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# Evidence That NADPH-Dependent Methemoglobin Reductase and Administered Riboflavin Protect Tissues From Oxidative Injury

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**NADPH-dependent methemoglobin reductase, first detected in erythrocytes sixty years ago, has subsequently been purified and characterized as a methylene blue reductase and a flavin reductase. The reductase plays no role in methemoglobin reduction under normal conditions, but its activity serves as the basis for the treatment of methemoglobinemia with methylene blue or flavin. On-going studies demonstrate that this cytosolic protein is also present in liver and that its primary structure distinguishes it from other known proteins. The bovine erythrocyte reductase tightly binds hemes, porphyrins, and fatty acids with resulting loss of activity. Pyrroloquinoline quinone serves as a high-affinity substrate of the reductase, suggesting that this naturally-occurring compound may be a physiological substrate. The ability of the reductase to catalyze the intracellular reduction of administered riboflavin to dihydroriboflavin suggested that this system might be exploited to protect tissues from oxidative damage. This hypothesis was supported by our finding that dihydroriboflavin reacts rapidly with Fe(IV)O and Fe(V)O oxidation states of hemeproteins, states that have been implicated in tissue damage associated with ischemia and reperfusion. Preliminary studies demonstrate that, as predicted, administration of low concentrations of riboflavin protects isolated rabbit heart from reoxygenation injury, rat lung from injury resulting from systemic activation of complement, and rat brain from damage caused by four hours of ischemia. Data from these animal studies suggest that flavin therapy holds promise in protecting tissue from the oxidative injuries of myocardial infarction, acute lung injury, stroke, and a number of other clinical conditions.**



**Key words: methemoglobin reductase, NADPH, riboflavin, oxidative injury**

## INTRODUCTION

In a volume dedicated to Ernst Jaffé, it is quite appropriate to review and discuss the methemoglobin reductases of erythrocytes, in that so many of us first learned about these reductases in the papers and reviews that he and his colleagues wrote over a number of decades. As illustrated in the 1966 review by Jaffé et al. [1], methemoglobin reduction in intact erythrocytes is catalyzed by an NADH-dependent system, but the rate of reduction can be enhanced greatly by the addition of methylene blue in a reaction catalyzed by an NADPH-dependent system. The present paper reviews the NADH-dependent and NADPH-dependent methemoglobin reductases and presents evidence that riboflavin therapy protects organs from ischemia and reperfusion injury by a mechanism which may involve NADPH-dependent methemoglobin reductase.

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## A REVIEW OF METHEMOGLOBIN REDUCTASES

### The NADH-Dependent Methemoglobin Reductase System

The ability of intact erythrocytes to reduce methemoglobin to hemoglobin was first described in 1891 by Dittrich [for a detailed review with reference citations, see ref. 2]. Studies from the laboratories of Warburg, Kiese, and Gibson in the 1930s and 1940s demonstrated that electrons for the reduction of methemoglobin could be derived from NADH generated during glycolysis. Gibson and coworkers reported that congenital enzymopenic methemoglobinemia was the result of an erythrocyte reductase deficiency, this being the first report of an enzyme deficiency giving rise to a congenital disease. The NADH-dependent methemoglobin reductase subsequently was isolated by Scott and coworkers, then further purified in other laboratories, and shown to be a flavoprotein with an FAD prosthetic group.

Hultquist and coworkers established that methemoglobin reduction in erythrocytes is mediated by cytochrome $b_5$. The NADH-dependent reductase functions as a cytochrome $b_5$ reductase and the resulting ferrous cytochrome $b_5$ reduces methemoglobin.

$$\text{NADH} + 2\ \text{Cyt } b_5\text{-Fe(III)} \xrightarrow{\text{Cyt } b_5 \text{ reductase}} \text{NAD}^+ + 2\ \text{Cyt } b_5\text{-Fe(II)} \quad (1)$$

$$\text{Cyt } b_5\text{-Fe(II)} + \text{Hb-Fe(III)} \longrightarrow \text{Cyt } b_5\text{-Fe(III)} + \text{Hb-Fe(II)} \quad (2)$$

Erthrocyte cytochrome $b_5$ and cytochrome $b_5$ reductase are cytosolic proteins that correspond closely in structure to the hydrophilic domains of liver microsomal cytochrome $b_5$ and cytochrome $b_5$ reductase. DNA studies suggest that a single gene codes for both the long and short forms of cytochrome $b_5$ reductase and a single gene codes for both forms of cytochrome $b_5$.

### The Early History of the NADPH-Dependent Methemoglobin Reductase System

NADPH-dependent "methemoglobin reductase" was first detected by Warburg on the basis of the protein's ability to catalyze a methylene blue-mediated reduction of methemoglobin in intact erythrocytes [3]. The reductase was first isolated by Kiese [4] and increasingly purer preparations of the enzyme were subsequently prepared in other laboratories [5–8].

The reductase functions as a methylene blue reductase and the resulting reduced form of the dye can rapidly reduce methemoglobin non-enzymatically [4, 9]. Similarly, the enzyme functions as a flavin reductase and the resulting dihydroflavin can rapidly reduce methemoglobin [5].

$$\text{NADPH} + \text{Flavin} \xrightarrow{\text{NADPH-dependent reductase}} \text{NADP}^+ + \text{Dihydroflavin} \quad (3)$$

$$\text{Dihydroflavin} + 2\ \text{Hb-Fe(III)} \longrightarrow \text{Flavin} + 2\ \text{Hb-Fe(II)} \quad (4)$$

The stimulation of methemoglobin reduction by methylene blue and by flavins is the basis for the therapeutic use of these compounds in the treatment of methemoglobinemia. Claims that this reductase plays an important role in methemoglobin reduction under normal conditions have been refuted on two counts. First, congenital deficiency of this reductase is not associated with methemoglobinemia [10]. Second, the $K_m$ of the reductase for flavins is at least 50-times higher than the normal concentration of free flavins in erythrocytes, and the extent to which methemoglobin reduction is mediated by such concentrations of flavin in vivo is considered to be insignificant [11].

### Structural Studies of NADPH-Dependent Methemoglobin Reductase

Hultquist and coworkers have purified and characterized two forms of the NADPH-dependent reductase from bovine erythrocytes [12–15]. The active form of the reductase is nearly colorless. In contrast, a green form of the protein exhibits no reductase activity but is highly reactive toward oxygen and peroxides. The green color of this form arises from the presence of a prosthetic group of unknown structure. The colorless and green proteins are monomers with protein moieties that are immunochemically cross-reactive and indistinguishable by electrophoretic migration, amino acid composition, and N-terminal sequence. The N-terminal sequence, VVK-KIALFGATGNTGLTTLAQAVQAGYEVTVLVRDP-, distinguishes the protein from other known mammalian proteins, including bovine erythrocyte glutathione S-transferase, which is a protein of similar subunit mass and similar binding properties [16]. The bovine reductase is homologous to NADPH-dependent reductase isolated from bullfrog erythrocytes [17].

### Detection of the NADPH-Dependent Reductase in Erythroid and Non-Erythroid Cells

Using antiserum prepared to highly purified bovine erythrocyte reductase, immunoreactive proteins have been detected in the cytosolic fractions of human, rabbit, rat, and bovine erythrocytes and in the cytosolic fractions of bovine and rat liver [15]. These results demonstrate that the NADPH-dependent reductase is not uniquely an erythroid protein. The bovine liver and erythrocyte proteins appear to have the same mass, as do the rat liver and erythrocyte proteins. Approximate molecular masses of

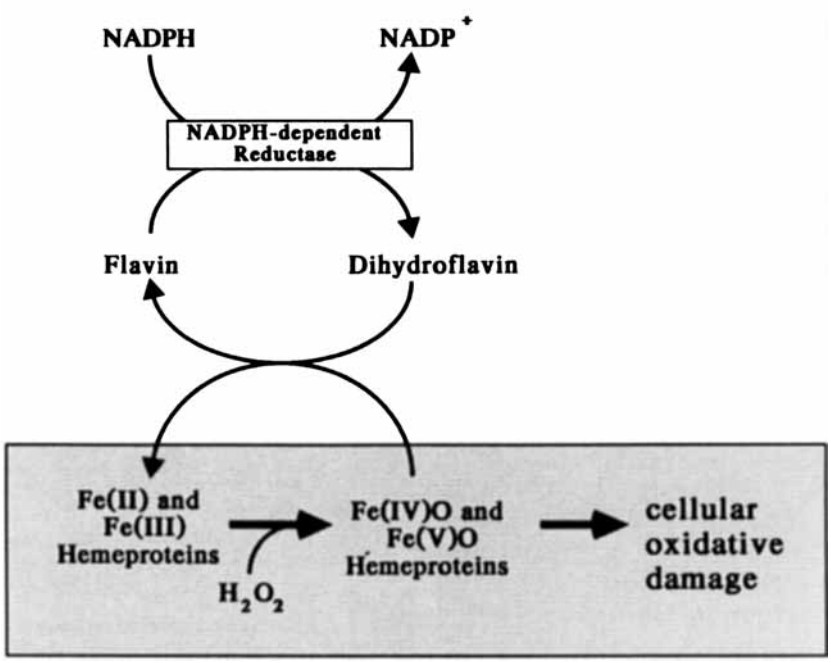


**Fig. 1. A proposed scheme for the participation of NADPH-dependent methemoglobin reductase and administered riboflavin in the protection of tissues from the oxidative damage resulting from reaction of peroxide with hemeproteins.**

the bovine, rat, rabbit, and human proteins were calculated to be 26,000, 27,000, 26,000, and 25,000, respectively. Quantitative immunoblotting indicates that the protein is present in bovine erythrocytes at a concentration of approximately 10 μM. Assay of reductase activity indicates a similar value in human erythrocytes [18].

### Characterization of the NADPH-Dependent Reductase as a Hemin-Binding Protein

The ability of intracellular free protohemin to damage cells is well recognized and intracellular hemin-binding proteins have been postulated to exist and to provide protection against such damage. Intracellular hemin-binding proteins have been proposed to play a role in hemin scavenging, storage, transport across intracellular membranes, regulation, and insertion into apoproteins.

The reductase form of the bovine erythrocyte enzyme has been shown to be a binding protein with a high affinity for protohemin and lower affinities for porphyrins [19]. Binding of hemins and porphyrins results in perturbation of the tetrapyrrole spectrum and non-competitive inhibition of reductase activity. Analysis of the binding data reveals that each reductase monomer binds a single tetrapyrrole and that the dissociation constant is 7 nM for the protohemin-reductase complex and 1 to 5 μM for the porphyrin-reductase complexes. Saturated and unsaturated fatty acids also inhibit activity and compete with protohemin for binding.

The affinity of the reductase for protohemin, its relatively high concentration in erythroid cells, and its presence in non-erythroid cells are all compatible with the idea that this protein functions as a hemin-binding protein. In addition to binding free protohemin, the reductase has also been observed to remove protohemin from glutathione S-transferase. Thus, addition of reductase to a hemin-complex of bovine erythrocyte glutathione S-transferase relieves the inhibition of the transferase. Since many cellular enzymes are inhibited by hemin, it is conceivable that the reductase may function to protect enzymes from such inhibition.

### Catalysis of PQQ Reduction by NADPH-Dependent Reductase

The NADPH-dependent reductase catalyzes the transfer of electrons from NADPH to pyrroloquinoline quinone (PQQ). Using a coupled reaction in which PQQ reduction is linked to the reduction of ferric cytochrome c in the absence of other electron acceptors or mediators, the apparent $K_m$ values of the reductase were calculated to be approximately 2 μM for PQQ and 0.4 μM for NADPH (Tang and Hultquist, unpublished data). This finding, in light of recently published evidence for the presence of PQQ in erythrocytes and other cells [20], suggests that PQQ may be involved with the functioning of the reductase in vivo.

### Catalysis of Fe(IV)O and Fe(V)O Reduction by NADPH-Dependent Reductase in the Presence of Riboflavin

Whereas the flavin reductase activity of NADPH-dependent reductase is sluggish and does not appear to play a significant role in methemoglobin reduction under normal conditions, one can not easily discount this activity as an artifact. Catalysis of flavin reduction does indeed occur in intact erythrocytes upon the administration of riboflavin, and the resulting flavin-mediated methemoglobin reductase activity has been exploited for the treatment of methemoglobinemia.

In searching for a physiological role for the dihydroflavin generated by the action of flavin reductase, dihydroriboflavin was observed to react very rapidly with the Fe(IV)O and Fe(V)O oxidation states of hemeproteins [21]. The Fe(IV)O state of myoglobin, generated by the reaction of ferrous myoglobin with peroxidase (Reaction 5), reacts with dihydroflavin to yield ferrous myoglobin (Reaction 6). Likewise, the Fe(V)O state of horseradish peroxidase, generated by reaction of ferric peroxidase with peroxide (Reaction 7), reacts with dihydroflavin to yield ferric peroxidase (Reaction 8).

$$\text{Mb-Fe(II)} + H_2O_2 \rightarrow \text{Mb-Fe(IV)O} + H_2O \quad (5)$$

$$\text{Mb-Fe(IV)O} + \text{dihydroflavin} \rightarrow \text{Mb-Fe(II)} + H_2O + \text{flavin} \quad (6)$$

$$\text{HRP-Fe(III)} + H_2O_2 \rightarrow \text{HRP-Fe(V)O} + H_2O \quad (7)$$

$$\text{HRP-Fe(V)O} + \text{dihydroflavin} \rightarrow \text{HRP-Fe(III)} + H_2O + \text{flavin} \quad (8)$$

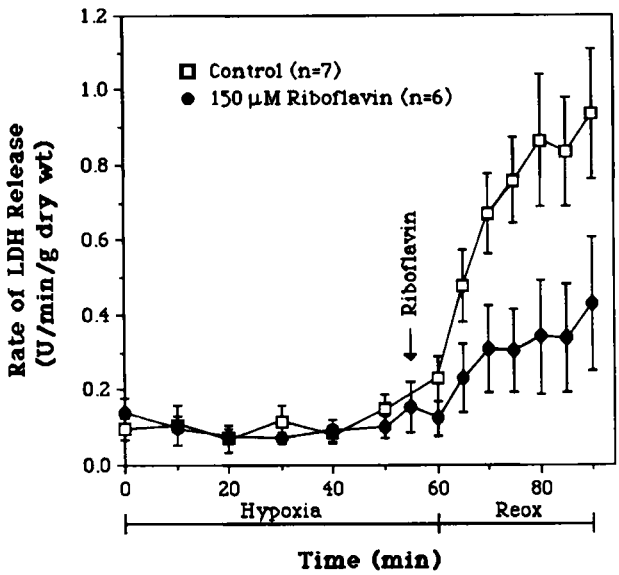


**Fig. 2. Effect of riboflavin on reoxygenation-induced injury to isolated rabbit hearts. Hearts were equilibrated for 30 min by retrograde perfusion with oxygenated (95% $O_2$ and 5% $CO_2$) physiologic saline solution (PSS), then perfused for 60 min with hypoxic (95% $N_2$ and 5% $CO_2$) PSS, and finally perfused with oxygenated PSS for 30 min. In 6 experiments, riboflavin (150 μM) was included in the PSS during the last 5 min of hypoxic perfusion (arrow) and throughout reoxygenation. Lactate dehydrogenase (LDH) release into the pulmonary artery effluent was measured as an indicator of damage. Data points are arithmetic means ± S.E.M. Control hearts (n = 7) showed considerable LDH release immediately after reoxygenation. Riboflavin administration provided significant protection against this reoxygenation injury.**

## PROTECTION OF CELLS AND ORGANS BY ADMINISTRATION OF RIBOFLAVIN

### A Proposed Mechanism for Protecting Cells From Oxidative Damage

Fe(IV)O and Fe(V)O complexes of hemeproteins may contribute appreciably to the oxidative damage of cells that occurs in injuries, medical procedures, aging, and a variety of pathological states, including ischemia, cardiac arrest, myocardial infarction, acute lung injury, and inflammatory diseases. Since NADPH-dependent reductase can generate dihydroflavin intracellularly and since this compound readily reduces the higher oxidation states of hemeproteins, these reactions together appear to constitute a mechanism by which a cell could protect itself from these extremely strong oxidizing agents. When Fe(IV)O and Fe(V)O reductions (Reactions 6 and 8) are coupled to the generation of dihydroflavin by flavin reductase (Reaction 3), the net reaction is the reduction of the higher oxidation states by NADPH.

Just as the intracellular concentration of free flavin determines the rate of methemoglobin reduction during riboflavin treatment of methemoglobinemia, so the rate of flavin-mediated reduction of Fe(IV)O and Fe(V)O complexes is expected to depend on the intracellular concentration of free flavin. Since the normal intracellular free flavin concentration is very small relative to the $K_m$ of the reductase for flavins, one would not expect this system to reduce the higher oxidation states efficiently under normal conditions. However, administration of a flavin, such as riboflavin, that quickly penetrates cells, would be expected to dramatically increase the rate of reduction. Thus, it was proposed that administration of riboflavin would protect cells and organs from oxidative damage. The mechanism proposed in Figure 1 depicts the sequential flow of electrons from NADPH to flavin to Fe(IV)O and Fe(V)O complexes, with NADPH-dependent reductase serving as catalyst.

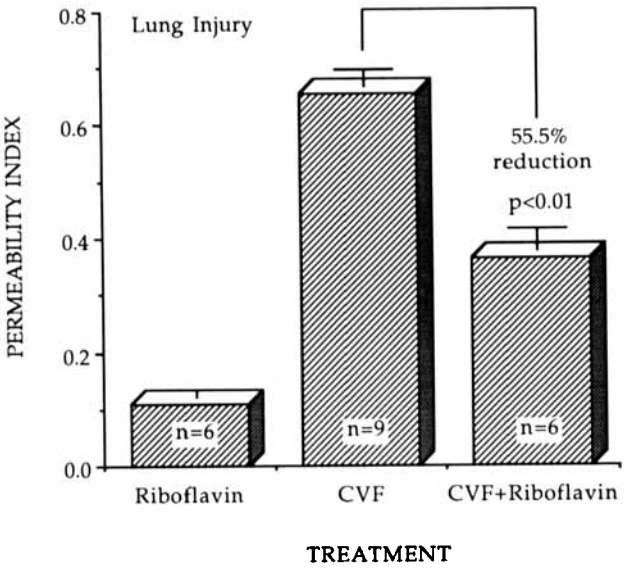


**Fig. 3. Protective effect of riboflavin on oxidant-mediated acute lung injury in the rat. Injury to lung vasculature was induced by intravenous injection of rats with 20 units of cobra venom factor (CVF)/kg body weight, which resulted in systemic complement activation [22]. The injury to lung vascular endothelial cells by complement-activated blood neutrophils was measured after 30 min. After exsanguination, lungs were removed and injury was assessed by measuring the increase in lung vascular permeability using $^{125}$I-bovine serum albumin. Ten minutes prior to CVF injection, nine Long-Evans rats were injected intraperitoneally with a riboflavin-saturated solution of 0.9% saline containing 50 mM glucose (6 μmol riboflavin/kg). Nine positive control rats received saline-glucose solution with no riboflavin 10 min before CVF injection. Six rats, serving as negative controls, were injected with saline instead of CVF. Data are presented as mean ± S.E.M. For calculation of % protection, the mean value for the negative controls was first subtracted from the mean values of the positive control and treatment groups. Administration of riboflavin was found to provide the lung vasculature with significant protection from oxidative damage.**

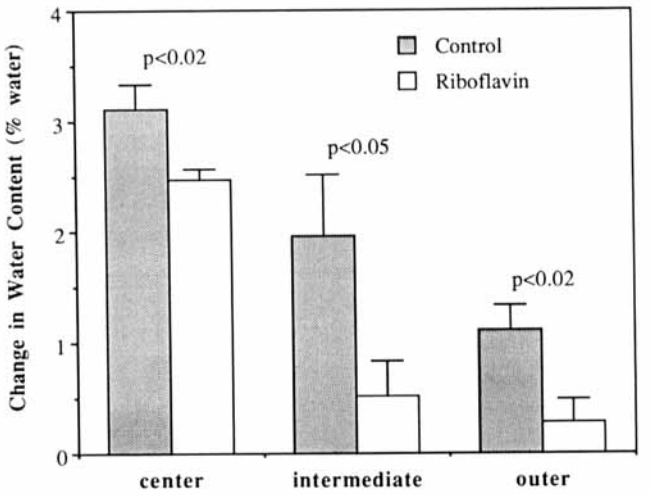


**Fig. 4. Effect of riboflavin treatment on ischemic injury to rat brain. Four Sprague-Dawley rats were pretreated with riboflavin (20 μmol/kg) by intraperitoneal injection of riboflavin-saturated saline solution one hour before the onset of ischemia. Four control rats received the identical volume of saline. Cerebral ischemia was produced by occlusion of the right middle cerebral artery under anesthesia with ketamine (50 mg/kg) and Rompun (10 mg/kg). Four hours later, the rats were killed, the brains were quickly removed, and the right and left cortexes were dissected into three zones. The center zone was a 7 mm diameter punch from the most ischemic tissue, the intermediate zone was a 10 mm diameter ring around the center zone, and the outer zone was the remaining cortex. The water content of each zone was calculated from the wet and dry weights of the tissue and the change in water content was calculated as the difference in water contents between the samples from ischemic and non-ischemic cortex for each zone. Data are presented as mean ± S.E.M. The significance of differences between the two groups was determined using a one-tailed Student's t-test. Riboflavin provided significant protection against ischemic damage in each of the areas of the brain.**

## Riboflavin Administration Protects Against Oxidative Damage in Models of Myocardial Infarction, Acute Lung Injury, and Stroke

In order to test the hypothesis that administration of flavin will protect cells and organs from oxidative damage, studies were designed to test the effect of riboflavin on the damage resulting from ischemia and reperfusion in animals and in isolated organs. Experiments by Mack and Shlafer demonstrate that riboflavin (150 μM) significantly reduces cardiac damage, assessed as lactate dehydrogenase release, to isolated rabbit hearts that were reoxygenated after 60 min of hypoxic perfusion (Fig. 2). In these studies riboflavin was administered after 55 min of hypoxia, corresponding to 5 min before reoxygenation. The protocol mimics a key pathophysiologic component of cardiac damage caused by the common clinical insult, ischemia and reperfusion. Biochemical and functional studies revealed that riboflavin causes no cardiotoxic effects.

Experiments by Seekamp and Till demonstrate the ability of riboflavin to protect rat lung from oxidative injury resulting from systemic activation of the complement system. Figure 3 illustrates the lung vasculature protection that is provided by intraperitoneal injection of riboflavin (6 μmol/kg body weight) 10 min prior to injection of cobra venom factor. Damage to the lung microvasculature (as measured by increased vascular permeability to radiolabeled bovine serum albumin) is diminished by riboflavin when neutrophil sequestration is initiated either by cobra venom factor or by hind limb ischemia and reperfusion. In these animal models for

acute lung injury, riboflavin provides protection at very low concentrations.

The ability of riboflavin to protect rat brain from the damage of ischemia is demonstrated in experiments by Betz and Ennis. Figure 4 illustrates that intraperitoneal injection of riboflavin (20 μmol/kg body weight) 1 hr prior to impairment of blood flow protects rat half-brains from 4 hr of ischemia. The degree of brain damage, measured as the increase in brain water content over a non-ischemic control, was reduced both in areas proximal and distal to the site of ischemia. In this animal model for stroke, very low concentrations of riboflavin protect the brain from swelling.

## DISCUSSION AND CONCLUSIONS

Despite the fact that the cellular function of NADPH-dependent "methemoglobin reductase" has not yet been established after more than 60 years of investigation, the studies reported here suggest that once again this enzyme can be exploited for therapeutic purposes. Our results with animal models suggest that flavin therapy may hold promise in protecting tissue from oxidative injury in a wide range of diseases, injuries, and medical procedures. Administration of flavin should be tested for its ability to protect heart tissue from reperfusion injury associated with myocardial infarction, cardiac arrest, and heart transplantation, to protect lung tissue from the oxidative damage associated with neutrophil-mediated acute lung injury, and to protect brain tissues from ischemic injury associated with stroke and head injury.

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## REFERENCES

1. Jaffé ER, Neumann G, Rothberg H, Wilson FT, Webster RM, Wolff JA: Hereditary methemoglobinemia with and without mental retardation: A study of three families. Am J Med 41:42, 1966.
2. Jaffé ER, Hultquist DE: Cytochrome $b_5$ reductase deficiency and enzymopenic hereditary methemoglobinemia. In Scriver CR, Beaudet AL, Sly WS, Valle D (eds): "The Metabolic Basis of Inherited Disease." 6th ed. New York: McGraw-Hill, 1989, p 2267.
3. Warburg O, Kubowitz F, Christian W: Über die katalytische Wirkung von Methylenblau in lebenden Zellen. Biochem Z 227:245, 1930.
4. Kiese M: Die Reduktion des Hämiglobins. Biochem Z 316:264, 1944.
5. Yubisui T, Matsuki T, Tanishima K, Takeshita M, Yoneyama Y: NADPH-flavin reductase in human erythrocytes and the reduction of methemoglobin through flavin by the enzyme. Biochem Biophys Res Commun 76:174, 1977.
6. Huennekens FM, Caffrey RW, Basford RE, Gabrio BW: Erythrocyte metabolism: Isolation and properties of methemoglobin reductase. J Biol Chem 227:261, 1957.
7. Shrago E, Falcone AB: Human-erythrocyte reduced triphosphopyridine nucleotide oxidase. Biochim Biophys Acta 67:147, 1963.
8. Scott EM, Duncan IW, Ekstrand V: The reduced pyridine nucleotide dehydrogenases of human erythrocytes. J Biol Chem 240:481, 1965.
9. Gibson QH: The reduction of methemoglobin in red blood cells and studies on the cause of idiopathic methemoglobinaemia. Biochem J 42:13, 1948.
10. Sass MD, Caruso CJ, Farhangi M: TPNH-methemogloblin reductase deficiency: A new red-cell enzyme defect. J Lab Clin Med 70:760, 1967.
11. Yubisui T, Takeshita M, Yoneyama Y: Reduction of methemoglobin through flavin at the physiological concentration by NADPH-flavin reductase of human erythrocytes. J Biochem (Tokyo) 87:1715, 1980.
12. DeFilippi LJ, Hultquist DE: The green hemoproteins of bovine erythrocytes: Purification and characterization. J Biol Chem 253:2946, 1978.
13. DeFilippi LJ, Hultquist DE: The green hemoproteins of bovine erythrocytes: Spectral, ligand-binding, and electrochemical properties. J Biol Chem 253:2954, 1978.
14. DeFilippi LJ, Ballou DP, Hultquist DE: Reaction of bovine erythrocyte green hemeprotein with oxygen and hydrogen peroxide. J Biol Chem 254:6917, 1979.
15. Quandt KS, Xu F, Chen P, Hultquist DE: Evidence that the protein components of bovine erythrocyte green heme binding protein and flavin reductase are identical. Biochem Biophys Res Commun 178:315, 1991.
16. Xu F, Hultquist DE: Bovine erythrocyte glutathione S-transferase: Purification, inhibition, and complex formation. Biochem. Int. 27:265, 1992.
17. Abe Y, Ito T, Okazaki T: Purification and characterization of NADPH-dependent methemoglobin reductase from the nucleated erythrocytes of bullfrog, *Rana catesbeiana*. J Biochem (Tokyo) 108:255, 1990.
18. Yubisui T, Matsuki T, Takeshita M, Yoneyama Y: Characterization of the purified NADPH-flavin reductase of human erythrocytes. J Biochem (Tokyo) 85:719, 1979.
19. Xu F, Quandt FS, Hultquist DE: Characterization of NADPH-dependent methemoglobin reductase as a heme-binding protein present in erythrocytes and liver. Proc Natl Acad Sci (USA) 89:2130, 1992.
20. Gallop PM, Paz MA, Flückiger R: The biological significance of quinonoid compounds in, on, and out of proteins. Biochem Mol Biol 1:357, 1990.
21. Xu F, Hultquist DE: Coupling of dihydroriboflavin oxidation to the formation of the higher valence states of hemeproteins. Biochem Biophys Res Commun 181:197, 1991.
22. Till GO, Johnson KJ, Kunkel R, Ward PA: Intravascular activation of complement and acute lung injury. Dependency on neutrophils and toxic oxygen metabolites. J Clin Invest 69:1126, 1982.