Nitrite uptake and metabolism and oxidant stress in human erythrocytes

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Received 9 June 2000; accepted in final form 7 August 2000

May, James M., Zhi-Chao Qu, Li Xia, and Charles E. Cobb. Nitrite uptake and metabolism and oxidant stress in human erythrocytes. Am J Physiol Cell Physiol 279: C1946–C1954, 2000.—Nitric oxide, when released into the bloodstream, is quickly scavenged by Hb in erythrocytes or oxidized to nitrite. Nitrite can also enter erythrocytes and oxidize Hb. The goals of this work were to determine the mechanism of erythrocyte nitrite uptake and whether this uptake causes oxidant stress in these cells. Erythrocytes took up 0.8 mM nitrite with a half-time of 11 min. Nitrite uptake was sensitive to temperature and to the pH and ionic composition of the medium but was not inhibited by the specific anion-exchange inhibitor DIDS. About 25% of nitrite uptake occurred on the sodium-dependent phosphate transporter and the rest as diffusion of nitrous acid or other species across the plasma membrane. Methemoglobin formation increased in proportion to the intracellular nitrite concentration. Nitrite reacted with erythrocyte ascorbate, but ascorbate loading of cells decreased nitrite-induced methemoglobin formation only at high nitrite concentrations. In conclusion, nitrite rapidly enters erythrocytes and reacts with oxyhemoglobin but does not exert a strong oxidant stress on these cells.

NITRIC OXIDE (NO) is released in the vascular bed by endothelial cell NO synthase and by the inducible form of the enzyme present in activated inflammatory cells (25). Much of the NO released into the bloodstream should be scavenged by Hb in erythrocytes (16) or converted to nitrite in the presence of molecular oxygen (13, 29). Because of its stability, nitrite has been used extensively to confirm the previous existence of NO (4, 22). Nitrite will also be removed from the bloodstream by erythrocytes, although the rapidity with which this uptake occurs, as well as its mechanism, is controversial. Using nitrite-induced formation of methemoglobin as a measure of nitrite uptake, Zavodnik et al. (40) found that rates of methemoglobin formation were similar in erythrocytes and in erythrocyte hemolysates. This led them to conclude that the cell membrane poses little barrier to the entry of nitrite. In contrast, microscopic photometric analysis of methemoglobin formation in single erythrocytes showed that nitrite enters the cells very slowly and not at all in the presence of glucose (36). In another study, the half-time for disappearance of 60 μM nitrite outside pig erythrocytes was reported to be ~12 min, which was considered slow relative to the circulation time (33).

In addition to wide variation in measurements of erythrocyte nitrite uptake, the mechanism by which nitrite enters erythrocytes is also unclear. In resealed erythrocyte ghosts, nitrite efflux was accelerated by other monovalent anions and inhibited by DIDS, which is a specific inhibitor of the anion-exchange (AE-1) protein in this cell (31). These findings implicate the latter protein in nitrite transport. However, the more recent studies of Zavodnik et al. (40) failed to show inhibition of nitrite-induced methemoglobin formation by DIDS when erythrocytes were incubated with nitrite. If nitrite does not enter erythrocytes by the AE-1 protein, then the means by which it is taken up remain to be established.

Once inside erythrocytes, nitrite reacts with oxyhemoglobin, which leads to the formation of nitrate and methemoglobin (10). The extent to which nitrite is reduced by the major low-molecular-weight antioxidants within erythrocytes is uncertain. Nitrite added to erythrocytes has been reported to oxidize GSH in rat erythrocytes (3). However, concern had been previously raised that GSH loss in such studies was due to its destruction after cell lysis (5). Ascorbic acid has long been known to reduce nitrite to NO (20, 27), and this has been proposed as a mechanism for extracellular NO regeneration in brain (26). However, ascorbate reduction of nitrite is rapid only at acid pH (9, 18, 27), and intracellular reaction of nitrite and ascorbate has not been demonstrated.

To address these issues, nitrite uptake and metabolism were studied in human erythrocytes. Erythrocytes removed nitrite from the medium in a dose-dependent manner that was very sensitive to temperature and pH, moderately sensitive to sodium and phosphate in the medium, and insensitive to DIDS. Together, these results favor diffusion of nitrite or nitrous acid (HNO2) across the erythrocyte membrane, with some uptake on...
the sodium-dependent phosphate transporter. Nitrite within erythrocytes reacted primarily with Hb but also depleted ascorbate when Hb was partly converted to methemoglobin. There was a slow but detectable reaction of ascorbate with nitrite that was associated with ascorbate depletion in intact cells. However, the high capacity for erythrocytes to scavenge nitrite is due largely to rapid uptake and reaction with Hb.

EXPERIMENTAL PROCEDURES

Materials. Sigma/Aldrich Chemical (St. Louis, MO) supplied dehydroascorbic acid (DHA), tetrapentylammonium bromide, sulfanilic acid, DIDS, sodium nitrite, HEPES, and sodium ascorbate. New England Nuclear Life Science Products (Boston, MA) supplied H32PO4.

Preparation of erythrocytes and resealed erythrocyte ghosts. Blood was drawn from volunteers just before each experiment and was anticoagulated with heparin. Except where noted, erythrocytes were prepared by three centrifugation washes in 10 volumes of PBS. The latter consisted of 140 mM NaCl and 1.5 mM Na2HPO4 in deionized water, pH 7.4. The buffy coat of leukocytes was carefully removed from the surface of the erythrocyte pellet with each wash.

Measurement of methemoglobin. Packed erythrocytes were hemolyzed by dilution with mixing in >10 volumes of deionized water. Methemoglobin in hemolysates was determined according to Winterbourn (38) as the difference in absorbance of diluted lysate between 577 and 630 nm, adjusted for the respective extinction coefficients of oxyhemoglobin and methemoglobin. In these crude hemolysates, there was a background of absorption at 630 nm due to cell membranes and cytosolic components. The level of this background present in control cells was subtracted from each determination of methemoglobin, and the results were expressed as the percent increase in methemoglobin relative to total Hb (oxyhemoglobin + methemoglobin).

Assay of intracellular ascorbate, GSH, GSSG, nitrite, and nitrate. The ascorbate content of erythrocytes was measured as previously described (23). This method uses filtration of diluted erythrocyte hemolysate through a Centricon YM-10 membrane (Millipore, Bedford, MA) to obtain a clear Hb-free filtrate that is suitable for direct assay of ascorbate by HPLC. The same method was used to prepare samples for measurement of the cellular contents of GSH (12) and GSSG (35). Concentrations of intracellular antioxidants, nitrite, and nitrate are expressed relative to the cytoplasmic space, which was taken as 70% of the packed cell volume (28).

Measurement of nitrite uptake. Erythrocytes at a 20% hematocrit were incubated at the indicated temperature in PBS that contained 5 mM D-glucose and 1.2 mM sodium nitrite, unless otherwise noted. At specified times, aliquots of mixed cells and buffer were removed, the cells were pelleted in a microfuge, and the nitrite concentration was determined in the supernatant. Intracellular nitrite was measured following ultrafiltration of hemolysates that had been hemolyzed by dilution in six volumes of water, as described above for assay of ascorbate and GSH. Nitrite was measured according to the method of Saville (30), with omission of ammonium sulfamate and mercuric chloride. The sensitivity of the assay was 0.5 μM with the use of nitrite standards. Nitrate was measured in the same samples as nitrite by the copper-cadmium method of Cortas and Wakik (8).

 Ionic dependence of nitrite uptake. Erythrocytes at a 20% hematocrit were washed three times by centrifugation in five volumes of buffers of different ionic compositions before measurement of nitrite uptake, as described in Measurement of nitrite uptake. All buffers contained 5 mM D-glucose and were adjusted to pH 7.4 just before the incubations. These buffers were PBS, PBS in which sodium had been replaced by potassium of the same molarity, and PBS in which phosphate had been replaced by 12.5 mM HEPES.

Measurement of radioactive phosphate uptake. Erythrocytes at a 2.5% hematocrit were incubated at room temperature in 1 ml of HEPES buffer that contained 5 mM D-glucose, 25 μM DIDS, 1 μCi of H32PO4, and the indicated concentration of nitrite. After 20 min, the cells were pelleted in a microfuge, washed once in 1 ml of ice-cold HEPES buffer, and lysed with 0.2 ml of 25% m-phosphoric acid. The suspension was mixed and then microfuged, and an aliquot of the supernatant was placed in 4 ml of liquid scintillation fluid and counted in a Packard CA-2000 liquid scintillation counter on settings for 32P with quench correction. Radioactivity was corrected for the results of a time 0 sample and normalized to the total radioactivity added.

Ascorbate and nitrite loading of erythrocytes. To increase intracellular concentrations of ascorbate in erythrocytes, cells at a 20% hematocrit were incubated for 15 min at 37°C in PBS containing 5 mM D-glucose and 400 μM DHA. DHA is taken up by the cells on the glucose transporter and immediately reduced to ascorbate, which is then trapped in the cells (37). We previously showed that ascorbate leakage from erythrocytes loaded in this manner is negligible over a 30-min incubation at 37°C (24). Loading of cells with nitrite was carried out by incubating control or DHA-treated cells with the indicated concentration of nitrite for an additional 60 min at 37°C. In some experiments, extracellular nitrite and nitrate concentrations were measured at this point. To determine intracellular nitrite and nitrate concentrations after nitrite loading, cells were pelleted in a microfuge, washed three times by centrifugation in 10 volumes of ice-cold PBS, and taken for determination of nitrite, nitrate, or methemoglobin. Use of ice-cold buffer prevented the efflux of nitrite during the centrifugation washes.

Electron paramagnetic resonance spectroscopy for detection of the ascorbate free radical. Changes in the concentration of the ascorbate free radical (AFR) were measured by electron paramagnetic resonance (EPR) spectroscopy. EPR data were acquired on a Bruker EMX 8/27 spectrometer equipped with a BVT300 variable-temperature controller. Sample temperature was maintained at 37°C during data acquisition by blowing precooled nitrogen into the cavity through the front optical port. X-band EPR spectra were collected using an ER041XG-DHA microwave bridge and ER4103TM/9614 cavity. Sample incubations were carried out in a Wilmad WG 804 aqueous-flow flat cell. The magnetic field was fixed at a value that corresponded to the maximum signal intensity of the low field resonance line of the AFR. The conversion time of the spectrometer was chosen to result in a sweep time of 167 s (since each data set contained 2,048 points, the timing resolution was ~0.062 s/point). Other spectrometer settings were as follows: 0.5 G modulation amplitude, 100 kHz modulation frequency, and 10 mW microwave power. The flat cell was preloaded with buffer or a suspension of erythrocytes at the indicated hematocrit to permit tuning of the spectrometer, and the experiment was initiated by starting the time sweep to record a baseline signal. After ~10 s, buffer or erythrocyte suspension that had been mixed with nitrite was injected, and the change in AFR signal amplitude was followed with time. The concentration of the AFR was determined by calibration of the instrument using the signal of 10 μM Tempol.

Statistical analysis. Values are means ± SE from the indicated number of experiments. Differences between treat-
ments were analyzed with the computer program SigmaStat 2.0 (Jandel Scientific, San Rafael, CA) using two-way ANOVA with post hoc testing by Tukey’s test or paired Student’s t-test as appropriate. P < 0.05 was considered significant.

RESULTS

Nitrite uptake by erythrocytes. As shown in Fig. 1A, nitrite was stable under the incubation conditions used in these studies. However, in the presence of 20% erythrocytes, the extracellular nitrite concentration fell progressively during incubation at 37°C, reaching ∼15% of the initial value at 60 min. Fitting the latter results with a monoexponential function and residual resulted in a half-time of 11 min. The disappearance of nitrite from the medium outside erythrocytes was mirrored in time by an increase in methemoglobin within the cells (Fig. 1B). When nitrite had fallen to low levels outside the cells, methemoglobin formation ceased. Cells incubated without nitrite formed little methemoglobin (Fig. 1B). Together, these results show that the cell-induced decrease in extracellular nitrite concentrations was due to its uptake and subsequent reaction with Hb.

Uptake of nitrite and nitrite-induced methemoglobin formation did not occur when uptake assays were carried out with cells on ice (results not shown). The rate of nitrite uptake by erythrocytes was sensitive to the pH of the medium and decreased as the pH was increased from 6.9 to 7.8 (Fig. 2). After a logarithmic transformation of the data, the slopes of the resulting lines were significantly different by two-way ANOVA between the pH 7.8 curve and the pH 6.9 curve. These results suggest that nitrite enters cells via a charge-sensitive mechanism.

The rate of removal of nitrite from the incubation medium by erythrocytes, measured over 10 min at 37°C, was linear up to an initial extracellular nitrite concentration of 1 mM (results not shown). Since ≥2 mM nitrite resulted in almost complete conversion of oxyhemoglobin to methemoglobin, nitrite uptake at >1 mM was not tested. Nonetheless, these results show that nitrite uptake is not a high-affinity process.

To evaluate a possible role for the erythrocyte AE-1 protein in nitrite uptake, the time dependence of nitrite uptake was followed in cells that had been pre-treated with the anion transport inhibitor DIDS. Neither nitrite uptake nor nitrite-induced methemoglobin formation was affected by 25 μM DIDS (results not shown). This indicates that nitrite uptake does not occur on the AE-1. The glucose transport inhibitors cytochalasin B (50 μM) and phloretin (100 μM) were also without effect on nitrite uptake by erythrocytes.

Ionic dependence of nitrite uptake. Nitrite uptake by erythrocytes was sensitive to the ionic composition of the incubation medium. As shown in Fig. 3A, replacing sodium in PBS with potassium significantly slowed the rate of nitrite entry into erythrocytes compared with a control incubation in PBS. Substituting HEPES for phosphate as the buffer anion enhanced nitrite uptake to a small but significant extent over a 30-min incubation (Fig. 3B). The inhibition by phosphate was further evaluated by measuring effects of increasing nitrite concentrations on 32Pi uptake. Uptake of 32Pi was linear with time under these conditions (results not shown). Since phosphate is known to be largely transported on the AE-1 protein (32), incubations also contained 25 μM DIDS to block this component. As shown
in Fig. 4, increasing concentrations of nitrite significantly inhibited $^{32}$P uptake in DIDS-treated cells, although the effect was partial and observed only at ≥2 mM nitrite. Phosphonoacetic acid, which inhibits the Na-P$_i$ cotransporter in renal brush-border membranes with an inhibition constant of 1.3 mM (34), had no effect on erythrocyte nitrite uptake at ≤15 mM (results not shown). Nonetheless, the inhibition of $^{32}$P$_i$ uptake by nitrite and the inhibition of nitrite uptake by phosphate suggest that nitrite can use the Na-P$_i$ cotransporter present in erythrocytes, albeit with a low affinity.

**Oxidant stress induced by nitrite.** The fate of nitrite incubated with erythrocytes was evaluated with the experiments shown in Figs. 5 and 6. Erythrocytes were first incubated with increasing concentrations of nitrite, washed by centrifugation to remove remaining extracellular nitrite, and taken for assay of intracellular nitrite and methemoglobin. To increase as much as possible the intracellular concentrations of nitrite in this experiment at a given initial extracellular nitrite concentration, the hematocrit was 10%, rather than 20%, as used in the experiment shown in Fig. 1. Nevertheless, intracellular nitrite concentrations did not increase appreciably until the extracellular nitrite concentration exceeded 200 μM (Fig 5A). On the other hand, consistent increases in methemoglobin content were observed after the cells were incubated with 100 μM extracellular nitrite (Fig. 5B). The sensitivity of methemoglobin formation to the intracellular nitrite concentration becomes more apparent when methemoglobin formation is expressed as a function of the measured intracellular nitrite concentration for the control sample (Fig. 5C). In three of the experiments shown in Fig. 5, intracellular nitrate was also measured and was unchanged by increasing extracellular nitrite concentrations (results not shown). At 1 mM added nitrite, almost one-half of the Hb in these cells...
was converted to methemoglobin during the 60-min incubation.

To determine whether intracellular ascorbate can affect intracellular nitrite or its reaction with Hb during nitrite uptake, erythrocytes were loaded with ascorbate by preincubation with 400 μM DHA. This treatment increased the intracellular ascorbate concentration from that of plasma (20–50 μM) to >1.5 mM (see legend to Fig. 8). Such a marked increase in the intracellular ascorbate concentration caused decreases in concentrations of intracellular nitrite (Fig. 5A) and methemoglobin (Fig. 5B), but statistical significance was observed only at the two highest initial nitrite concentrations.

Incubation of erythrocytes with increasing concentrations of nitrite for 60 min resulted in corresponding increases in extracellular nitrite concentrations (Fig. 6). The increase in extracellular nitrite indicates oxidation of intracellular nitrite to nitrate and release of nitrate from the cells. To assess how much nitrite had not been taken up by the cells, extracellular nitrite concentrations were also measured in these experiments. Less than one-half of the total nitrite added was measured as nitrite and nitrate, suggesting that there were other products of nitrite oxidation or that nitrite reacted with cellular proteins, such as Hb. Loading the cells with ascorbate by a 15-min preincubation with 400 μM DHA (Fig. 5) was without effect on the increases in extracellular nitrite or nitrate (not shown).

The mechanism by which ascorbate decreased intracellular nitrite and methemoglobin formation (Fig. 5) was investigated. It is possible that the ascorbate-induced decrease in intracellular nitrite was due to interference by ascorbate with the assay for nitrite. Incubation of 1.0 mM nitrite with an equimolar concentration of ascorbate at pH 7.4 for 30 min in PBS at 37°C caused a 12 ± 2% (n = 5, P < 0.01) decrease in the nitrite concentration compared with that in a control incubation. This decrease was not abolished by destruction of the remaining ascorbate with ascorbate oxidase just before assay of nitrite, which indicates that the ascorbate effect was not due to direct interference by ascorbate with the nitrite assay. Reaction of nitrite and ascorbate at pH 7.4 in PBS was assessed using EPR spectroscopy to detect the appearance of the AFR. When 1 mM ascorbate was incubated in oxygenated PBS at pH 7.4, an AFR concentration of ~0.6 μM was observed (Fig. 7A, bottom trace). When 4 mM nitrite was also included at the same pH, a small but consistent increase in the AFR signal was observed (Fig. 7A; n = 3). At pH 5.0 the ascorbate-nitrite reaction was severalfold greater in buffer (not shown). The AFR signal was also apparent in intact cells incubated with 2.5 mM nitrite, and this signal was increased in cells that had been loaded with ascorbate by incubation with 400 μM DHA (Fig. 7B). In contrast to the results in buffer, the increase in the intracellular AFR signal was gradual over the first 90 s of incubation, which probably reflects entry of nitrite into the cells.

The reaction of nitrite with ascorbate within cells was also evident as a decrease in the intracellular ascorbate concentration, although only at very high loading concentrations of nitrite. As shown in Fig. 8A, incubation of cells with increasing concentrations of
nitrite induced a fall in intracellular ascorbate that became significant at 5 mM nitrite in control and DHA-treated cells (Fig. 8A). These results support the notion of reaction of ascorbate with nitrite or its breakdown products. The highest concentration of nitrite had only a small effect on the GSH content of control (A, n = 4), GSH (B, n = 5), and GSSG (C, n = 7) were measured. Ascorbate results are shown as a fraction (Fx) of control. Initial ascorbate concentrations were 24 ± 13 μM in control cells and 1.7 ± 0.3 mM in ascorbate-loaded cells. *P < 0.05 compared with cells incubated in the absence of nitrite by 2-way ANOVA.

Discussion

Erythrocytes have long been thought to scavenge nitrite that is generated from oxidation of NO in the bloodstream (10). For this reason, it is debatable whether any nitrite can be detected in blood plasma. Published measurements of nitrite in serum or plasma range from undetectable (2, 11) to 0.4 μM (17, 29) to as high as 8.8 μM (1). Scavenging of nitrite by erythrocytes could also be important when the local nitrite concentration in blood increases dramatically in response to NO release from endothelial or inflammatory cells. We found that erythrocytes can rapidly take up and metabolize nitrite at concentrations much greater than expected in vivo with little evidence of oxidant stress, aside from methemoglobin formation. At extracellular nitrite concentrations expected in blood in vivo (i.e., ≤100 μM), there was little intracellular nitrite accumulation (Fig. 5A) or methemoglobin formation (Fig. 5B). The lag in methemoglobin formation with increasing extracellular nitrite probably relates to the function of methemoglobin reductase (39) to recycle methemoglobin. When the capacity of this enzyme is exceeded, nitrite accumulates within the cells and methemoglobin is formed (Fig. 5C).

At least part of the capacity for erythrocytes to scavenge nitrite relates to rapid uptake of extracellular nitrite. In our experiments, erythrocytes took up 500 μM nitrite with a half-time of 11 min (Fig. 1), in agreement with previous results from uptake studies using pig erythrocytes (33) and from studies in human erythrocytes in which nitrite uptake was inferred from the rate of methemoglobin formation (40). In the latter study, it was concluded that the erythrocyte membrane posed little barrier to nitrite entry, since rates of methemoglobin formation in hemolysates were similar to those in intact cells (40). Our result showing that intracellular AFR is generated in response to uptake of extracellular nitrite over a 2-min period (Fig. 7B) also suggests rapid transmembrane equilibration of nitrite followed by a slower, but irreversible, reaction with Hb that allows progressive uptake of nitrite from the medium. Thus the erythrocyte membrane does present a barrier to nitrite entry but probably does not limit the reaction of nitrite with Hb.

Nitrite appears to cross the erythrocyte membrane by more than one mechanism. Simple transmembrane diffusion of NO₂⁻ is probably minimal because of the negative charge of nitrite. Another possibility suggested by Zhao et al. (41) is that nitrite solutions contain small amounts of gaseous nitrogen oxides, such as nitric oxide (NO), nitrogen dioxide (NO₂), dinitrogen trioxide (N₂O₃), and dinitrogen tetroxide (N₂O₄), which would readily diffuse across the cell membrane. Diffusion of HNO₂, which has a pKₐ of 3.2 (19), is also plausible. At pH 7.4, the HNO₂ concentration of a 1 mM solution of NO₂⁻ would be ~60 nM (41). Since the HNO₂ concentration would increase logarithmically with a decrease in pH, this could account in part for our observation that a decrease in pH of the medium increases nitrite uptake (Fig. 2). The effect of pH on nitrite transport could also be due to a pH-dependent change in function of a transport protein.

In the erythrocyte the most likely protein-dependent route for entry of a monoanion such as nitrite is the AE-1 protein. However, our results measuring nitrite
uptake as the disappearance of nitrite from the incubation medium showed no inhibition by DIDS. This finding confirmed that of Zadvodnik et al. (40), who used methemoglobin formation to reflect nitrite uptake. Under the conditions employed in the present studies, DIDS should completely block transport on AE-1 (32). It was previously shown in pancreatic acinar cells that DIDS-sensitive bicarbonate transporters were the major route of entry of nitrite (41), but this does not appear to be the case for human erythrocytes. Previous measurements of nitrite efflux in erythrocyte ghosts indicated that DIDS was inhibitory (31). Whereas AE-1 is not involved in nitrite influx, it could still be involved in nitrite efflux (31).

At least part of the uptake of nitrite by erythrocytes in our experiments occurred on the Na-P_i cotransport system that has been described in these cells (32). The initial rate of nitrite uptake was inhibited by ~25% by removal of sodium from the incubation medium and by inclusion of phosphate (Fig. 3). There was also a corresponding inhibition by nitrite of DIDS-insensitive {superscript}32Pi uptake by erythrocytes (Fig. 4), which coincides with at least partial uptake on this transporter. Shoemaker et al. (32) found 75% of phosphate uptake by erythrocytes to occur on the AE-1 protein and 20% on the Na-P_i cotransport system. In contrast to the present results, phosphate uptake on the latter protein decreases with a decrease in medium pH (32). It is possible that the opposite effect observed in the present studies was due to enhanced diffusion of HNO_2 with decreasing medium pH and that this obscured a decrease in nitrite transport on the Na-P_i cotransport system. The erythrocyte Na-P_i cotransporter appears to differ from the well-characterized renal brush-border membrane protein in its sensitivity to inhibitors. Neither arsenate (32) nor phosphonoacetic acid (present studies) inhibited phosphate transport in erythrocytes, whereas both are known inhibitors of the renal brush-border membrane Na-P_i cotransporter (34). Nonetheless, on the basis of the extent of inhibition of nitrite uptake by the addition of phosphate and removal of sodium (Fig. 3), nitrite uptake on the erythrocyte Na-P_i cotransport system could account for as much as 25% of nitrite uptake at pH 7.4. The remainder appears to be due to diffusion of HNO_2 and gaseous species across the membrane.

Once taken up by erythrocytes, nitrite reacted largely with Hb, resulting in methemoglobin formation (Figs. 1 and 5). Little nitrite or nitrate accumulated in the cells, even at very high extracellular nitrite concentrations relative to those expected in plasma. In addition to increased amounts of methemoglobin within the cells, raising the nitrite concentration in the medium also increased nitrate outside the cells (Fig. 6). This indicates oxidation of nitrite in conjunction with methemoglobin formation. The failure to account for all the nitrite added to the cells as nitrite or nitrate is unexplained but could be due to reactions of nitrite with cell proteins, including Hb, which would form nitrosohemoglobin (7).

The ability of ascorbate to reduce nitrite to NO is well established, and this has prompted speculation that recycling of nitrite to NO by ascorbate in the brain could be of physiological importance (26). However, the ascorbate-nitrite reaction is typically measured at low pH, where it is rapid (18, 27). Low pH facilitates the formation of species such as H_2NO_2^- and N_2O_3, which will nitrosoate the ascorbate anion, with subsequent release of NO (27). We found that ascorbate did react directly with nitrite at pH 7.4, albeit slowly, on the basis of the generation of the AFR (Fig. 7A). It has long been known that DHA is generated in this reaction, and it was predicted that the AFR would be an intermediate (9). Our EPR findings of a pH-dependent increase in the AFR after mixing nitrite and ascorbate confirm this prediction. Subsequent reaction of the AFR with nitrite or with another molecule of the AFR serves to generate DHA. It is also likely, but less certain, that the ability of nitrite to generate the AFR in intact cells (Fig. 7B) is due to direct reaction of nitrite and ascorbate. The latter effect could be due in part to reaction of ascorbate with other oxidants generated from nitrite within the cells. Scavenging of these products by ascorbate would tend to inhibit subsequent methemoglobin formation (see below).

We found that increasing the ascorbate content of cells decreased the amount of methemoglobin formed when nitrite was added to the medium (Fig. 5B). However, this decrease was evident only at high loading concentrations of nitrite (>400 μM), when >20% of cellular Hb had been oxidized to methemoglobin. There are two plausible explanations for the effect of ascorbate to decrease nitrite-dependent methemoglobin formation. The first relates to direct reaction of ascorbate with nitrite or with its breakdown products. The ascorbate-induced decrease in methemoglobin formation was associated with a corresponding decrease in intracellular nitrite concentrations (Fig. 5A). This, along with the observations that ascorbate reacts with nitrite or its metabolites in buffer at pH 7.4 (Fig. 7A), that it generates the AFR within cells (Fig. 7B), and that it decreases ascorbate concentrations in intact cells (Fig. 8A), suggests that the ascorbate effect on methemoglobin formation is due to removal of nitrite within the cell. NO generated in the reaction of ascorbate and nitrite should react with any remaining oxyhemoglobin. Our finding of a decrease in methemoglobin formation in intact cells loaded with ascorbate could indicate that NO is not the major product of the reaction of ascorbate with nitrite or with its breakdown products. More likely, it could indicate reactions of NO other than those leading to methemoglobin formation. For example, NO generated in this reaction might serve to S-nitrosylate Hb, and S-nitrosohemoglobin could then form a reserve of NO that could be released under the proper conditions (14).

The second explanation for why ascorbate decreased nitrite-induced methemoglobin formation has to do with interference by ascorbate with the reaction between nitrite and oxyhemoglobin. Reaction of nitrite with oxyhemoglobin is thought to occur via a two-step
process: a slow initial phase followed by a more rapid autocatalytic phase (10). In solutions of Hb, ascorbate has been shown to inhibit the autocatalytic phase of Hb oxidation by nitrite (6, 10, 15). It appears that reaction of ascorbate with superoxide, hydrogen peroxide, or peroxynitrite generated during this phase accounts for the inhibition (10). This effect could therefore account for at least part of the ability of ascorbate to decrease methemoglobin formation that we observed. However, in the intact cell, methemoglobin reductase recycles the Fe\(^{3+}\) in methemoglobin to Fe\(^{2+}\) which then can again bind oxygen to form oxyhemoglobin (21). These considerations also suggest that use of methemoglobin formation in intact erythrocytes to measure nitrite or NO generation outside the cells is at best only qualitative, since it depends on cellular uptake and methemoglobin reduction. Clearly, the cell system is quite complex. Our failure to find significant effects of ascorbate on methemoglobin formation at any but very high nitrite concentrations argues that the physiological relevance of the effect is small. Furthermore, since the cellular contents of GSH and GSSG were minimally affected by high concentrations of extracellular nitrite (Fig. 8, B and C), the oxidant stress induced by external nitrite was not sufficiently severe to overwhelm this cellular defense mechanism.

In conclusion, erythrocytes take up nitrite by at least two different mechanisms: one involving diffusion of HNO\(_2\) and one involving the Na-F\(_2\) cotransporter. When the capacity for oxyhemoglobin to consume nitrite within cells is decreased, high levels of intracellular ascorbate can partially prevent Hb oxidation due to nitrite or is oxidative products. This effect is due to direct reaction of ascorbate with nitrite or its breakdown products and/or to inhibition of Hb oxidation by ascorbate. Ascorbate reacts with intracellular nitrite at physiological pH. However, this reaction is slow relative to the ability of erythrocytes to scavenge nitrite. Notwithstanding methemoglobin formation, which is reversible, nitrite concentrations expected in vivo exert little oxidant stress in erythrocytes.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-50435.

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