Reduction and uptake of methylene blue by human erythrocytes

James M. May, Zhi-chao Qu, and Charles E. Cobb

1Medicine and 2Molecular Physiology and Biophysics, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6303

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May, James M., Zhi-chao Qu, and Charles E. Cobb. Reduction and uptake of methylene blue by human erythrocytes. Am J Physiol Cell Physiol 286: C1390–C1398, 2004. First published February 18, 2004; 10.1152/ajpcell.00512.2003.—A thiazine dye reductase has been described in endothelial cells that reduces methylene blue (MB), allowing its uptake into cells. Because a different mechanism of MB uptake in human erythrocytes has been proposed, we measured MB uptake and reduction in this cell type. Oxidized MB (MB\(^+\)) stimulated reduction of extracellular ferricyanide in a time- and concentration-dependent manner, reflecting extracellular reduction of the dye. Reduced MB was then taken up by the cells and partially oxidized to MB\(^+.\) Both forms were retained against a concentration gradient, and their redox cycling induced an oxidant stress in the cells. Whereas concentrations of MB\(^+\) \(<5\ \mu M\) selectively oxidized NAD(P)H, higher concentrations also oxidized both glutathione (GSH) and ascorbate, especially in the absence of d-glucose. MB\(^+\)-stimulated ferricyanide reduction was inhibited by thiol reagents with different mechanisms of action. Phenylarsine oxide, which is toxic for cell-free thiols in proteins, inhibited MB\(^+\)-dependent ferricyanide reduction more strongly than it decreased cell GSH and pentose phosphate cycle activity, and it did not affect cellular NADPH. Open erythrocyte ghost membranes facilitated saturable NAD(P)H oxidation by MB\(^+\), which was abolished by pretreating ghosts with low concentrations of trypsin and phenylarsine oxide. These results show that erythrocytes sequentially reduce and take up MB\(^+\), that both reduced and oxidized forms of the dye are concentrated in cells, and that the thiazine dye reductase activity initially responsible for MB\(^+\) reduction may correspond to MB\(^+\)-dependent NAD(P)H reductase activity in erythrocyte ghosts.

THE REDOX DYE METHYLENE BLUE (MB\(^+\)) has long been used clinically to reverse methemoglobinemia caused by genetic deficiencies and metabolic poisoning (43). For example, MB\(^+\) has been used as an antidote to paraquat poisoning (14), to treat isosamidase encephalopathy (1, 17), and to maintain blood pressure in patients in septic shock (33) and during orthotopic liver transplantation (16). Until recently, the mechanism by which MB\(^+\) is taken up and converted to its active reduced form (MBH) seemed to be established, at least in erythrocytes. Sass et al. (36) showed that MB\(^+\) added to erythrocytes in suspension was taken up by the cells. During 20 min of incubation, cells increased the concentration of the dye 150- to 200-fold compared with extracellular MB\(^+\) concentrations. It was proposed that MB\(^+\) freely diffuses across the erythrocyte membrane and is rapidly reduced to MBH, which is then trapped in the cell (36). On the basis of their studies in pulmonary arterial endothelial cells, however, Merker and colleagues (3, 26, 27, 30) concluded that MB\(^+\) is reduced at the exocellular cell surface. The resulting MBH diffuses into the cell, where it is oxidized to MB\(^+\), which is then trapped in the cell. Direct evidence for this conclusion is that the cells reduce a cell-impermeant polymer of toluidine blue at rates comparable to those of MB\(^+\) (3). Indirect evidence derives from the ability of MB\(^+\) to stimulate the reduction of ferricyanide (3, 27). Ferricyanide, which does not cross the cell membrane, owing to its size and charge (41), is directly reduced by MBH. Thus MB\(^+\)-dependent ferricyanide reduction must necessarily be due to MBH outside the cell or to trans-plasma membrane reduction of ferricyanide by intracellular MBH. Furthermore, entry of the positively charged MB\(^+\) across the cell membrane will likely be slower than that of the more lipophilic MBH (34). These considerations are at odds with the earlier conclusions drawn from erythrocytes regarding which form of the dye is transported and whether MB\(^+\) is reduced at the exocellular cell surface, within the cells, or both. It is also possible that the transmembrane thiazone dye reductase activity described in endothelial cells (3) is absent in erythrocytes.

A cell surface site for MB\(^+\) reduction also brings into question the long-held notion that MB\(^+\) is directly reduced within cells by NADPH (29, 36). Clearly, NADPH generated in the pentose phosphate cycle is required for erythrocyte reduction of MB\(^+\), because cells of patients with glucose 6-phosphate dehydrogenase deficiency have impaired ability to take up the dye (36) and to carry out MB\(^+\)-dependent methemoglobin reduction (43). This could be explained if NADPH or reducing equivalents derived from NADPH served as the cofactor for a transmembrane thiazone dye reductase that reduces MB\(^+\) on the cell surface. However, the substrate specificity of this activity has not been determined.

MBH that has entered or has been generated within cells can be oxidized by molecular oxygen (9, 25), but its major fate in cells is to reduce Fe\(^3+\) in prosthetic groups of numerous cellular proteins and enzymes (25). In erythrocytes, the major acceptor for electrons from MBH is Fe\(^3+\) (in) (hem)oglobin (37), whereas in other cells, MBH donates electrons to heme groups of cytochrome c (25), guanylate cyclase (8), and nitric oxide synthase (24). Although MB\(^+\) accumulates in certain as yet unidentified organelles in endothelial cells (27), whether MB\(^+\) or MBH accumulates in erythrocytes is unclear. Although Sass et al. (36) could detect little MB\(^+\) in diluted suspensions of erythrocytes treated with MB\(^+\), blue staining of erythrocytes has been proposed as the basis for a clinical test for glucose 6-phosphate dehydrogenase deficiency (32).

Because the mechanisms of uptake or reduction of MB\(^+\) are relevant to its clinical use as well as in providing insight into
cellular metabolism redox dyes, we studied the effects of MB⁺ in human erythrocytes. We report that, as in other cell types, there is extracellular reduction of MB⁺ and that MB⁺ accumulates as MB⁺ and MBH in erythrocytes as a function of its extracellular concentration. At MB⁺ concentrations >5 μM, MB⁺ generates an oxidant stress in the cells that consumes ascorbic acid. The transmembrane reduction of extracellular MB⁺ seems to be mediated by a protein, because it is sensitive to inhibition by thiol reagents and enzyme digestion in erythrocyte ghosts devoid of cytoplasm.

MATERIALS AND METHODS

Materials. MB⁺, diethylmaleate (DEM), N-ethylmaleimide (NEM), phenylarsine oxide (PAO), and other analytical reagents were purchased from Sigma (St. Louis, MO). Diethylmaleate and PAO were initially dissolved in DMSO, such that the highest concentration of the latter was 0.2% at diethylmaleate and PAO concentrations of 0.5 mM and 50 μM, respectively. New England Nuclear Life Science Products (Boston, MA) supplied [1-¹⁴C]glucose.

Preparation of erythrocytes and erythrocyte ghosts. Erythrocytes were prepared from blood obtained by venipuncture from healthy volunteers. The cells were washed three times in 10 volumes of PBS that contained 140 mM NaCl and 12.5 mM Na₂PO₄, pH 7.4. Theuffy coat of white cells was removed with each wash. Where indicated, cells at a 5% packed cell volume were loaded with ascorbate by incubation for 15 min at 37°C with 0.2 mM freshly prepared dehydroascorbic acid (DHA). Leaky or clear erythrocyte ghosts were prepared from intact cells as previously described (40).

Assay of ferricyanide reduction. Reduction of extracellular ferricyanide to ferrocyanide was measured in aliquots of cell supernatants by the ortho-phenanthroline method of Avron and Shavit (2). Corrections were made as necessary in a paired sample for the absorbance generated in the presence of cells by the same concentration of MB⁺ without ferricyanide. At 20 μM MB⁺, this value was <20% of that observed in the presence of ferricyanide. Results are expressed relative to the intracellular water space, which was taken as 70% of the packed cell volume (31).

Uptake of MB⁺. The uptake and/or reduction of MB⁺ by erythrocytes was measured as the decrease in absorbance of cell supernatants after removal of cells by microfuging at 13,600 g for 1 min. Absorbance of MB⁺ was measured at 610 nm, and the MB⁺ concentration was calculated by using a measured extinction coefficient of 8.8 mM⁻¹-cm⁻¹ in PBS.

The intracellular content of MB⁺ and MBH was assessed as follows. Cells at a 10% packed cell volume were loaded with 40 μM MB⁺ in PBS containing 5 mM t-glucose for 30 min at 37°C. The cells were then pelleted in the cold in a microfuge, and the supernatant was then removed for measurement of extracellular MBH and MB⁺. The former was determined on the basis of the absorbance at 610 nm, and the latter was calculated as the difference between total dye concentration (determined after addition of 2 mM ferricyanide) and MB⁺ alone. The cells were then hemolyzed with 10 volumes of deionized water that contained either no additives or 2 mM ferricyanide. The hemolysate was ultrafiltrated through a Centricon YM-10 membrane (Amicon, Beverly, MA) by performing centrifugation at 3°C for 10 min at 5,000 g. The concentration of MB⁺ was calculated on the basis of the absorbance at 610 nm. Dye that was bound to cell protein or lipid was measured as follows. The remaining hemolysate was treated with 0.1 ml of metaphosphoric acid, diluted 10-fold with 100 mM sodium phosphate (pH 8.0), and microfuged. The concentration of MB⁺ was determined on the basis of the absorbance at 610 nm. Each sample obtained from cells treated with MB⁺ was corrected for absorbance in a paired sample not exposed to MB⁺ that had or had not been treated with ferricyanide, as appropriate. This correction was <15% of the reading in MB⁺-treated cells.

Measurement of the ascorbate free radical by electron paramagnetic resonance spectroscopy. Time-dependent changes in ascorbate free radical (AFR) concentration were measured by electron paramagnetic resonance spectroscopy as previously described (21).

Assay of radiolabeled glucose metabolism in human erythrocytes. Glucose metabolism through the pentose phosphate pathway was measured as the generation of [¹⁴C]CO₂ from [U-¹⁴C]glucose, as previously described in erythrocytes (6).

Other assays. Intracellular concentrations of glutathione (GSH) were measured according to the method of Hissin and Hilf (11), as previously described (22). The recycling method of Zerez et al. (44) was used to measure pyridine nucleotide concentrations in 2-mlsylates of erythrocytes, except that a concentration of 1 mM (rather than 2 mM) phenazine ethosulfate was used, as recommended by Wagner and Scott (42). Intracellular ascorbic acid was measured as previously described (18) by performing HPLC with electrochemical detection (23), except that tetrapentylammonium bromide was used as an ion pair reagent. Intracellular concentrations of GSH, pyridine nucleotides, and ascorbate were expressed relative to the intracellular water space as noted in Assay of ferricyanide reduction.

Data analysis. Results are shown as means ± SE. Differences between treatments were analyzed with SigmaStat 2.0 software (Jandel Scientific, San Rafael, CA). Comparisons of more than two treatments were made by using either one- or two-way ANOVA with post hoc testing by the Dunnett test. P < 0.05 was considered significant.

RESULTS

Stimulation of erythrocyte ferricyanide reduction by MB⁺. To determine the extent to which erythrocytes reduce MB⁺ to MBH that can be detected outside the cells, we measured the effect of MB⁺ on time-dependent reduction of extracellular ferricyanide. As noted earlier, ferricyanide is directly reduced by MBH. Because of its size and trivalent negative charge, however, ferricyanide does not enter erythrocytes (41), so it encounters only extracellular MBH. In the absence of MB⁺, erythrocytes reduced ferricyanide in a time-dependent manner (Fig. 1A, ○). When 10 μM MB⁺ was also present, ferricyanide reduction was increased by two- to threefold compared with samples treated with ferricyanide alone (Fig. 1A, □), depending on the time of sampling.

Ferricyanide reduction increased in a concentration-dependent manner with increasing amounts of MB⁺ (Fig. 1B, ○). For example, at 20 μM MB⁺, rates were more than five times greater than with ferricyanide alone. When cells were incubated in the absence of both D-glucose and MB⁺, basal rates of ferricyanide reduction decreased by 30% (Fig. 1B, □). MB⁺-stimulated ferricyanide reduction decreased by ~50% in the absence of D-glucose across the range of MB⁺ concentrations (Fig. 1B, □). These results show that erythrocyte reduction of both ferricyanide and MB⁺ requires glucose metabolism for full activation. Glucose was superior to other energy substrates, because 10 mM concentrations of pyruvate and inosine sustained rates of ferricyanide reduction stimulated by 10 μM MB⁺ that were only 16 and 67%, respectively, of those observed with D-glucose.

Correlation of MB⁺ uptake with ferricyanide reduction. The reduction and uptake of MB⁺ were measured as the decrease in its concentration in the incubation medium with time. In the presence of D-glucose, the MB⁺ concentration outside the cells gradually decreased during 30 min of incubation (Fig. 2A, ○). This could be due either to reduction of MB⁺ to the colorless MBH outside the cells or to uptake of MB⁺ or MBH into the
cells, or both. When d-glucose was omitted from the incubation, the extracellular MB⁺ concentration did not change (Fig. 2A, □). This finding is in agreement with the decrease in MB⁺-stimulated ferricyanide reduction in the absence of d-glucose, shown in Fig. 1A. These results confirm those shown in Fig. 1B in that reducing equivalents from glucose metabolism are necessary for sustained reduction of MB⁺.

When ferricyanide was included in incubations containing d-glucose, extracellular MB⁺ concentrations also did not change (Fig. 2A, △). That is, net MB⁺ uptake into the cells did not occur in the presence of ferricyanide, despite the fact that ferricyanide reduction was enhanced by MB⁺ under these conditions (Fig. 1). The failure of extracellular MB⁺ concentrations to decrease shows that there is little or no uptake of the dye as MB⁺ by the cells. Whether there is uptake of the dye as MBH was addressed by incubating cells under the conditions shown in Fig. 2A with 14 μM MB⁺ in the presence or absence of 2 mM ferricyanide. After 30 min, 2 mM ferricyanide was added to incubations not already containing it. The cells were pelleted in the microfuge, and aliquots of the supernatant were obtained for assay for extracellular MB⁺ and ferrocyanide. As expected, cells exposed to MB⁺ in the presence of ferricyanide showed no change in extracellular MB⁺ concentration compared with that initially present in the medium (Fig. 2B, compare first and fourth bars), whereas cells exposed to MB⁺ alone showed a 79% decrease in extracellular MB⁺ compared with MB⁺ plus ferricyanide (Fig. 2B, compare second and fourth bars). Cells incubated for 30 min with MB⁺ and then treated with ferricyanide just before assay for MB⁺ showed a 50% decrease in extracellular MB⁺ compared with cells treated with ferricyanide during the 30-min incubation with MB⁺ (Fig. 2B, compare fourth and fifth bars). As expected, ferricyanide reduction was tripled by coinubcation with MB⁺ (Fig. 2C, compare third and fourth bars), but there was little ferricyanide reduction when ferricyanide was added after 30 min of incubation with MB⁺ (Fig. 2C, compare fourth and fifth bars). These results show that 63% of the MB⁺ initially added is actually taken up by the cells during the 30-min incubation in the absence of ferricyanide, whereas 37% of that present outside the cells is MBH.

The question of whether the dye accumulated in the cells as MB⁺ or as MBH was addressed by measuring MB⁺ in erythrocyte hemolysates, as shown in Table 1. After 30 min of incubation of 10% packed cells with ~38 μM MB⁺, the cells took up and/or reduced 71 ± 6% of the dye. One-half of the intracellular dye was free, and one-half was bound to proteins and/or lipids in the hemolysate. The fact that acid treatment released the dye suggests that binding was ionic, but it was not possible to determine the redox state of the bound dye. The cells concentrated free MB⁺ 14-fold, free MBH 4.3-fold, and bound dye 18-fold. These findings confirm a marked accumulation of both forms of the dye against a concentration gradient.

Sources of cellular reducing equivalents for MB⁺. The source of reducing equivalents for MB⁺-stimulated ferricyanide reduction was investigated by measuring changes in cell concentrations of NAD(P)H in response to increasing amounts of MB⁺. As shown in Fig. 3A, NADPH (○) decreased and NADP⁺ (□) increased in response to treatment with MB⁺, even in the presence of d-glucose. Approximately one-half of the NADPH was oxidized by the lowest concentration of MB⁺, or 1 μM. Similar results were observed for NADH, with a decrease in NADH (○) and a compensatory increase in NAD⁺ (□) over the same MB⁺ concentration range (Fig. 3B). These results show that erythrocyte pyridine nucleotide metabolism is exquisitely sensitive to MB⁺ and that the reduced forms of both major nucleotides are decreased in a parallel manner.

To determine whether and to what extent MB⁺ caused generalized oxidant stress, erythrocyte GSH and ascorbate concentrations were measured. Whereas MB⁺ did not affect erythrocyte GSH in the presence of d-glucose (Fig. 4A, ○), in the absence of d-glucose, GSH decreased in response to increasing concentrations of MB⁺, reaching ~50% at 20 μM MB⁺ (Fig. 4A, □). Endogenous erythrocyte ascorbate concentrations were decreased by MB⁺, even in the presence of d-glucose (Fig. 4B, ○). The decrease in ascorbate in the absence of d-glucose was greater than in its presence, although there was little decrease in ascorbate at MB⁺ concentrations <5 μM.
Evidence for direct oxidation of ascorbate by MB⁺ was obtained in buffer and in erythrocytes. Incubation of 1 mM ascorbate in PBS resulted in an AFR signal, which was quantified over time by measuring the amplitude of the low-field peak of the AFR spectrum (Fig. 5, inset). The AFR concentration in buffer alone was stable for 10 min at ~2.7 μM and was increased to ~4 μM by 10 μM MB⁺ (Fig. 5A). Next, erythrocytes were loaded with ascorbate by incubation with 250 μM DHA. This generates an intracellular ascorbate concentration of ~1–1.5 mM in erythrocytes (20). No AFR was detected in these cells during incubation in medium without MB⁺. When cells were treated with 10 and 100 μM MB⁺, however, there was a concentration-dependent (although not linear) increase in AFR that was sustained for ≥10 min (Fig. 5B). These results show that MB⁺ oxidizes ascorbate and generates the AFR in intact cells loaded with ascorbate.

**Role of cell protein thiols in MB⁺ reduction.** If MB⁺ is reduced outside erythrocytes, then the mechanism might involve a transmembrane thiazine dye reductase, as suggested by Merker and colleagues (26, 27). Therefore, three thiol reagents with different mechanisms of action were used to probe the protein in the erythrocyte membrane. DEM selectively depletes GSH in erythrocytes after conjugation with GSH by glutathione S-transferase (4). NEM irreversibly alkylates GSH and protein thiols (15). PAO is an arsenical with specificity for reversible reaction with vicinal dithiols (7). In the experiments listed in Table 2, the activity of the thiazine dye reductase in intact cells was measured indirectly as ferricyanide reduction. When cells were incubated for 30 min with DEM or NEM, basal rates of ferricyanide reduction were decreased by 30–
40% but were not significantly affected by PAO. MB⁺-stimulated ferricyanide reduction was again decreased by 30–40% by DEM and NEM, but it was completely ablated by PAO. That is, PAO treatment decreased ferricyanide reduction due to

![Fig. 3. Oxidation of NAD(P)H by MB⁺. Erythrocytes at a 5% packed cell volume were incubated at 37°C in PBS that contained 5 mM D-glucose and the indicated concentration of MB⁺. After 30 min, the cells were washed 3 times by centrifugation and taken for assay of reduced and oxidized pyridine nucleotides. A: NADPH and NADP⁺; B: NADH and NAD⁺. Results are from 3 experiments with NADPH and 5 experiments with NADH.](image)

![Fig. 4. Generation of oxidant stress by MB⁺. Erythrocytes at a 5% packed cell volume were incubated for 30 min at 37°C in PBS in the absence or presence of 5 mM D-glucose and the indicated concentration of MB⁺. This was followed by 3 cell washes, centrifugation, and measurement of GSH (A) and ascorbate (B) in the cell pellets. Results are from 4 experiments with GSH and 5 experiments with ascorbate.](image)

![Fig. 5. Oxidation of ascorbate (ASC) by MB⁺. A: oxidation of 1 mM ascorbate was measured in PBS that contained either no additives or 10 μM MB⁺, as indicated. The concentration of the ascorbate free radical (AFR) was determined by analysis of the low-field peak height in the AFR spectrum (inset, arrow) and is shown as a function of time. B: erythrocytes at a 40% packed cell volume were incubated at 37°C in PBS that contained 5 mM D-glucose and 0.25 mM docosahexaenoic acid (DHA). After 15 min, the indicated concentration of MB⁺ was added, and the sample was mixed and injected into a flat cell for recording at 37°C as described in A. AFR concentration is shown per total volume; to obtain intracellular AFR concentrations, multiply by 3.6.](image)

Table 2. Inhibition of FC reduction and oxidation of GSH by thiol reagents

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FC reduction, μmol·ml⁻¹·30 min⁻¹</th>
<th>GSH, mM</th>
</tr>
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<tbody>
<tr>
<td>No additions</td>
<td>5.4 ± 0.2†</td>
<td>1.83 ± 0.05</td>
</tr>
<tr>
<td>FC alone (2 mM)</td>
<td>14.5 ± 1.3*</td>
<td>1.69 ± 0.10</td>
</tr>
<tr>
<td>MB⁺ (10 μM) + FC</td>
<td>2.9 ± 0.2†</td>
<td>0.49 ± 0.09†</td>
</tr>
<tr>
<td>DEM (0.5 mM) + FC</td>
<td>8.1 ± 0.7†</td>
<td>0.53 ± 0.11†</td>
</tr>
<tr>
<td>DEM + FC + MB⁺</td>
<td>2.8 ± 0.3†</td>
<td>0.43 ± 0.05†</td>
</tr>
<tr>
<td>NEM (0.5 mM) + FC</td>
<td>7.1 ± 1.3†</td>
<td>0.40 ± 0.08†</td>
</tr>
<tr>
<td>NEM + FC + MB⁺</td>
<td>4.6 ± 0.3†</td>
<td>1.04 ± 0.12†</td>
</tr>
<tr>
<td>PAO (10 μM) + FC</td>
<td>5.0 ± 0.8†</td>
<td>1.11 ± 0.03†</td>
</tr>
<tr>
<td>PAO + FC + MB⁺</td>
<td>1.8 ± 0.2†</td>
<td>1.83 ± 0.05</td>
</tr>
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Values are means ± SE from at least 4 experiments for each determination. Erythrocytes at a 5% packed cell volume were incubated at 37°C in PBS that contained the indicated agent and 5 mM D-glucose, followed at 30 min by addition of 2 mM ferricyanide (FC) where noted. After 30 min of incubation at 37°C, the cells were pelleted in a microfuge. Aliquots of the supernatant were assayed for ferrocyanide, and the cell pellets were washed 3 times by centrifugation and taken for assay of glutathione (GSH). *P < 0.05 compared with sample treated with MB⁺ and FC in the same column. †P < 0.05 compared with sample treated with MB⁺ and FC in the same column. DEM, diethylmaleate; NEM, N-ethylmaleimide; PAO, phenylarsine oxide.
MB\(^+\) at the same rates as observed with either PAO plus ferricyanide or ferricyanide alone. The effect of PAO on ferricyanide reduction was mirrored by its ability to inhibit MB\(^+\) uptake. In the studies shown in Fig. 2A, PAO was also included in parallel incubations. Whereas the extracellular concentration of MB\(^+\) decreased by 80\% in cells incubated for 30 min with 10 \(\mu\)M MB\(^+\) alone (Fig. 2A, ○), it decreased by only 10 ± 2\% in cells incubated with 50 \(\mu\)M PAO and 10 \(\mu\)M MB\(^+\) (\(n = 6\) experiments; \(P < 0.05\)). This shows that PAO inhibited MB\(^+\)-dependent ferricyanide reduction and MB\(^+\) uptake to a similar extent, which is compatible with inhibition of a common step in both pathways.

As shown in the second column of Table 2, GSH concentrations in the cells were unaffected by either MB\(^+\) or ferricyanide alone, but they were decreased by ~80\% by both DEM and NEM. In contrast, PAO decreased GSH by only 45\% in the absence of MB\(^+\) and by 39\% in its presence. These differential changes in MB\(^+\)-stimulated ferricyanide reduction and GSH content suggest that PAO has effects on MB\(^+\) uptake or reduction that are not directly dependent on GSH. Therefore, PAO was used to define the mechanism of MB\(^+\) uptake and reduction in subsequent studies.

PAO was first used to assess the extent to which MB\(^+\)-dependent ferricyanide reduction might be mediated by the transmembrane ferricyanide reductase activity that uses intracellular ascorbate as a source of electrons (31). To allow direct comparison, results were expressed as fractions of ascorbate- or MB\(^+\)-stimulated activity, corrected for basal ferricyanide reduction (Fig. 6). At a 20 \(\mu\)M concentration, MB\(^+\) increased ferricyanide reduction 2.6-fold compared with treatment of cells with ferricyanide alone, from 6.0 ± 0.5 to 15.9 ± 1.5 \(\mu\)mol/ml cells\(^{-1}\)·30 min\(^{-1}\). MB\(^+\)-stimulated ferricyanide reduction decreased significantly at 10 \(\mu\)M PAO and was decreased maximally by >90\% at 50 \(\mu\)M PAO. Preloading the cells with 0.5 mM DHA to yield intracellular ascorbate concentrations of ~1.5–2 mM (20) increased ferricyanide reduction 4.8-fold, from 4.6 ± 1.0 to 22 ± 3.8 \(\mu\)mol/ml cells\(^{-1}\)·30 min\(^{-1}\). Ascorbate-stimulated ferricyanide reduction was significantly decreased with 20 \(\mu\)M PAO, but it was inhibited by only 27\% with 100 \(\mu\)M PAO. This contrasts with nearly complete inhibition of MB\(^+\)-stimulated ferricyanide reduction and suggests that the two activities are not mediated by the same protein.

The extent to which PAO inhibited MB\(^+\)-stimulated ferricyanide reduction by its effects on intracellular metabolism was examined. As shown in Fig. 7A, treatment of cells with PAO alone had no effect on cellular NADPH content. Activity of the pentose phosphate cycle was inhibited by PAO, but this reached significance only at PAO concentrations of ≥100 \(\mu\)M (Fig. 7B). Whereas MB\(^+\)-stimulated ferricyanide reduction was dependent on NADPH and cellular glucose metabolism, the inhibition of this activity by PAO was not.

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**Fig. 6. Phenylarsine oxide (PAO) inhibition of ascorbate- and MB\(^+\) dependent ferricyanide reduction.** Erythrocytes at a 5\% packed cell volume were loaded with ascorbate by incubation for 15 min at 37°C in PBS that contained 5 mM D-glucose and 0.5 mM DHA. Different aliquots of cells were also treated with 10 \(\mu\)M MB\(^+\) for 30 min under the same conditions without DHA. PAO was added to all samples at the indicated concentration for 30 min, after which ferricyanide was added to a concentration of 2 mM. The incubations were continued for another 30 min, the cells were pelleted in a microfuge, and aliquots of the supernatant were obtained for assay of ferricyanide. Results are from 6 experiments and are expressed as a fraction of the result in a sample not incubated with PAO, corrected for the baseline rate of ferricyanide reduction. \(*P < 0.05\) compared with control in original data.

**Fig. 7. Effects of PAO on cellular NADPH content and on the pentose phosphate cycle.** A: erythrocytes at a 5\% packed cell volume were incubated at 37°C in PBS that contained 5 mM D-glucose and the indicated concentration of PAO. After 30 min, the cells were washed 3 times by centrifugation and taken for assay of NADPH and NADP\(^+\). Results are based on 4 experiments. B: erythrocytes at a 10\% packed cell volume were incubated at 37°C in PBS that contained 8 mM NaHCO\(_3\), 0.5 mM D-glucose, and the indicated concentration of PAO. After 30 min, 0.05 \(\mu\)Ci of D-[1-\(^{14}\)C]glucose was added, and accumulation of \(^{14}\)CO\(_2\) was measured. Results are from 3 experiments. \(*P < 0.05\) compared with sample not exposed to PAO.
Together, these results support the notion that at least part of the effect of PAO on MB²⁺-stimulated ferricyanide reduction and MB²⁺ uptake and/or reduction is due to modification of the transmembrane thiazine dye reductase. This possibility was explored with the use of erythrocyte ghosts.

**Involvement of a membrane protein in MB²⁺ reduction.** Erythrocyte ghosts lacking hemoglobin and other cytosolic components were prepared and incubated with increasing concentrations of MB²⁺ in the presence of NADH or NADPH as electron donors. Because of the low absorbance of MB²⁺ and MBH at 340 nm (results not shown), it was possible to follow NAD(P)H oxidation at this wavelength in the presence of MB²⁺. Rates of NADH and NADPH oxidation were linear for ≥3 min (results not shown) and increased with increasing MB²⁺ concentrations (Fig. 8A). The relative increases in NADH and NADPH oxidation rates were similar, although the rate for NADPH appeared to level off. For both nucleotides, the rates were corrected for direct oxidation of NADH and NADPH by MB²⁺, which were linear and 22 ± 4% and 16 ± 3% of the total rate at 10 μM MB²⁺, respectively. These results show a saturable increase in NAD(P)H oxidation that is best explained by the activity of a membrane-bound enzyme. To further investigate this protein, we studied its sensitivity to trypsin and to PAO.

When ghosts were treated with trypsin for 30 min before assay of NADH oxidation, the rate of oxidation was markedly decreased at trypsin concentrations as low as 10 μg/ml (Fig. 8B). Similarly, NADH oxidation was strongly inhibited by preincubation of cells with low concentrations of PAO for 30 min before assay of NADH oxidation (Fig. 8C). Together, these results suggest that MB²⁺ facilitation of NAD(P)H oxidation by erythrocyte ghosts is protein dependent. Treatment of intact cells with 100 μg/ml trypsin had no effect on the rate of NADH-dependent MB²⁺ reduction by the ghosts (results not shown).

**DISCUSSION**

Human erythrocytes reduce MB²⁺ to MBH that can be detected and quantified outside cells by measurement of ferricyanide reduction (Fig. 1). In contrast to earlier conclusions (36), there is no evidence that MB²⁺ itself is taken up by the cells, because no dye is internalized when it is maintained as MB²⁺ in the presence of ferricyanide (Fig. 2A). This contrasts with the behavior of anthracycline doxorubicin (34) and xanthones (12), which are positively charged at physiological pH but cross the erythrocyte membrane with half-times of 1 to 10 min, respectively. Our results show that MBH rather than MB²⁺ crosses the cell membrane, as diagrammed in Fig. 9. These findings support the conclusions of Bongard et al. (3) that MBH is generated at the exofacial cell surface and is either oxidized to MB²⁺ when ferricyanide is present or crosses the cell membrane to enter cells in the absence of ferricyanide.

Both MB²⁺ and MBH are concentrated in erythrocytes (Table 1). The presence of MB²⁺ in erythrocytes in this and a previous study (32), as well as in endothelial cells (27), shows that intracellular MBH undergoes oxidation with trapping of MB²⁺ in the cells. Oxidation of MBH by Fe³⁺ in hemoglobin (37) and in Fe³⁺-containing enzymes is responsible for the beneficial effects of MB²⁺, such as reduction of methemoglobin and activation of guanylate or nitric oxide synthases. If these pathways are saturated, however, MBH will react with molecular oxygen to generate superoxide and other reactive oxygen species (ROS) that can be damaging to the cells (25, 39) (Fig. 9). This mechanism is present in erythrocytes, because MB²⁺ concentrations of ≥5 μM increase oxygen uptake (9, 29) and oxidize ascorbate and GSH (Figs. 4 and 5). We (19) recently reported that a similar range of MB²⁺ concentrations also oxidized ascorbate and GSH in cultured endothelial cells.

![Fig. 8. NAD(P)H oxidation by erythrocyte ghosts.](image-url)
associated with oxidation of dihydrofluorescein. The latter provides strong evidence for the generation of excess ROS (10). The present study shows in addition that excess ROS are due not as much to the initial reduction of MB\(^+\) at the cell surface as to its redox cycling.

Redox cycling of MB\(^+\) is readily quantified as MB\(^+\)-stimulated ferricyanide reduction. At an initial extracellular concentration of \(\sim 10 \mu\text{M}\), correcting for basal rates of ferricyanide reduction and considering that 1 mole of MBH reduces 2 moles of ferricyanide, erythrocytes at a 5% packed cell volume reduce \(\sim 88 \text{ nmol of } \text{MB}^+\) for 30 min in the absence of D-glucose and 225 nmol in the presence of D-glucose (Fig. 1B). The latter is \(\sim 20\) times the amount of MB\(^+\) in the system. This is likely the reason that MBH is also strongly concentrated against a gradient in the cells. Paradoxically, it is likely the high capacity of the cells to reduce MB\(^+\) and generate MBH, not the direct effects of MB\(^+\) itself, that causes excess oxidant stress. That both NADH and NADPH are severely depleted by low extracellular MB\(^+\) concentrations (Fig. 3) demonstrates their crucial role in MB\(^+\) reduction. In addition, our results point to contributions by both ascorbate and GSH in MB\(^+\) recycling (Fig. 4). Although MB\(^+\) can be reduced directly by both ascorbate and GSH in MB\(^+\) recycling (Fig. 4). Although MB\(^+\) can be reduced directly by both ascorbate (Fig. 5A; see Ref. 35) and GSH (13), the glucose dependence and depletion of both at higher MB\(^+\) concentrations may also reflect ascorbate scavenging of intracellular ROS, with subsequent GSH-dependent reduction of ascorbate (Fig. 4).

Whereas activation of the pentose phosphate cycle by NADP\(^+\) generated by NADPH oxidation is clearly required to sustain MB\(^+\) reduction (5, 38), a role for NADP has been suggested only indirectly in studies using glycolytic inhibitors in erythrocytes (37) and in pulmonary artery endothelial cells (26). Our results, showing nearly complete oxidation of NADH by low concentrations of MB\(^+\) (Fig. 3B), indicate that NADH generated in glycolysis also plays a role. The differential sensitivity to oxidation of NAD(P)H compared with ascorbate and GSH by MB\(^+\) in glucose-utilizing erythrocytes suggests, in fact, that low concentrations of MB\(^+\) might be a good tool with which to selectively deplete the pyridine nucleotides.

The present findings are in accord with the results in endothelial cells pointing to a transmembrane thiazine dye reductase as the initial mechanism for MB\(^+\) reduction to MBH, which then diffuses into the cells. That this activation is mediated by a protein is indicated by its sensitivity to inhibition by thiol reagents. This may in part reflect the dependence of a crucial protein thiol on GSH or on the cell redox state, because DEM should have some specificity for GSH alone. The results with PAO also suggest that there are sensitive vicinal thiols on the protein, especially because PAO inhibited MB\(^+\)-dependent ferricyanide reduction more strongly than it decreased GSH. The specificity of PAO is further indicated by its lack of effect on NADPH or on pentose phosphate cycle activity at concentrations effective for inhibition of MB\(^+\)-stimulated ferricyanide reduction (Fig. 7). PAO strongly inhibited MB\(^+\)-dependent NADH oxidation in erythrocyte ghosts (Fig. 8C), which supports the notion that the ghost activity might correspond to reduction of MB\(^+\) by cells.

The identity of the putative transmembrane thiazine dye reductase remains to be established. Merker et al. (28) concluded that because it was not activated by intracellular ascorbate in endothelial cells, it did not correspond to the transmembrane ascorbate-dependent ferricyanide oxidoreductase. We provide additional evidence that the two activities are mediated by different proteins, with the observation that MB\(^+\) reductase is much more sensitive than the ascorbate-dependent activity to inhibition by PAO (Fig. 6). It is possible that the thiazine dye reductase corresponds to the MB\(^+\) reductase activity that we were able to measure in erythrocyte ghosts. The latter likely reflects the activity of an enzyme, because it was saturable with MB\(^+\) and inhibited by both trypsin and PAO. Inhibition by trypsin in ghosts but not in intact cells suggests that the protein is mostly exposed on the cytosolic membrane face. Because either NADH or NADPH was a substrate for the ghost activity, the observed depletion of NAD(P)H in intact cells by low concentrations of extracellular MB\(^+\) (Fig. 3) suggests that pyridine nucleotides are substrates for the initial extracellular reduction of MB\(^+\). It is possible to compare the rates of ghost NAD(P)H-dependent MB\(^+\)-reductase with those of ferricyanide reduction in cells, because the latter reflect only rates of MB\(^+\) reduced at the cell surface. At 10 \(\mu\text{M MB}\(^+\), ghosts oxidized \(\sim 10 \text{ nmol-mg protein}\)^{-1}\(\text{min}^{-1}\) of NADH or NADPH (Fig. 8A). Considering that 1 ml of packed erythrocytes yields \(3.5 \text{ mg of } \text{ghost protein}\), this would correspond to a rate of 35 nmol/ml packed red cells\(^{-1}\)min\(^{-1}\). Because 2 moles of ferricyanide reduces 1 mole of NAD(P)H, the rate of MB\(^+\)-dependent NAD(P)H oxidation induced by ferricyanide in intact cells would be \(\sim 79 \text{ nmol-mg protein}\)^{-1}\(\text{min}^{-1}\) from the 10-min point shown in Fig. 1A. Thus the ghost activity accounts for roughly one-half of the rate of ferricyanide reduction in intact cells. This seems to be a reasonable conclusion because the MB\(^+\)-reductase activity might not be fully active in ghosts compared with intact cells. Whereas the evidence that the ghost activity and that measured in cells reflects activity of the same protein is only circumstantial at this point, erythrocyte ghosts provide a system in which to test this possibility. Furthermore, the presence of sensitive thiols on the protein will be useful in its purification, reconstitution, and ultimate identification.

In conclusion, we have confirmed the presence of a transplasma membrane thiazine dye reductase in erythrocytes that reduces extracellular MB\(^+\) to MBH, which then is taken up by the cells and undergoes redox cycling. The latter generates an
oxidant stress that also depletes low-molecular-weight antioxidants. MB+ reductase activity is sensitive to inhibition by thiol reagents in intact cells, suggesting that it is a protein. This activity may correspond to MB+-dependent NAD(P)H reductase activity in erythrocyte ghosts, which would aid in its identification.

GRANTS

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