may be pertinent to recall that deactivation of glutathione reductase in hemolysates following treatment with the HMP intermediate 6-phosphogluconate was also negated by the inclusion of NADP in the mixture (18). Deactivation of glutathione reductase by the present procedure therefore differed from flavin/apoenzyme dissociations in free solution which depend on physicochemical factors to promote separation of

Table IV. Effect of pH on the Rate of Glutathione Reductase Deactivation by ACD/Inosine/Adenine[a]

Time[c] (hr)	EGRAC pH 4.0[b] Control[d]	EGRAC pH 4.0[b] Treated[d]	EGRAC pH 6.0[b] Control[d]	EGRAC pH 6.0[b] Treated[d]
0	1.17	1.23	1.26	1.27
1	1.76	4.40	1.33	1.17
2	2.16	4.14	1.33	1.33
4	1.70	—	1.34	1.42
7	1.16	—	1.29	2.00
19	1.10	—	1.09	4.08

[a] Four volumes of washed red cell suspension in saline (approximately 50%) treated with 1 volume of ACD/inosine (25 m*M*)/adenine (12.5/m*M*).
[b] pH of ACD/inosine/adenine before addition to red cell.
[c] Time of incubation.
[d] Data from single experiment only. Confirmed by similar experiments not quoted.

Table V. Adenosine Phosphates in Red Cells Treated with ACD/Inosine/Adenine for 90 min at 37°C[a]

Treatment	EGRAC	Adenosine phosphates[b]			
		ATP	ADP	AMP	Total
Control	0.99 ± 0.07	5835 ± 257	554 ± 67	43 ± 14	6431 ± 327
ACD/inosine/adenine[c]	4.02 ± 0.50	4209 ± 383	1400 ± 383	727 ± 108	6336 ± 240
ACD/inosine/adenine + methylene blue[d]	1.13 ± 0.12	5151 ± 306	981 ± 50	255 ± 55	6387 ± 240
Control	1.11 ± 0.09	5071 ± 360	490 ± 87	66 ± 14	5604 ± 300
pH 4.0[e]	5.47 ± 1.13	3281 ± 240	1646 ± 209	860 ± 252	5787 ± 293
pH 6.0[e]	1.13 ± 0.08	5103 ± 360	225 ± 22	35 ± 3	5076 ± 360

[a] Mean ± SEM of three determinations.
[b] In nmol/g hemoglobin.
[c] Four volumes of washed erythrocytes in saline (approximately 50%) treated with 1 volume of ACD/inosine (25 m*M*)/adenine (12.5 m*M*).
[d] As in footnote *c* + 0.2 m*M* methylene blue.
[e] pH of ACD solution.

the flavin (21–23). Insofar as the degree of activation of erythrocyte glutathione reductase in hemolysates by FAD is a reflection of riboflavin status (7), EGRAC above 1.3 have been correlated with an increasing severity of riboflavin deficiency. The deactivation of washed erythrocytes *in vitro* by incubation with ACD/inosine/adenine solution therefore appeared to simulate riboflavin deficiency even to a degree greater than observed in vitamin-deprived subjects.

The effect of pH on the metabolic deactivation of glutathione reductase, over the limited range studied, appeared to be one of rate, and the reduction of intracellular pH by citrate buffer greatly accelerated deactivation. It should be emphasized that significant change in the EGRAC required the presence of inosine and/or adenine in addition to the ACD buffer system. Deactivation developed much more slowly at the higher pH but, as shown in Table IV, the EGRAC eventually reached a high value. No hemolysis or other obvious damage was observed within the pH range studied. Samples of whole blood treated with deactivation mixture also developed elevated EGRAC, but the deactivation was very slow and resembled the change in washed red cells treated with deactivation mixture at pH 6.0. Thus, the use of nonphysiologic conditions of pH may be regarded as a convenient experimental approach to a phenomenon which proceeds, albeit slowly, at a more physiologic value. It is also noteworthy that, irrespective of the artificial nature of the treatment, the EGRAC of riboflavin-replete red cells could be drastically increased by a relatively mild procedure which apparently depends upon metabolic processes within the intact cell. Hence, the activity of the enzyme could be modulated by factors other than riboflavin availability. Elevated EGRAC values may therefore indicate defects in flavin metabolism other than a direct deficiency of riboflavin and this may have a bearing on certain patients.

A standard mixture of adenine and inosine was used routinely in these experiments. Both compounds were roughly equally effective in initiating deactivation but relatively little additive effect was observed. This contrasted markedly to the stimulation of ATP synthesis in stored erythrocytes by a similar procedure where adenine greatly enhanced the effect of inosine alone (1, 2). A 10-fold dilution of the additives reduced the rate of deactivation but elevated EGRAC still resulted and increased even higher on extended incubation.

The possibility that one or more adenosine phosphates acted directly on the reductase was considered. ATP, ADP, and, to a lesser extent, AMP compete with FAD for binding sites on the "stripped" reductase (5, 24) whereas ATP and ADP are established competitive inhibitors of glutathione reductase (25). Any mechanism for the displacement of FAD from glutathione reductase by adenosine phosphates would seem to require a significant increase in the basal level of either ATP or total erythrocyte adenosine phosphates. As the present deactivation developed against a small decline in the ATP concentration and a virtually unchanged total adenosine phosphate level, none of these components appeared to be the immediate cause of the deactivation.

Conditions have been described which permitted the preparation *in vitro* of intact red cells in which glutathione reductase displayed a marked reversible deactivation. Using the criterion of the EGRAC index as a reflection of riboflavin deficiency, cells so treated resemble cells from riboflavin-deficient subjects and could possibly prove useful as a model for such a deficiency. It should be noted, however, that such a model is incomplete as defective glutathione reductase activity is only one component of an authentic riboflavin deficiency (26). Furthermore, the fate of FAD under the present deactivating conditions is unknown and the milieu within the present glutathione reductase-deactivated red cell may differ from that in authentic riboflavin-deficient cells. Nevertheless, the ease with which such a quasi riboflavin deficiency can be developed suggests that caution be exercised in the interpretation

of the EGRAC as exclusively diagnostic for the riboflavin status of the subject.

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1. Nakao M. ATP-requiring phenomena in red cell membranes. In: Yoshikawa H, Rapoport SM, Eds. Cellular and Molecular Biology of Erythrocytes. Baltimore: University Park Press, pp35–54, 1974.
2. Nakao M, Nakao T, Tatibana M, Yoshikawa H. Phosphorus metabolism in human erythrocytes. III. Regeneration of adenosine triphosphate in long stored erythrocytes by incubation with inosine and adenine. J Biochem **47**:661–671, 1960.
3. Glatzle D, Koerner WF, Christeller S, Wiss O. Method for the detection of biochemical riboflavin deficiency. Stimulation of NADPH-dependent glutathione reductase from human erythrocytes by FAD in vitro. Investigation of the vitamin B_2 status of healthy people and geriatric patients. Int J Vit Res **40**:166–183, 1968.
4. Tillotson JA, Baker ME. An enzymatic measurement of the riboflavin status in man. Am J Clin Nutr **25**:425–443, 1972.
5. Beutler E. Effect of flavin compounds on glutathione reductase activity: in vivo and in vitro studies. J Clin Invest **48**:1957–1966, 1969.
6. Bamji MS. Glutathione reductase activity in red blood cells and riboflavin nutritional status in humans. Clin Chim Acta **26**:263–269, 1969.
7. Nichoalds GE, Lawrence JD. Assessment of status of riboflavin nutriture by assay of erythrocyte glutathione reductase activity. Clin Chem **20**:624–628, 1974.
8. Podrazky V, Steven FS. Reversible inhibition by its coenzyme as a source of error in glutathione reductase assays. Clin Chim Acta **137**:349–354, 1984.
9. Beutler E, Duron O. Effect of pH on preservation of red cell ATP. Transfusion **5**:17–24, 1964.
10. Beutler E. In: Beutler E, Ed. Red Cell Metabolism. A Manuel of Biochemical Methods. 2nd ed. New York: Grune and Stratton, pp104–108, 1975.
11. Jaworek D, Gruber W, Bergmeyer UH. Adenosine 5′-diphosphate and adenosine 5′-monophosphate. In: Bergmeyer UH, Ed. Methods of Enzymatic Analysis. 2nd ed. New York: Academic Press, pp2127–2131, 1974.
12. van Kampen EJ, Zijlstra WG. Standardization of hemoglobinometry. II. The hemiglobincyanide method. Clin Chim Acta **6**:538–544, 1961.
13. Vir SC, Love AHG, Thompson W. Riboflavin status in pregnancy. Am J Nutr **34**:2699–2705, 1981.
14. Heunnekens FM, Nurk E, Gabrio BW. Erythrocyte metabolism: I. Purine nucleoside phosphorylase. J Biol Chem **221**:971–981, 1956.
15. Szeinberg A, Marks PA. Substances stimulating glucose catabolism by the oxidative reactions of the pentose phosphate pathway in human erythrocytes. J Clin Invest **40**:914–924, 1961.
16. Keiner MJ, Alexander NM. Methylene blue directly oxidizes glutathione without the intermediate formation of hydrogen peroxide. J Biol Chem. **260**:15168–15171, 1985.
17. Glader BE, Conrad ME. Cyanate inhibition of erythrocyte glucose 6-phosphate dehydrogenase. Nature **237**:336–337, 1972.
18. Carson PE, Frischer H, Noyes CM. Regulation of glutathione reductase activity. J Lab Clin Med **74**:860, 1969.
19. Carson PE. Clinical, metabolic and molecular consequences of genetic disorders of the pentose phosphate pathway. Proc R Soc Med **63**:175, 1970.
20. Wood LA, Beutler E. Effect of ascorbate on the maintenance of 2,3-diphosphoglycerate (2,3-DPG) in stored red cells. Br J Haematol **25**:611–618, 1973.
21. Warburg O, Christian W. Bemerkung ueber gelbe Fermente. Biochem Z **298**:368–377, 1938.
22. Stritmatter P. The nature of the flavin binding in microsomal cytochrome b_5 reductase. J Biol Chem **236**:2329–2335, 1961.
23. Massay V, Curti B. A new method for the preparation of D-amino acid oxidase apoprotein and a conformational change after its combination with flavin adenine dinucleotide. J Biol Chem **241**:3417–3423, 1966.
24. Beutler E. Glutathione reductase: Stimulation in normal subjects by riboflavin supplementation. Science **165**:613–615, 1969.
25. Icen A. Glutathione reductase of human erythrocytes. Purification and properties. Scand J Clin Lab Invest 96(suppl):5–87, 1967.
26. Prentice AM, Bates CJ. A biochemical evaluation of the glutathione reductase (EC 1.6.4.2) test for riboflavin status. 1. Rate and specificity of response in acute deficiency. Br J Nutr **45**:37–52, 1981.