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Similarities in the Metabolism of Alloxan and Dehydroascorbate in Human Erythrocytes

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ABSTRACT. The β-cell toxin alloxan is reduced within cells to dialuric acid, which may then decompose to release damaging reactive oxygen species. We tested whether such redox cycling of alloxan occurs in the human erythrocyte, a cell with stronger antioxidant defenses than β-cells. Erythrocytes incubated with increasing concentrations of alloxan progressively accumulated dialuric acid, as measured directly by HPLC with electrochemical detection. At concentrations up to 2 mM, alloxan decreased cellular GSH slightly, but did not affect erythrocyte contents of ascorbate or α-tocopherol. Intracellular H_2O_2 generation, measured as inhibition of endogenous catalase activity in the presence of 3-amino-1,2,4-triazole (aminotriazole), was decreased by alloxan. Despite its failure to induce significant oxidant stress in erythrocytes, 2 mM of alloxan doubled the activity of the hexose monophosphate pathway (HMP). This likely reflected consumption of reducing equivalents during reduction of alloxan to dialuric acid. Alloxan pretreatment enhanced the ability of erythrocytes to reduce extracellular ferricyanide while protecting α-tocopherol in the cell membrane from oxidation by ferricyanide. Ninhydrin, a hydrophobic derivative of alloxan, showed similar effects, but caused progressive GSH depletion and cell lysis at concentrations above 50 μM. The ability of alloxan to enhance ferricyanide reduction and to spare α-tocopherol suggests that dialuric acid or other reducing species within the cells can protect or recycle α-tocopherol and donate electrons to a transmembrane transfer process. This behavior resembles that observed for the dehydroascorbate (DHA)/ascorbate pair, and leads to the unexpected conclusion that alloxan increases the reducing capacity of the erythrocyte. BIOCHEM PHARMACOL **55**;8:1301–1307, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. alloxan; dialuric acid; ascorbate; dehydroascorbate; α-tocopherol; human erythrocytes

Alloxan [2,4,5,6-(1*H*,3*H*)pyrimidinetetrone], perhaps best known as a β-cell toxin, resembles dehydroascorbate, the two-electron oxidation product of ascorbate or vitamin C, in several respects. Both compounds have ring structures that contain a vicinal triketone configuration (Fig. 1). Both are transported on the GLUT family of facilitative glucose transporters with much higher affinities than their reduced forms [1–4]. Once inside the cells, the central carbonyl of both compounds is readily reduced by cellular thiols [5–7], as depicted in Fig. 1. Both dialuric acid and ascorbate are oxidized in the presence of transition metal ions, such as Fe^{3+}, and generate superoxide, hydrogen peroxide, and eventually the hydroxyl radical [8–10]. However, dialuric acid is more reactive than ascorbate, and decomposes in oxygenated buffers, even in the absence of transition metals [8]. This oxidative decomposition of dialuric acid is thought to account for alloxan toxicity in the β-cell, which has low levels of superoxide dismutase and GSH [11]. Because superoxide dismutase and GSH can stabilize dialuric acid *in vitro* [12], alloxan may have less toxicity in cells with greater antioxidant reserves, such as human erythrocytes.

In human erythrocytes, GSH-dependent reduction of DHA† to ascorbate enhances the ability of cells to reduce extracellular ferricyanide [7, 13]. This ferricyanide reductase activity is thought to be mediated by a transmembrane oxidoreductase activity (EC 1.6.99.3) [14, 15]. The ascorbate generated by reduction of DHA protects the cell membrane from peroxidative damage, since ascorbate sealed within erythrocyte ghosts spares α-tocopherol in the ghost membranes from oxidation by ferricyanide [16]. By these measures, redox cycling of DHA and ascorbate within erythrocytes appears to be beneficial. On the other hand, enhanced redox cycling of alloxan and unstable dialuric acid could generate significant oxidant stress in the cell. Whether the alloxan/dialuric acid couple acts as a surrogate for DHA/ascorbate could provide insight into cellular mechanisms of protection against intracellular oxidant stress, and also has relevance to the substrate specificity of the transmembrane oxidoreductase. The present studies were carried out to address these issues.

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† *Abbreviations:* aminotriazole, 3-amino-1,2,4-triazole; DHA, dehydroascorbate; and HMP, hexose monophosphate pathway.

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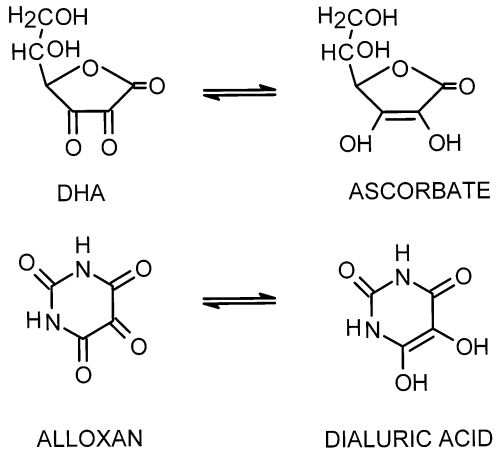

FIG. 1. Structural similarities and reduction of alloxan and DHA.

MATERIALS AND METHODS

Erythrocytes were prepared from heparinized blood that had been drawn from normal volunteers. Erythrocytes were separated from plasma and other cellular elements by three centrifugation washes in 10 vol. of PBS. The latter consisted of deionized water containing 140 mM of NaCl and 12.5 mM of Na_2HPO_4, pH 7.4.

Ascorbate and dialuric acid concentrations were measured by HPLC with electrochemical detection. Packed cells (0.2 mL) were diluted with 0.6 mL of PBS that contained 1 mM of dithiothreitol, frozen in dry ice–acetone, and allowed to thaw on ice. Dithiothreitol was included to prevent oxidation of dialuric acid during sample preparation. This precaution was probably not necessary, since results similar to those shown in Fig. 2A were observed in the absence of dithiothreitol. The hemolysate was transferred to a Centricon-10 filter apparatus (Amicon, Inc.) and ultrafiltered [17] to remove cell membranes and cytosolic proteins. The clear ultrafiltrate was placed on ice, and assayed immediately by HPLC for ascorbate and dialuric acid. The method was modified from one published previously for the separation of ascorbic and isoascorbic acids [18]. The mobile phase consisted of 75% acetonitrile and 25% deionized water that contained 20 mM of dihydrogen sodium phosphate, pH 4.6, before addition of acetonitrile. The mobile phase was oxidized before sample injection using an ESA model 5020 guard cell set at +0.5 V. Separation was carried out on a 4.6 × 150 mm Alltech Adsorbosil NH_2 column (5 μm), with a 4-mm guard column that contained C_{18} packing material. Peaks were detected using an ESA model 5100A detector and model 5010 analytical cell with the first electrode set at zero and the second detecting electrode set at +0.4 V. Dialuric acid standards were prepared by reduction of 0.1 mM of alloxan by dithiothreitol (1 mM). Reduction was followed to completion at 273 nm using an extinction coefficient of 16,000 M^{-1} cm^{-1} for dialuric acid [19]. At a flow rate of 2 mL/min, ascorbate was eluted at 5.2 to 5.6 min, and dialuric acid was eluted at 7.2 to 7.6 min, with complete separation of the peaks (results not shown). Cellular GSH content was measured using the fluorometric assay of Hissin and Hilf [20]. This assay detects only reduced GSH. Both ascorbate and GSH concentrations are expressed per milliliter of erythrocyte cytoplasm, which was taken as 70% of the packed cell volume [13].

Erythrocytes were processed for measurement of α-tocopherol according to the method of Lang *et al.* [21]. Erythrocyte α-tocopherol was measured by HPLC with electrochemical detection on a Waters DeltaPak C_{18} column (300 μm, 5 μm) with a 4-mm guard column of the same packing material. The mobile phase was 95% methanol and 5% water that contained 50 mM of sodium acetate. An ESA model 5021 conditioning cell, preceded by a graphite in-line filter, was placed just after the analytical column and set in the reducing mode at −0.5 V. An ESA model 5011 analytical cell was used for detection, with the first electrode in the analytical cell set at −0.5 V, and the second detecting electrode set at 0.6 V. Reduction of the post-column mobile phase was used to confirm that α-tocopherol had not been oxidized to α-tocopherolquinone during sample processing [22]. At a flow rate of 1 mL/min, γ-tocopherol eluted at 5.6 to 5.9 min, and α-tocopherol eluted at 6.1 to 6.4 min. The assay sensitivity for α-tocopherol was 2–5 pmol. For consistency, the α-tocopherol results are expressed per milliliter of erythrocyte cytoplasm.

Intracellular H_2O_2 was measured by its ability to inactivate endogenous erythrocyte catalase in the presence of aminotriazole [23]. Cell lysis and assay of H_2O_2 were carried out as described by Ou and Wolff [24], except that 10 μL of erythrocytes at a 5% hematocrit was added to the lysis buffer, and only 25 μL of the hemolysate was added to 975 μL of the FOX1 reagent [25]. After a 30-min incubation at room temperature, the O.D. of the sample was measured at 560 nm and compared with standards of H_2O_2 [25]. The reading in each sample was corrected for that of a blank that did not contain H_2O_2, and the percent inhibition of catalase in an unknown erythrocyte sample was calculated relative to that observed in an erythrocyte sample in which catalase was inhibited completely by 1 mM of sodium azide.

The ability of erythrocytes to reduce extracellular ferricyanide was measured as previously described [2], using 1,10-orthophenanthroline to detect ferrocyanide in the assay of Avron and Shavit [26]. Erythrocyte reduction of nitroblue tetrazolium was measured as deposition of the reduced diformazan in erythrocyte ghost membranes, as previously described [15]. Following incubation with nitroblue tetrazolium, the cells were washed three times in 5 vol. of PBS, and unsealed ghosts were prepared by the method of Steck and Kant [27]. The ghosts were resuspended in 5 mM of sodium phosphate buffer, pH 8.0, and the O.D. at 560 nm was measured on a spectrophotometer. Absorbance due to deposition of the diformazan of nitroblue tetrazolium was calculated by subtracting absorbance in a paired sample of membranes from cells that were not exposed to nitroblue tetrazolium.

Generation of $^{14}CO_2$ from D-[1-^{14}C]glucose (New England Nuclear) in the HMP of erythrocytes was measured as described previously for rat adipocytes [28] with the following modifications. A 1-mL suspension of erythrocytes in PBS that contained 8 mM of sodium bicarbonate (pH 7.4) was incubated at 37° in a 7-mL polyethylene scintillation vial (PicoPrias vial, Packard Instrument Co.). After 1 hr, the suspension was acidified with 0.1 mL of 8 N H_2SO_4 during connection with another scintillation vial. This collection vial contained a piece of Whatman No. 3 filter paper (0.5 cm square) that had been saturated with 25 μL of 6 N NaOH. The acidified cells and collecting vial were incubated for an additional hour at 37°. The vial containing the filter paper was removed and filled with 5 mL of scintillation fluid, and the sample was counted in a Packard 2000CA liquid scintillation counter with quench and luminescence corrections. Release of $^{14}CO_2$ over 1 hr was calculated based on the specific activity of the [1-^{14}C]glucose in the incubation, and expressed per milliliter of packed cells.

Results are shown as means ± SEM, except where otherwise indicated. Linear or nonlinear curve-fitting was carried out with the graphical software package FigP (Biosoft). Statistical significance was assessed by one-way ANOVA using the statistical software package Sigmastat 2.0 (Jandel Scientific).

RESULTS

Incubation of erythrocytes with increasing amounts of alloxan followed by centrifugation washes to remove extracellular reagent resulted in the progressive accumulation of dialuric acid within the cells (Fig. 2A). This shows that the cells had taken up alloxan and converted it to dialuric acid. As also shown in Fig. 2B, up to 2 mM of alloxan had no consistent effect on the intracellular concentration of ascorbate, although there was a significant downward trend in the cell content of GSH, even in the presence of 5 mM of D-glucose. Under the same conditions, 1 mM of alloxan was without effect on the membrane content of α-tocopherol, which was 1.3 ± 0.4 nmol/mL in control cells and 1.9 ± 0.4 nmol/mL in treated cells.

Alloxan metabolism was accompanied by activation of the erythrocyte HMP, since release of $^{14}CO_2$ from D-[1-^{14}C]glucose was increased (Fig. 3). This suggests that the cells had the metabolic capacity to convert alloxan to dialuric acid, and that the latter either was stabilized within the cells or decomposed without exceeding the antioxidant defenses of the cells. In an attempt to detect decomposition of dialuric acid to H_2O_2, we measured intracellular generation of the latter as inhibition of endogenous catalase in the presence of aminotriazole. However, as shown in Fig. 4, increasing amounts of alloxan resulted in a small but progressive decrease in intracellular H_2O_2 generation. This indicates not only that catalase was not required to decompose any H_2O_2 released by dialuric acid, but that dialuric acid actually lowered basal rates of H_2O_2 generation in the cells.

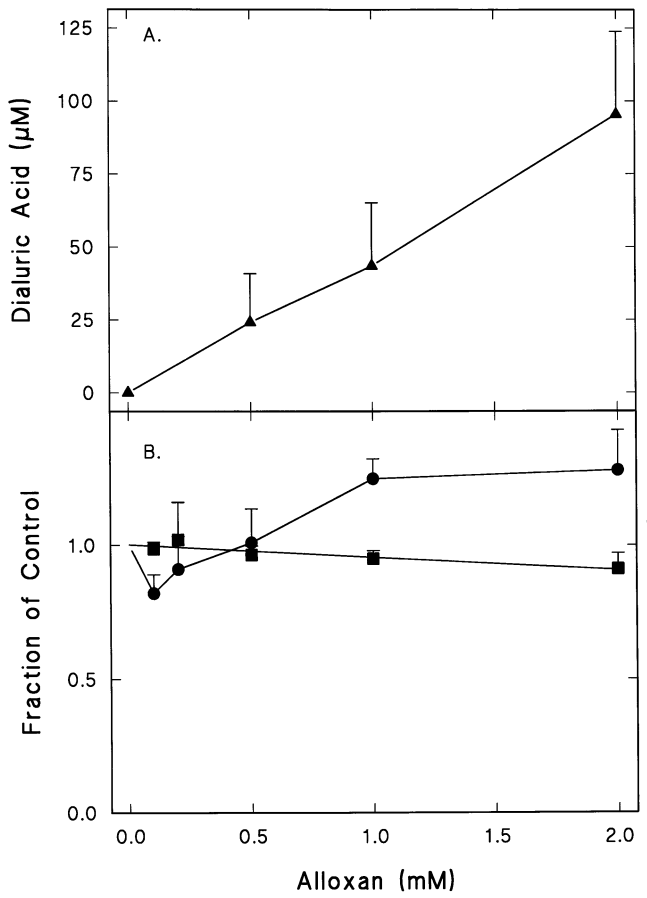

FIG. 2. Lack of effect of alloxan preincubation on erythrocyte ascorbate and GSH. Erythrocytes at a 10% hematocrit were incubated for 15 min at 37° with the indicated concentration of alloxan in PBS that contained 5 mM of glucose, were washed three times in 2 mL of PBS, and were taken for assay of intracellular dialuric acid (panel A, triangles, N = 3), ascorbate (panel B, circles, N = 8), and GSH (panel B, squares, N = 3). Data for panel B are normalized to the initial cell concentration of each antioxidant, which was 36 ± 6 μM for ascorbate and 1.9 ± 0.1 mM for GSH. The GSH data were fit by linear regression, and the slope of the resulting line was significantly less than zero ($P < 0.01$). Values are means ± SEM.

Further evidence that alloxan had been stabilized in a reduced form within the cells was obtained by measuring the ability of alloxan-loaded cells to reduce extracellular ferricyanide. Erythrocytes incubated for 15 min at 37° with alloxan and then washed to remove extracellular agent had an increased ability to reduce ferricyanide compared with control cells. Enhanced ferricyanide reduction was sustained over a subsequent 40-min incubation (Fig. 5). When measured at 30 min in the presence of increasing concentrations of alloxan, ferricyanide reduction increased in a saturable manner (Fig. 6). Such alloxan-dependent ferricyanide reduction was half-maximal at 300 μM of alloxan, and plateaued at twice the basal rate. Alloxan did not reduce ferricyanide in the absence of erythrocytes (results not shown). Cells that had been preincubated with alloxan

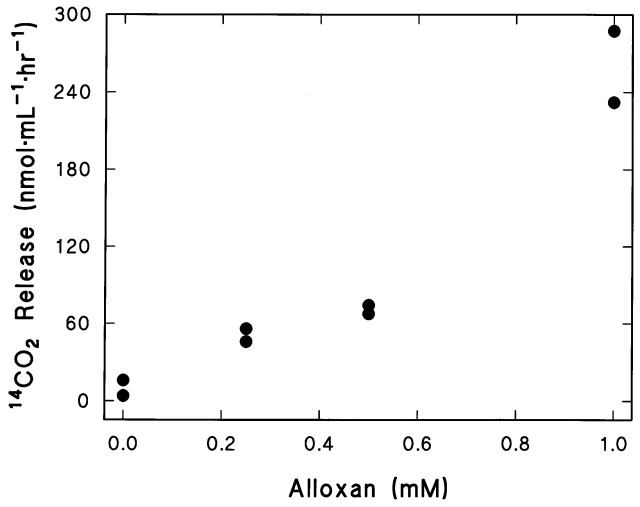

FIG. 3. Stimulation of the HMP by alloxan. Erythrocytes at a 10% hematocrit were incubated with 0.5 mM of glucose, 0.05 μCi of [1-^{14}C]glucose, and the indicated concentration of alloxan in PBS that contained 8 mM of sodium bicarbonate, pH 7.4. After 1 hr at 37°, the amount of $^{14}CO_2$ that had been liberated was measured as described under Materials and Methods. Data are shown from 2 experiments.

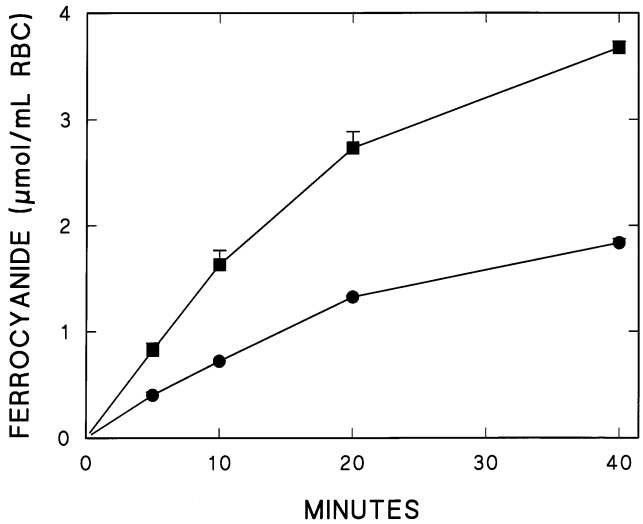

FIG. 5. Time course of alloxan stimulation of ferricyanide reduction by erythrocytes. Erythrocytes at a 10% hematocrit were incubated for 15 min at 37° in the absence (circles) or presence (squares) of 1 mM of alloxan in PBS that contained 5 mM of D-glucose. The cells were washed three times by centrifugation in 10 vol. of PBS, resuspended to a 5% hematocrit in PBS that contained 5 mM of D-glucose and 1 mM of potassium ferricyanide, and incubated at 37°. At the indicated times, aliquots of cells and buffer were removed, the cells were pelleted in a microfuge, and duplicate aliquots of the supernatant were assayed for ferrocyanide. Data are shown (± range) from an experiment representative of three such performed.

also showed enhanced reduction of nitroblue tetrazolium, as measured by deposition of the diformazan in the erythrocyte membrane (Fig. 6). This effect was also saturable, with a half-maximal effect above basal at an initial extracellular alloxan concentration of 430 μM. The ability of erythrocytes preincubated with alloxan to reduce both extracellular ferricyanide and nitroblue tetrazolium suggests that dialuric acid or another reductant had been generated within the cells. To confirm the specificity of this effect for the triketone moiety, two analogues of alloxan were tested in the erythrocyte system: ninhydrin and barbituric acid.

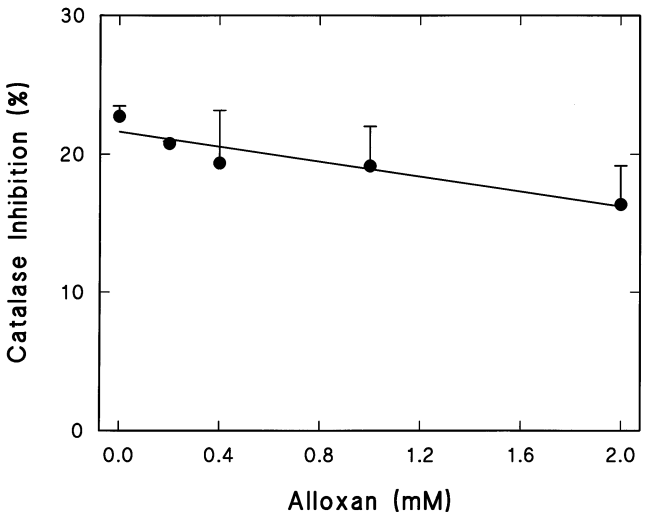

FIG. 4. Inhibition of intracellular H_2O_2 production by alloxan. Erythrocytes at a 1% hematocrit were incubated at 37° for 30 min in PBS that contained 5 mM of glucose and 50 mM of aminotriazole. The extent of erythrocyte catalase inhibition was measured in 3 experiments at the indicated alloxan concentrations. Values are means ± SEM. The line shows the linear fit of the data ($P = 0.02$).

Ninhydrin also reduced extracellular ferricyanide, but was many-fold more potent than alloxan in this regard. As shown in Fig. 7, ninhydrin pretreatment of cells resulted in a progressive increase in ferricyanide reduction up to a ninhydrin concentration of 50 μM. Above this concentration, ferricyanide reduction decreased, and the cells began to lyse (results not shown). As shown in Fig. 7, ninhydrin preincubation caused a greater decrease in intracellular GSH concentrations than did much higher concentrations of alloxan. Barbituric acid, which lacks the central carbonyl group of the alloxan triketone structure (Fig. 1), did not enhance ferricyanide reduction (results not shown).

A comparison of the effects of alloxan, ninhydrin, and DHA on ferricyanide reduction and release of $^{14}CO_2$ from D-[1-^{14}C]glucose are shown in Table 1. At optimal concentrations of each agent, the ability of ascorbate to reduce extracellular ferricyanide was several-fold greater than that of either alloxan or ninhydrin. On the other hand, the same concentrations of each agent enhanced glucose utilization through the HMP to a similar extent.

Incubation of erythrocytes with optimal concentrations of DHA, alloxan, and ninhydrin also protected α-tocopherol in the erythrocyte membrane from oxidative loss during treatment with extracellular ferricyanide (Table 1). In control cells, about half the content of α-tocopherol was lost following ferricyanide exposure, but in cells that had

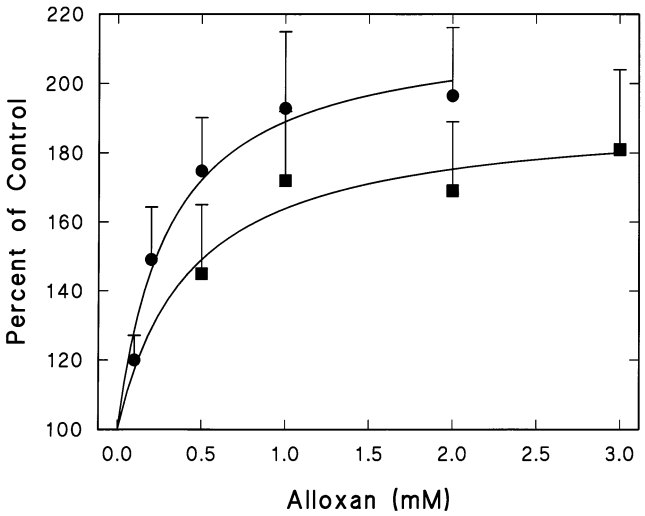

FIG. 6. Stimulation of ferricyanide and nitroblue tetrazolium reduction by alloxan. Measurement of ferricyanide reduction (circles, N = 3) was carried out for 30 min as described in the legend to Fig. 5 in 10% cells that had been loaded with the indicated concentration of alloxan. For measurement of nitroblue tetrazolium reduction (squares, N = 3), 20% erythrocytes were incubated with the indicated concentration of alloxan for 1 hr at 37° in PBS that contained 5 mM of D-glucose and 116 μM of nitroblue tetrazolium. The cells were washed three times by centrifugation in 5 vol. of PBS, and deposition of the diformazan of nitroblue tetrazolium was measured in ghost membranes as described under Materials and Methods. Data are shown as means ± SEM relative to a control incubation, with the drawn lines representing hyperbolic fits to the data. Baseline values for ferricyanide reduction were 3.9 ± 0.8 μmol · (mL erythrocytes)$^{-1}$ · hr^{-1}, and for nitroblue tetrazolium deposition, 3.5 ± 1.3 absorbance units at 560 nm · (mL erythrocytes)$^{-1}$ · hr^{-1}.

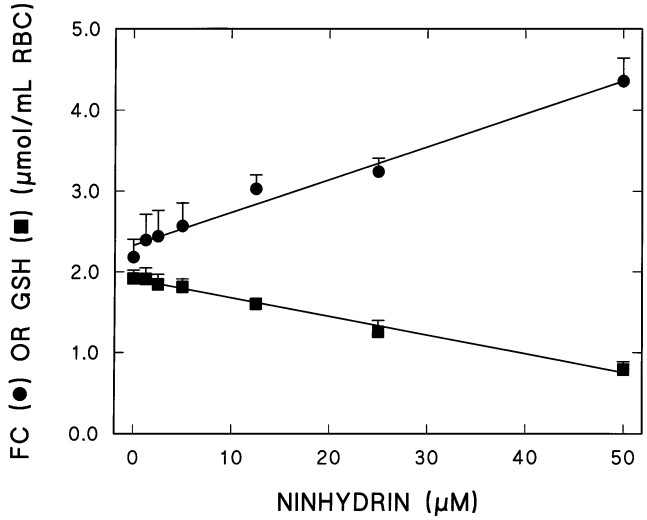

FIG. 7. Effects of ninhydrin on erythrocyte GSH content and ferricyanide reduction. Erythrocytes were incubated at a 5% hematocrit in PBS that contained 5 mM of D-glucose and the indicated concentration of ninhydrin. After 15 min at 37°, the cells were washed three times in 5 vol. of PBS. A cell aliquot was taken for assay of GSH and another was taken for measurement of ferricyanide reduction (FC), as described in the legend to Fig. 5. Data (means ± SEM) are shown from 3 experiments, with the drawn lines representing linear fits to the data ($P < 0.001$ for both).

been preincubated with the agents, no loss of α-tocopherol was observed.

DISCUSSION

Instead of inducing an oxidant stress, alloxan enhanced the ability of erythrocytes to reduce the mild oxidants ferricyanide and nitroblue tetrazolium, and protected α-tocopherol in the cell membrane against oxidation by extracellular ferricyanide. Incubation of erythrocytes with alloxan resulted in the appearance of dialuric acid within the cells, which probably reflected uptake of alloxan on the glucose transporter [3, 4], intracellular reduction to dialuric acid, and trapping of the latter within cells. There are several mechanisms by which erythrocytes can reduce alloxan, each of which ultimately requires NADPH. First, GSH can directly reduce alloxan to dialuric acid [12]. GSH, in turn, is recycled from GSSG by glutathione reductase, using reducing equivalents from NADPH. Second, alloxan can be reduced directly by NADPH *in vitro* [29]. Third, NADPH-dependent reduction of alloxan is facilitated by the thioredoxin system [30, 31], which has been detected in erythrocytes [32]. Depletion of NADPH during alloxan reduction by any of these mechanisms will activate glucose utilization in the HMP, as observed in this study and in a previous study [33]. Thus, glucose is the ultimate electron donor to alloxan and was included in all incubations.

At alloxan concentrations of 2 mM or less, we found no evidence that the HMP was activated by H_2O_2 or by other unmeasured oxidants that were generated during redox cycling of alloxan and dialuric acid. Erythrocyte antioxidants were either not affected (ascorbate, α-tocopherol) or decreased only slightly (GSH) by alloxan, and intracellular H_2O_2 generation was not detected by the aminotriazole-catalase inhibition assay. Although these results do not rule out generation of H_2O_2 in amounts that could be consumed solely by the glutathione peroxidase/reductase system [34], it seems more likely that dialuric acid was stabilized by GSH [12] or by other cellular thiols [35, 36].

Uptake and reduction of DHA by erythrocytes lowered GSH in the absence of glucose [7]. Similarly, the primary oxidant stress induced by alloxan in erythrocytes was the initial reduction of alloxan by GSH, which lowered GSH only slightly at 5 mM of glucose. In contrast to alloxan, ninhydrin decreased erythrocyte GSH content, and caused cell lysis at concentrations above 50 μM. We suspect that the greater potency of ninhydrin was due to its hydrophobicity and rapid diffusion across the cell membrane. Alloxan, on the other hand, competes with glucose for uptake on the glucose transporter [37, 38]. This limitation likely contributes to the lack of oxidant damage observed in this study. Nonetheless, at diabetogenic concentrations in blood, alloxan causes a fall in blood GSH [39, 40], which is due almost completely to a fall in erythrocyte GSH content. At higher alloxan concentrations, therefore, diffusion

TABLE 1. Ferricyanide oxidation of erythrocytes loaded with alloxan, ninhydrin, and DHA

Treatment	Ferrocyanide ($\mu mol \cdot mL^{-1}$)	$^{14}CO_2$ release ($nmol \cdot mL^{-1} \cdot hr^{-1}$)	α-Tocopherol (fraction of control)
Control	2.1 ± 0.2	33.3 ± 17	0.51 ± 0.1
Alloxan (1 mM)	3.3 ± 0.3*	253 ± 18*	0.94 ± 0.07*
Ninhydrin (50 μM)	5.7 ± 1.3*	267 ± 33*	0.97 ± 0.06*
DHA (0.5 mM)	12.0 ± 1.4*	273 ± 33*	0.97 ± 0.04*

Ferricyanide reduction was measured in 5 experiments at 30 min as described in the legend to Fig. 5, following loading of the cells for 30 min with the indicated concentration of each agent. Release of $^{14}CO_2$ from cells incubated with D-[1-^{14}C]glucose was measured in 3 experiments in the presence of each agent as described in the legend to Fig. 3. Protection of α-tocopherol from oxidation by ferricyanide was measured by loading cells at a 40% hematocrit for 30 min at 37° with each agent in PBS that contained 5 mM of D-glucose. The cells were washed four times by centrifugation in 10 vol. of PBS, and incubated at a 5% hematocrit in the presence or absence of 1 mM of ferricyanide in PBS containing 5 mM of D-glucose. After 30 min, the cells were washed twice in PBS and taken for assay of α-tocopherol. For 3 experiments, the α-tocopherol content of cells treated with each agent is expressed as a fraction of the α-tocopherol in the paired control sample not exposed to ferricyanide.

Values are means ± SEM.

*$P < 0.05$ compared with the control.

of alloxan across the cell membrane will become significant and will overwhelm even the ability of the erythrocyte to regenerate GSH.

The uptake, reduction, and stabilization of alloxan within erythrocytes mirrors the behavior of DHA. Since intracellular ascorbate can reduce extracellular ferricyanide [2, 13] and nitroblue tetrazolium [15], it was not surprising that washed erythrocytes that had been preincubated with alloxan also reduced these oxidants. Alloxan was less efficient at ferricyanide reduction than was ascorbate, since it caused a similar activation of the HMP at concentrations that resulted in less than a third the extent of ferricyanide reduction as ascorbate. Nonetheless, ferricyanide reduction by dialuric acid reflects a transmembrane electron transfer, which is thought to be mediated by one or more transmembrane oxidoreductase activities [41, 42]. Whereas NADH is the usual cofactor for membrane-bound ferricyanide reductases [43], we showed ascorbate to be the major electron donor in erythrocytes to extracellular ferricyanide [15]. The present results extend the substrate specificity of this transfer to the reduced forms of compounds having an "active" triketone structure (Fig. 1).

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