Ascorbic Acid Recycling Enhances the Antioxidant Reserve of Human Erythrocytes†

James M. May,*,‡,§ Zhi-chao Qu,‡ and Richard R. Whitesell§

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

Received March 24, 1995; Revised Manuscript Received June 1, 1995⊗

ABSTRACT: The role of ascorbate transport and metabolism in the response of human erythrocytes to an extracellular oxidant stress was investigated. Rates of entry and exit of [^{14}C]dehydroascorbate from erythrocytes were more than 10-fold greater than those of [^{14}C]ascorbate. Both the reduced and oxidized forms of the vitamin were transported largely by the glucose transporter. Inside erythrocytes, dehydroascorbate was converted to ascorbate, increasing intracellular ascorbate concentrations 2−3-fold over those in the medium. In such ascorbate-loaded cells, the membrane-impermeant oxidant ferricyanide induced a transmembrane oxidation of intracellular ascorbate to dehydroascorbate. The latter escaped the cells on the glucose transporter, which resulted in a halving of the net entry of [^{14}C]dehydroascorbate in the presence of ferricyanide. Treatment of ascorbate-loaded cells with H_2O_2 and Cu^{2+} also oxidized ascorbate and induced efflux of [^{14}C]dehydroascorbate. Ferricyanide-dependent intracellular oxidation of ascorbate resulted in a corresponding reduction of extracellular ferricyanide, which served as an integrated measure of ascorbate recycling. Ferricyanide reduction was proportional to the loading concentration of dehydroascorbate and was enhanced when loss of dehydroascorbate from cells was decreased, either by blockade of the glucose transporter or by concentrating the cells. Selective depletion of cellular ascorbate lowered rates of ferricyanide reduction by two-thirds, suggesting that ascorbate rather than NADH is the major donor for the transmembrane ferricyanide oxidoreductase activity. On the basis of the ascorbate-dependent rate of ferricyanide reduction, erythrocytes at a 45% hematocrit can regenerate the ascorbic acid present in whole blood every 3 min. Erythrocyte ascorbate recycling may thus contribute more to the antioxidant reserve of blood than is evident from plasma ascorbate concentrations alone.

Ascorbic acid is considered the most important antioxidant in human plasma (Ames et al., 1987; Frei et al., 1989), where it circulates at concentrations of 20−60 μM in unsupplemented individuals (Dhariwal et al., 1991; Loh & Wilson, 1971; Robertson et al., 1991; Evans et al., 1982). The oxidized form of the vitamin, dehydroascorbate (DHA)[1], is present at concentrations much lower than those of ascorbate and is often undetectable (Vinson et al., 1989; Sinclair et al., 1991; Jennings et al., 1987; Stankova et al., 1984). The mechanisms by which ascorbate is maintained in blood are not well understood. Since humans are unable to synthesize the vitamin, its circulating concentrations depend upon the interplay of several different factors, including intestinal absorption, oxidation and reduction in blood, tissue uptake and release, and renal losses. Most cells and tissues take up reduced ascorbic acid by active transport, resulting in intracellular concentrations that are often many-fold higher than those in serum (Rose, 1988). Erythrocytes may carry as much as 30% of the ascorbate in whole blood (Evans et al., 1982), but the intracellular erythrocyte concentration of ascorbate is similar to that in serum (Evans et al., 1982; Jacob et al., 1987; Okamura, 1979). Whereas reduced ascorbate is thought to enter and leave erythrocytes slowly by simple diffusion (Hornig et al., 1971; Hughes & Maton, 1968; Wagner et al., 1987), DHA is rapidly transported into erythrocytes, primarily by the GLUT1 glucose transporter (Vera et al., 1993; Bianchi & Rose, 1986; Rose, 1988). Once inside erythrocytes, DHA is reduced to ascorbate (Christine et al., 1956; Okamura, 1979; Wagner et al., 1987; Iheanacho et al., 1993), which is effectively trapped within the cell (Wagner et al., 1987). The ability of erythrocytes to recycle DHA to ascorbate in this manner may add to the antioxidant reserve of blood by providing a renewable source of reducing equivalents.

Several groups have reported that mild oxidants such as ferricyanide (Orringer & Roer, 1979; Balmukhanov et al., 1980; Schipfer et al., 1985; Tsybyshev & Kuznetsov, 1988), methemoglobin (McGown et al., 1990), and ferricytochrome c (Tomoda et al., 1980), when present outside erythrocytes, are reduced by electrons transferred across the erythrocyte plasma membrane. This transmembrane electron transfer is thought to be mediated by an oxidoreductase (EC 1.6.99.3) that uses intracellular NADH as the electron donor to an extracellular electron acceptor such as ferricyanide (Zamudio et al., 1969; Crane et al., 1985). Ferricyanide does not oxidize hemoglobin in intact erythrocytes, and thus is considered cell-impermeant (Székely et al., 1952; Mishra & Passow, 1969). Intracellular ascorbate may also contribute

† This work was supported in part by a pilot and feasibility study from the Vanderbilt Clinical Nutrition Research Unit (DK 26657).

* Correspondence should be addressed to this author at 715 Medical Research Building II, Vanderbilt University School of Medicine, Nashville, TN 37232-6303. Phone: 615-936-1653. Fax: 615-936-1667.

‡ Department of Medicine.

§ Department of Molecular Physiology and Biophysics.

⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1995.

[1] Abbreviations: DHA, dehydroascorbate; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; Tempol, 2,2,6,6-tetramethyl-4-hydroxypiperidine-*N*-oxyl.

0006-2960/95/0434-12721$09.00/0 © 1995 American Chemical Society

to the transfer of reducing equivalents across the cell membrane, since incubation of erythrocytes with DHA has been shown to accelerate extracellular ferricyanide reduction (Orringer & Roer, 1979; Schipfer et al., 1985). There is controversy, however, regarding whether ascorbate-dependent electron transfer is mediated by the actual movement of ascorbate across the membrane (Orringer & Roer, 1979) or by the transmembrane oxidoreductase (Schipfer et al., 1985; Grebing et al., 1884; Goldenberg et al., 1983).

The present studies were undertaken to determine the mechanism of oxidant-induced ascorbate transport in human erythrocytes, and whether ascorbate recycling within erythrocytes contributes to the antioxidant reserve capacity of whole blood.

EXPERIMENTAL PROCEDURES

Materials. Solid [1-^{14}C]ascorbic acid (4.7 Ci/mol) and 3-*O*-[^{3}H]methyl-D-glucose (87.6 Ci/mmol) were obtained from New England Nuclear. Ascorbic acid oxidase, DHA, D-isoascorbate, and 2,2,6,6-tetramethyl-4-hydroxypiperidine-*N*-oxyl (Tempol)[1] were obtained from Sigma Chemical Co., St. Louis, MO.

Cell Preparation. Human erythrocytes were prepared from freshly drawn heparinized blood from normal volunteers. The cells were washed 3 times in 10 volumes of phosphate-buffered saline (PBS)[1] that consisted of 140 mM NaCl and 12.5 mM sodium phosphate, pH 7.4. Sometimes cells were stored up to 24 h in autologous serum at 4 °C before use.

Preparation of [^{14}C]Ascorbic Acid. To prevent its oxidation, solid [^{14}C]ascorbic acid was aliquoted under nitrogen and stored at −20 °C until use. Just before each experiment, a separate aliquot of the radiolabeled ascorbic acid was dissolved in PBS. Where noted, 0.1 mM GSH was also present in this buffer to keep ascorbate in the reduced form (Winkler, 1987). This was confirmed by thin-layer chromatography, in which a 3 h old sample of [^{14}C]ascorbate in the GSH-containing buffer was applied to a silica gel G-60 thin-layer plate. The plate was developed in ascending fashion in a bath of chloroform/methanol/H_2O (65:25:4). A single radioactive spot was observed with $R_f = 0.33$. This spot stained immediately positive with 0.1% saturated $AgNO_3$ in acetone (Finn & Johns, 1980), and its R_f corresponded to that of crystalline ascorbic acid.

Measurements of Radiolabeled Ascorbate and Hexose Transport. For measurement of radiolabeled ascorbate or DHA influx into erythrocytes, 0.01 mL of packed cells was rapidly mixed at the indicated temperature with 4 volumes of PBS containing either 5 mM D-glucose or 2.5 mM adenosine and the indicated concentrations of [^{14}C]ascorbate or [^{14}C]DHA. In experiments in which [^{14}C]DHA uptake was measured, radiolabeled DHA was generated from [^{14}C]-ascorbate by the action of ascorbate oxidase (2−5 units/mL) present just before and during mixing with cells. This treatment quantitatively converted [^{14}C]ascorbate to [^{14}C]-DHA, as determined by thin-layer chromatography (data not shown). During uptake assays of [^{14}C]ascorbate or [^{14}C]-DHA, a tracer concentration of 3-*O*-[^{3}H]methylglucose (3 μM) was also included in the radioactive solution to determine the function of the glucose transporter and to estimate the intracellular water space. The uptake assays were continued for the indicated time and then rapidly diluted with 1 mL of ice cold PBS containing 0.1 mM phloretin. A low temperature and the presence of phloretin were found to effectively prevent efflux of [^{14}C]DHA and 3-*O*-methylglucose on the glucose transporter during subsequent washes (data not shown). The cell suspensions were microfuged, and the buffer was removed within 1 min. The wash with ice-cold PBS−phloretin was repeated, and the packed cells were lysed with the addition of 0.6 mL of 6% (w/v) trichloroacetic acid. The lysate was mixed and microfuged, and 0.5 mL of the supernatant was counted for radioactivity by dual-label methods. The amount of ascorbate/DHA transported into cells was calculated relative to the measured equilibrium distribution space of 3-*O*-methylglucose in the same sample.

Efflux of [^{14}C]ascorbate (or [^{14}C]DHA) from erythrocytes was measured as follows. Erythrocytes in PBS containing 5 mM D-glucose at a 20% hematocrit were loaded to the indicated initial concentration with [^{14}C]DHA ([^{14}C]ascorbate plus 5 units/mL ascorbate oxidase) for 10−30 min at the indicated temperature. The extracellular radioactivity was removed by washing the cells 3 times by centrifugation in 10 volumes of PBS containing D-glucose. It has been previously shown under such conditions that [^{14}C]DHA is reduced to [^{14}C]ascorbate, which is retained within cells during the washes (Okamura, 1979; Wagner et al., 1987). Packed cells were pipetted into microfuge tubes, and efflux was initiated by vigorously diluting the cells with 20−100 volumes of room temperature PBS containing the indicated additives. The assay was terminated by another 5-fold dilution with ice-cold PBS containing 0.1 mM phloretin. In some assays the radioactivity remaining inside the cells was determined following lysis as described above for the influx assay, whereas in most assays aliquots of the medium were counted for radioactivity released from the cells. The latter approach was generally more sensitive to low efflux rates. The amount of radiolabel that escaped the cells was divided by that present in the cells before the start of the assay. This fraction was subtracted from unity to obtain the fraction of radioactivity remaining in the cells at the given time point.

Measurement of Ferricyanide Reduction. Washed erythrocytes were preincubated as indicated, and then washed an additional 3 times in 5−10 volumes of PBS containing either 5 mM D-glucose or 2.5 mM adenosine. Experiments revealed a source of intracellular reducing equivalents was required to sustain ferricyanide reduction: either 5 mM D-glucose or 2.5 mM adenosine was equally effective (data not shown). To measure ferricyanide reduction, an aliquot of the packed cells (0.1 mL) was diluted to 1 mL with PBS containing 1 mM ferricyanide and the indicated additives. Incubations were performed in a shaking water bath at 37 °C. At various times (usually 5, 10, 20, and 40 min), 0.2 mL aliquots of cells and buffer were removed and microfuged for 1 min. An aliquot of 0.1 mL of the supernatant was sampled and the ferrocyanide content was measured by the assay of Avron and Shavit (1963). Ferrocyanide was determined from its optical density at 510 nm, using an extinction coefficient of 10 500 $M^{-1}\cdot cm^{-1}$ for the 1,10-phenanthroline−ferrocyanide complex generated by oxidation of a known amount of ascorbate. Correction was made for the absorbance of the cell-conditioned buffer, which was probably due to a small amount of hemolysis over the time of the assay. The ferrocyanide generated was expressed relative to the intracellular water space of the erythrocytes,

considered to be 70% of the packed cell volume (Orringer & Roer, 1979).

Prior to some experiments, endogenous erythrocyte ascorbate was depleted by repeated washing with the nitroxide spin-label 2,2,6,6-tetramethyl-4-hydroxy-piperidine-*N*-oxyl (Tempol)[1] (Mehlhorn, 1991; Winkler et al., 1994). Following the initial centrifugation washes to remove serum, erythrocytes were diluted to a hematocrit of 10% at 37 °C with PBS containing 1 mM Tempol. After 5 min, the suspension was centrifuged, and the supernatant was removed. The Tempol washes were repeated twice, followed by three centrifugation washes in 10 volumes of PBS without Tempol. Glucose was absent during all of these steps.

Determination of Intracellular Erythrocyte Ascorbate Concentrations. Erythrocytes were lysed according to the method described by Iheanacho et al. (1995), which avoids denaturation of hemoglobin and subsequent Fe^{3+}-mediated oxidation of ascorbate. Packed erythrocytes (0.2 mL) were diluted with 0.4 mL of PBS, frozen in dry ice−acetone, and thawed over several minutes by incubation in a water bath at 37 °C. The hemolyzed cell solution was transferred to a Centricon-10 filter (Amicon, Inc., Beverly, MA) and centrifuged at 4 °C for 30 min at 5000*g*. The clear ultrafiltrate (0.4−0.5 mL) was diluted with 90% methanol containing 1 mM EDTA, and assayed for ascorbate by HPLC using the ion-pairing method of Pachla and Kissinger (1979). Separation was accomplished 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 consisted of 70 mM ammonium acetate, 1 mM tridecylamine, and 15% methanol, pH 5.2. At a flow rate of 1 mL/min ascorbate was eluted as a doublet at 5−6 min, with UV detection at 254 nm. The assay sensitivity was 50 pmol/sample. Although this method does not detect dehydroascorbate or 1,3-diketogulonic acid, when radiolabeled these derivatives were identified by scintillation counting of the HPLC column fractions. Elution of [^{14}C]dehydroascorbate occurred in the void volume, and 1,3-diketogulonic acid was found at 8−9 min.

Other Assays and Data Analysis. The NADH content of washed erythrocytes was assayed by the method of Zerez et al. (1987). Erythrocyte GSH was assayed by the fluorometric method of Hissen and Hilf (1976). Statistical comparisons were made using the paired Student's "*t*"-test.

RESULTS

Measurements of [^{14}C]Ascorbate and [^{14}C]DHA Influx into Erythrocytes. The uptakes of ascorbate and DHA into erythrocytes were first studied in the absence of an oxidant stress. Entry of [^{14}C]ascorbate into erythrocytes was very slow relative to that of [^{14}C]DHA. In the experiment shown in Figure 1, [^{14}C]ascorbate accumulated to an intracellular concentration about 35% of that of the intracellular space marker 3-*O*-methylglucose. In other experiments in which the intracellular accumulation of 100 μm unlabeled ascorbate was measured directly, ascorbate concentrations reached about 70% of extracellular ascorbate after 40 min of incubation (Table 1). This difference in uptake rates was typical from cells of different donors. In contrast to the slow uptake of [^{14}C]ascorbate, that of DHA was quite rapid, even when measured at 23 °C (Figure 1). DHA was taken up by the cells to concentrations usually 2−4-fold higher than those present in the medium within the first 2 min, although more

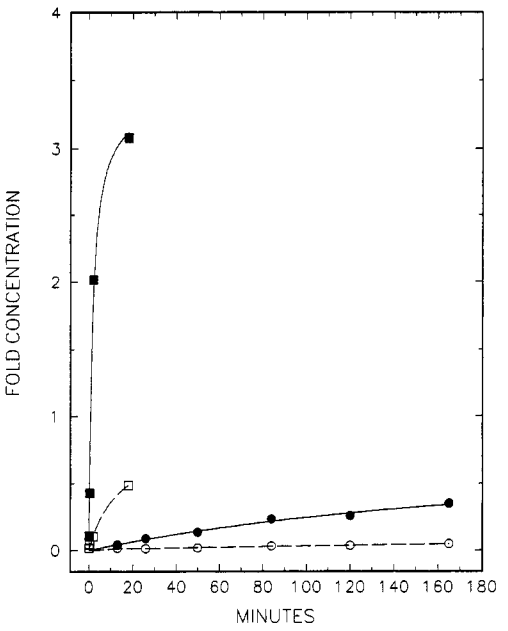

FIGURE 1: Influxes of radiolabeled ascorbate and DHA into erythrocytes. The uptake of 23 μM [^{14}C]ascorbate was measured in the absence (solid circles) and presence (open circles) of 10 μM cytochalasin B at 37 °C. In another experiment, the uptake of 24 μM [^{14}C]DHA into 20% hematocrit erythrocytes was measured as described for [^{14}C]ascorbate, except that the incubation temperature was 23 °C and that the medium contained 4 units/mL ascorbate oxidase and no GSH. The closed squares indicate rates of [^{14}C]-DHA uptake in control cells, and the open squares show rates observed in the presence of 10 μM cytochalasin B. Similar results were obtained in two other experiments.

Table 1: Effects of Various Treatments on Intracellular Erythrocyte Ascorbate Concentrations[a]

treatment	DHA (100 μM)	ascorbate content (μM)	*N*
none	−	20 ± 3	4
none	+	285 ± 27	4
cytochalasin B (10 μM)	+	176 ± 31	2
ascorbate (100 μM)	−	70 ± 3	2
ascorbate + cytochalasin B	−	50 ± 8	2
$CuSO_4$ (0.1 mM)	+	153 ± 18	2
glucose oxidase (0.88 unit/mL)	+	71 ± 10	2
ferricyanide (1 mM)	+	47 ± 10	3

[a] Washed 20% hematocrit erythrocytes were incubated with the indicated additions for 40 min at 37 °C. The cells were pelleted by centrifugation and washed once in 5 volumes of PBS before assay of intracellular ascorbate contents. Results are shown from "*N*" experiments. All values are significantly different than DHA-loaded cells at the $p < 0.05$ level.

slowly thereafter. Uptakes of both ascorbate and DHA were inhibited by 10 μM cytochalasin B (Figure 1), a potent and specific inhibitor of the erythrocyte GLUT1 glucose transporter (Taverna & Langdon, 1973). The influx of 3-*O*-[^{3}H]-methylglucose in the same cells was essentially complete after 30 s, and was retarded over 80% by 10 μM cytochalasin B (not shown). Cytochalasin E at the same concentration had no effect (data not shown). Transported DHA accumulated as ascorbate within cells, as shown in Table 1. After 40 min of incubation, accumulation relative to the extracellular DHA concentration was about 3-fold, but more than 10-fold relative to the original intracellular ascorbate content. Cytochalasin B inhibition of unlabeled DHA accumulation was less pronounced at the longer uptake times and higher temperature of the experiments shown in Table

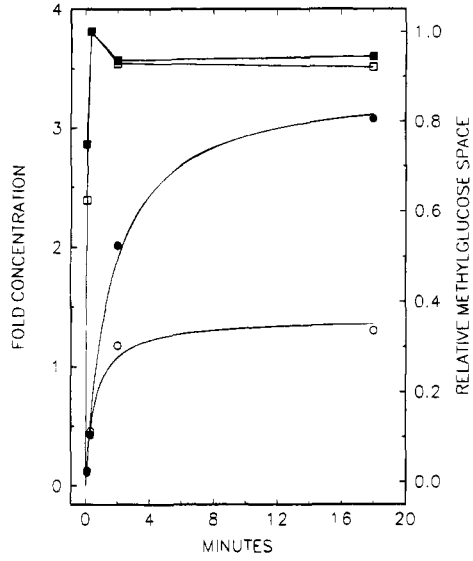

FIGURE 2: Effects of ferricyanide treatment on [^{14}C]DHA and 3-*O*-[^{3}H]methylglucose influx into erythrocytes. Uptake of 24 μM [^{14}C]-DHA was determined in the absence (closed circles) and presence (open circles) of 1 mM ferricyanide. The results are expressed relative to the equilibrium 3-*O*-methylglucose space measured in the same samples and scaled along the left *y*-axis. Uptake of 3-*O*-[^{3}H]methylglucose was also measured in the absence (closed squares) and presence (open squares) of 1 mM ferricyanide and scaled along the right *y*-axis. The 3-*O*-methylglucose uptake data are expressed relative to the maximal intracellular hexose concentration. All data are taken from the same experiment, which is representative of four such performed.

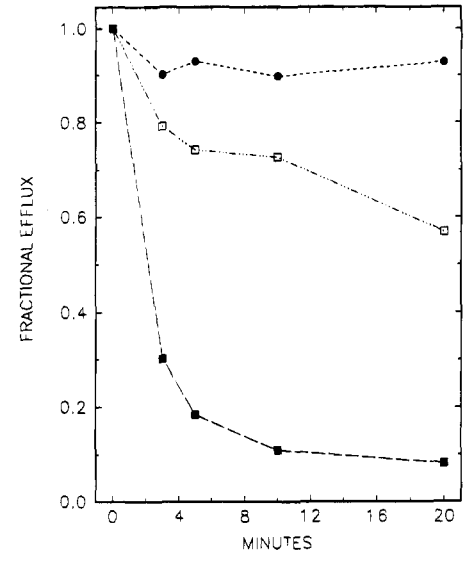

FIGURE 3: Effluxes of [^{14}C]ascorbate and [^{14}C]DHA from [^{14}C]-ascorbate-preloaded erythrocytes. Efflux of radiolabel from cells originally loaded with 24 μM [^{14}C]DHA was measured at 37 °C in PBS containing 5 mM D-glucose (solid circles), in the same buffer with 1 mM ferricyanide (solid squares), or in the same buffer with 1 mM ferricyanide and 16 μM cytochalasin B (open squares). The experiment shown is representative of three performed.

1, since initial rates of uptake were not measured. These results suggest that ascorbate and DHA entered cells with greatly different efficiencies, and that transported DHA was retained within cells following conversion to ascorbate.

We next studied the effect of ferricyanide on [^{14}C]DHA uptake into erythrocytes. It is known that ferricyanide quantitatively oxidizes [^{14}C]ascorbate to [^{14}C]DHA (Orringer & Roer, 1979). We confirmed this finding by thin-layer chromatography and HPLC (results not shown). Therefore, it was not possible to study [^{14}C]ascorbate uptake in the presence of extracellular ferricyanide. We found that ferricyanide decreased the net uptake of [^{14}C]DHA by about 50% compared to control in each of several experiments, one of which is depicted in Figure 2. Again, the presence of 10 μM cytochalasin B decreased [^{14}C]DHA uptake to less than 20% of control rates, whether or not ferricyanide was present (data not shown). This indicates that most transport occurred on the glucose transporter. The rapid accumulation of intracellular 3-*O*-methylglucose was unaffected by ferricyanide (Figure 2), suggesting that the ferricyanide-induced decrease in [^{14}C]DHA uptake was not due to impairment of the glucose transport mechanism. The cause of the ferricyanide-induced decrease in [^{14}C]DHA uptake became apparent from measurement of [^{14}C]DHA efflux, as described below.

Measurements of [^{14}C]Ascorbate and [^{14}C]DHA Efflux from Erythrocytes. In Figure 3, it can be seen that cells preloaded with [^{14}C]ascorbate (loaded as [^{14}C]DHA) lost radiolabel very slowly ($t_{1/2} \approx 150$ min). Efflux of radiolabel was increased over 10-fold when cells were incubated with 1 mM extracellular ferricyanide (Figure 3), indicating an acceleration of efflux of either [^{14}C]ascorbate or [^{14}C]DHA. Since [^{14}C]ascorbate does not appreciably leave erythrocytes without extracellular ferricyanide, the most likely explanation for the ferricyanide-induced efflux of radiolabel is that the oxidant converted intracellular [^{14}C]ascorbate to [^{14}C]DHA, which in turn rapidly left the cells on the glucose transporter. Involvement of the glucose transporter is strongly suggested by the observation that cytochalasin B inhibited most of the efflux of radiolabel (Figure 3). Ferricyanide-induced efflux of [^{14}C]DHA can also account for the 50% inhibition of [^{14}C]-DHA influx by ferricyanide shown in Figure 2. The rapid influx of [^{14}C]DHA observed without ferricyanide (Figures 1 and 2) may be aided by intracellular reduction of [^{14}C]-DHA to [^{14}C]ascorbate, which slows the rate of [^{14}C]DHA backflux out of cells on the glucose transporter. When extracellular ferricyanide is present, intracellular [^{14}C]ascorbate is oxidized to [^{14}C]DHA, and the latter escapes the cells on the glucose transporter. This effect decreases the net influx of [^{14}C]DHA. Such a mechanism is also supported by the finding that a 40 min ferricyanide treatment decreased the intracellular ascorbate concentration in ascorbate-loaded cells to about 25% that of cells loaded with ascorbate alone (Table 1).

We also found that other types of oxidant stress caused oxidation of intracellular [^{14}C]ascorbate and release of [^{14}C]-DHA from cells. Both Cu^{2+} ions and H_2O_2 increased release of radiolabel from [^{14}C]ascorbate-loaded cells (Figure 4), and lowered intracellular ascorbate concentrations proportionately (Table 1). By analogy to the previous results with ferricyanide, these data indicate that intracellular ascorbate was oxidized to DHA and that it escaped the cells on the glucose transporter. In support of this mechanism, the efflux of radiolabel from [^{14}C]ascorbate-loaded cells induced by the glucose oxidase/glucose system was decreased about 80% by the presence of cytochalasin B (not shown). The H_2O_2-induced efflux of radiolabel from erythrocytes was not due to enhanced activity of the glucose transporter, since in other experiments concentrations of H_2O_2 up to 1 mM were without effect on initial rates of 3-*O*-methylglucose transport in erythrocytes (not shown). Thus, the oxidant-induced

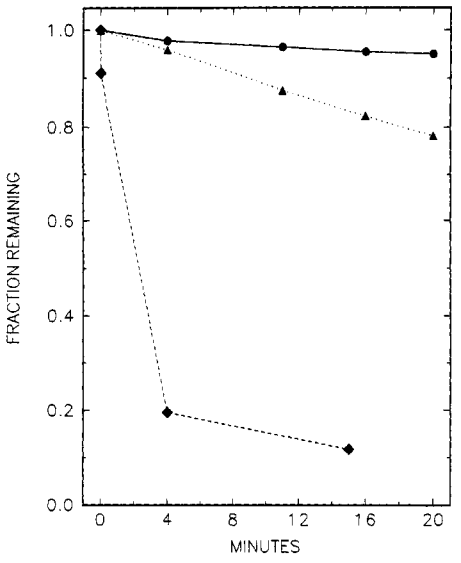

FIGURE 4: Acceleration of [^{14}C]DHA efflux from erythrocytes by Cu^{2+} and H_2O_2. Efflux of [^{14}C]DHA from 10% erythrocytes that had been loaded to an intracellular [^{14}C]ascorbate concentration of 46 μM was measured at 23 °C. The circles show exit in 5 mM D-glucose alone, the triangles show exit with 0.1 mM $CuSO_4$ added, and the diamonds show exit with 0.88 unit/mL glucose oxidase present.

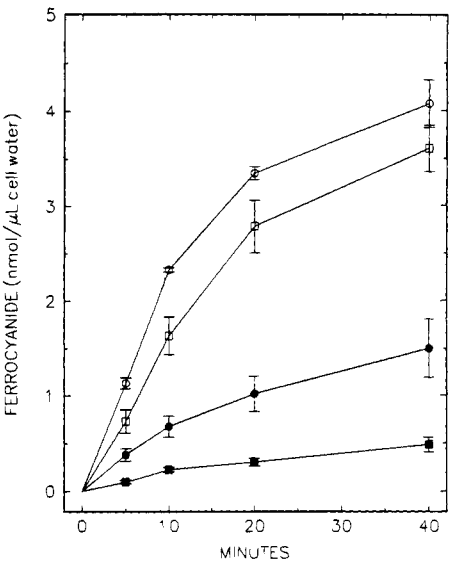

FIGURE 5: Effects of ascorbate depletion and repletion on ferricyanide reduction. Erythrocytes were subjected to the Tempol wash steps described under Experimental Procedures in the presence or absence of 1 mM Tempol. Cells from each treatment were resuspended to a 10% hematocrit in PBS containing 5 mM D-glucose and 1 mM ferricyanide, in the presence or absence of 100 μM DHA. At the indicated times, aliquots were taken for assay of ferricyanide reduction. The symbols correspond to the following: open circles, no Tempol, plus DHA; open squares, Tempol, plus DHA; closed circles, no Tempol, no DHA; closed squares, Tempol, no DHA. The data are means ± SE from four experiments.

release of intracellular radiolabel from [^{14}C]ascorbate-loaded cells is not unique to ferricyanide, indicating that such release may be expected when the cells are exposed to an overwhelming oxidant stress.

Dynamics of Extracellular Ferricyanide Reduction by Erythrocytes. The results thus far suggest that the oxidant stress caused by extracellular ferricyanide induces conversion of intracellular [^{14}C]ascorbate to [^{14}C]DHA, since the latter can rapidly leave the cells via the glucose transporter. We next wanted to determine the extent to which increasing the ascorbate content of cells contributes to their capacity to respond to an oxidant stress. It was convenient in these experiments to use ferricyanide both to oxidize ascorbate and to estimate the cumulative recycling of ascorbate. The latter is reflected in the amount of ferrocyanide generated by ferricyanide reduction in the extracellular medium. However, using ferricyanide reduction as an integrated measure of ascorbate recycling is complicated by the fact that both intracellular NADH (Zamudio et al., 1969; Mishra & Passow, 1969) and endogenous ascorbate will donate electrons to ferricyanide. To eliminate any contribution from endogenous ascorbate, we employed the cell-permeant nitroxide Tempol to selectively oxidize intracellular ascorbate to DHA (Mehlhorn, 1991; Winkler et al., 1994), followed by dilution and wash steps to allow efflux and removal of DHA. In experiments not shown, three Tempol washes removed about 95% of the radioactivity from [^{14}C]ascorbate-loaded cells. Similarly, in freshly prepared erythrocytes, such Tempol treatment decreased the intracellular ascorbate concentration to 4−6% that of washed cells not exposed to Tempol, and was without effect on intracellular GSH or NADH concentrations (data not shown). Thus, this agent does appear to selectively decrease the intracellular ascorbate content when the resulting DHA is allowed to leave the cell before it can oxidize GSH.

In Figure 5 is depicted a comparison of the time course of ferricyanide reduction in such Tempol-treated cells compared to the time course in cells not depleted of ascorbate. Although both time courses were curvilinear, it is apparent that Tempol treatment decreased the amount of ferricyanide reduced at each time point to about one-third that observed in untreated cells carried through the same wash steps. This residual ferricyanide reducing capacity, which may reflect the NADH-dependent component, can be corrected for by subtracting it from ferricyanide reduction in cells not depleted of ascorbate.

The effects of DHA addition during ferricyanide reduction are also shown in Figure 5 for both control and Tempol-treated cells. In the absence of cells, 100 μM DHA had no effect on the rate of ferricyanide reduction (not shown). Therefore, the additional reducing capacity observed in cells that had taken up DHA must have resulted from its intracellular conversion to ascorbate (see Table 1). In control cells, ferricyanide reduction was more than tripled by DHA addition, and in Tempol-treated cells, it was increased almost to the level seen in DHA-treated control cells (Figure 5). The latter shows that ascorbate-depleted cells retain the capacity to convert DHA to ascorbate, as long as glucose is present. In the absence of glucose, ferricyanide reduction is minimally affected by DHA loading, and GSH concentrations fall by 70−80% (data not shown). Overall, the results suggest that the ability of erythrocytes to reduce extracellular ferricyanide corresponds to their intracellular ascorbate content, and that ascorbate may be the major electron donor for extracellular ferricyanide.

When allowed sufficient time to enter cells, both ascorbate and its stereoisomer D-isoascorbate also enhanced erythrocyte ferricyanide reduction. In such an experiment, 15% erythrocytes were incubated in PBS containing 0.1 mM GSH for 1 h at 37 °C in the presence or absence of 100 μM ascorbate or D-isoascorbate, and washed to remove extracellular reductant. Ferricyanide reduction in both ascorbate- and

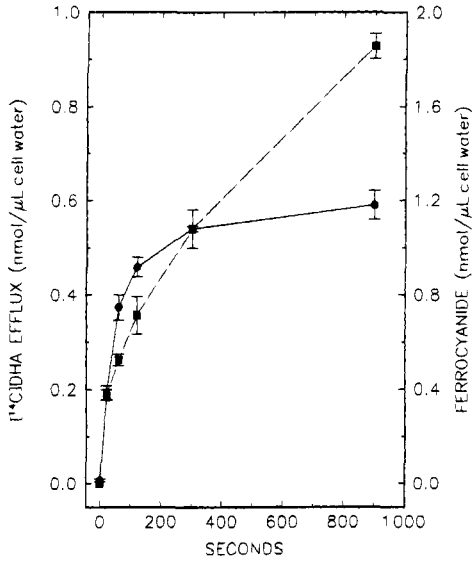

FIGURE 6: Relative rates of [^{14}C]DHA efflux and ferricyanide reduction. Erythrocytes that had been depleted of ascorbate by Tempol treatment were divided in half, and one portion was reloaded with [^{14}C]DHA to an intracellular concentration of 311 μM [^{14}C]ascorbate. Both sets of cells were resuspended to a 5% hematocrit and incubated at 37 °C for the times indicated with 1 mM ferricyanide and 5 mM D-glucose in PBS. At each time point, ferricyanide reduction was corrected for the amount of ferricyanide reduced in ascorbate-depleted cells that were not loaded with [^{14}C]-ascorbate. The squares represent ferricyanide reduction, and the circles represent [^{14}C]DHA efflux. The results are shown as means ± SE from three experiments.

D-isoascorbate-loaded cells was similar, and more than twice that observed in control cells over a 40 min period of sampling (results not shown).

To compare the time dependence of ferricyanide-induced ascorbate oxidation and subsequent loss of DHA from the cells, these two activities were measured in the same cells loaded with relatively high concentrations of [^{14}C]ascorbate (Figure 6). Several features of this experiment are important to point out. First, the potential contributions of endogenous ascorbate and NADH were removed by using ascorbate-depleted cells and by subtracting at each time point the ferricyanide reduction observed in cells not loaded with ascorbate from the ascorbate-dependent values. Second, the cells were diluted to a 5% hematocrit during the assay to minimize any reuptake of [^{14}C]DHA lost from cells. Third, the use of different scales for the right- and left-hand *y*-axes in Figure 6 assumes that each molecule of ascorbate reduces two molecules of ferricyanide. It is evident from Figure 6 that the initial rate of [^{14}C]DHA release was at least as great as the rate of ferricyanide reduction. After about 3 min, the net loss of [^{14}C]DHA from the cells ceased, leaving an intracellular concentration of [^{14}C]DHA or [^{14}C]ascorbate of 24−30 μM. Recycling of this residual ascorbate very likely accounts for the continued increase in ferricyanide reduction after equilibration of radiolabel across the cell membrane. These data indicate that under conditions of extreme cell dilution, the [^{14}C]DHA generated by oxidation of [^{14}C]-ascorbate leaves the cells as fast as it is produced. However, residual intracellular [^{14}C]DHA can be converted to [^{14}C]-ascorbate to sustain further ferricyanide reduction.

Whether such loss of DHA on the glucose transporter can modify the rate of ferricyanide reduction was further assessed in the experiment shown in Table 2. In cells preloaded with

Table 2: Effects of Ascorbate/DHA Transport on Ferricyanide Reduction[a]

cell concn	cytochalasin B	ferrocyanide generated (nmol·μL^{-1}·min^{-1})
concentrated	−	0.34 ± 0.03
	+	0.37 ± 0.04
Dilute	−	0.17 ± 0.01[b]
	+	0.26 ± 0.02[c]

[a] Erythrocytes were loaded with 200 μM DHA as described under Experimental Procedures. Equal volumes (0.15 mL) of packed, washed, erythrocytes were incubated in a total of 0.3 mL ("concentrated") or 3 mL ("dilute") of PBS containing 2.5 mM adenosine and 1 mM ferricyanide for 5 min at 37 °C before assay of ferrocyanide reduction. Cells were also incubated in the presence or absence of 16 μM cytochalasin B, as indicated. The data from five experiments are expressed as mean ± SE. [b] $p < 0.01$ compared to control cells. [c] $p < 0.05$ in cytochalasin B-treated samples compared to the respective control.

200 μM DHA, the intracellular DHA or ascorbate concentrations were maintained either by keeping the cells concentrated or by using cytochalasin B to block loss of ascorbate or DHA on the glucose transporter. Cells at a 5% hematocrit had only about half the capacity to reduce ferricyanide as the same number of cells at a 50% hematocrit (Table 2). When cytochalasin B was added to the cells before dilution, the decrease in ferricyanide reduction due to cell dilution was halved (Table 2). Cytochalasin B did not significantly increase ferricyanide reduction in 50% cells. This may indicate that ferricyanide did not lower the intracellular ascorbate concentration below a saturating level in the concentrated cells. These results suggest that maintaining intracellular ascorbate, either by decreasing the in-to-out concentration gradient (concentrated cells) or by preventing DHA loss (diluted cells in the presence of cytochalasin B), increases the capacity for extracellular ferricyanide reduction.

The capacity of erythrocytes to recycle ascorbate under *in vivo* conditions was investigated. In freshly prepared 45% cells incubated at 37 °C, the initial rate of ferricyanide reduction corresponded to a rate of ascorbate regeneration of 39 ± 3 nmol·(mL of cell water)$^{-1}$·min^{-1} (mean ± SD). This value has been corrected for ascorbate-independent ferricyanide reduction in Tempol-treated cells, and represents half the total amount of ferricyanide reduced in a given time period. When cells were incubated overnight at 3 °C in autologous serum, their capacity to reduce ferricyanide was markedly diminished to 6 ± 1.5 nmol·(mL of cell water)$^{-1}$·min^{-1}. This decrease likely reflects depletion of endogenous ascorbate, since overnight incubation typically decreases ascorbate content by 50−80%, and since such stored cells were fully able to reduce ferricyanide after ascorbate loading (results not shown).

DISCUSSION

In this work, we measured the transmembrane fluxes of radiolabeled ascorbate and DHA in human erythrocytes. As expected, DHA transport was rapid and mediated both into and out of cells by the glucose transporter. Because ascorbate movement is so slow relative to that of DHA, it had been previously ascribed to simple diffusion (Hughes & Maton, 1968; Wagner et al., 1987). However, our results suggest that ascorbate is also transported on the glucose transporter, although with a much lower efficiency than either DHA or D-glucose. Whereas ascorbate had not equilibrated

across the membrane after 3 h of incubation, DHA accumulated to concentrations several-fold greater than present in the medium within several minutes (Figure 1). This concentrative uptake of DHA was almost certainly due to its reduction to ascorbate and subsequent retention within the cells, as previously shown by direct measurements (Okamura, 1979; Iheanacho et al., 1995) and again in this study (Table 1). The implication of this result is that erythrocytes will effectively scavenge DHA released in the vascular bed in areas of oxidant stress and recycle the DHA to ascorbate. Such a mechanism has been suggested for human neutrophils (Washko et al., 1993), but may be quantitatively much more important in erythrocytes. Additionally, exogenous H_2O_2 in amounts sufficient to overwhelm erythrocyte catalase (Winterbourn & Stern, 1987; Agar et al., 1986) and GSH (Meister, 1994) will oxidize erythrocyte ascorbate, reflected in our experiments as loss of [^{14}C]DHA on the glucose transporter (Figure 4). This might also occur in areas of H_2O_2 release by neutrophils. Thus, not only can erythrocytes scavenge extracellular DHA by taking it up and recycling it to ascorbate, but their own ascorbate stores can be used in the defense against oxidant stress.

The mechanism of oxidant-induced ascorbate recycling proposed by Orringer and Roer (1979) involved efflux of ascorbate from cells, extracellular ascorbate oxidation, uptake of the resulting DHA on the glucose transporter, and intracellular reduction of DHA to ascorbate. This hypothesis was attractive because it explained observations that electron acceptors known to remain outside cells were reduced in the presence of erythrocytes (Mishra & Passow, 1969; McGown et al., 1990; Tomoda et al., 1980). In fact, extracellular ferricyanide does induce a rapid efflux of radioactivity from [^{14}C]ascorbate-loaded cells (Figure 3) that corresponds to the amount of ferricyanide reduced (Figure 6). Several aspects of our results suggest that this efflux occurs as [^{14}C]DHA rather than as [^{14}C]ascorbate, however. The rate of ferricyanide-induced exit of radiolabel from erythrocytes mirrors the rapid influx of [^{14}C]DHA rather than the slow uptake of [^{14}C]ascorbate (Figure 1). A novel ascorbate transporter that is both activated by ferricyanide and inhibited by cytochalasin B seems unlikely, since other types of oxidant stress also increase radiolabel efflux from [^{14}C]ascorbate-loaded cells (Figure 4). Further, ferricyanide reduction by ascorbate-loaded cells is increased by treatment with cytochalasin B (Table 2), rather than decreased, as predicted if ferricyanide reduction requires efflux of ascorbate from cells on a cytochalasin B-sensitive ascorbate transporter.

Instead of extracellular oxidation of ascorbate, our results support the model shown in Figure 7, in which ferricyanide causes oxidation of intracellular ascorbate to DHA, which then rapidly leaves the cell on the glucose transporter. The model predicts that the extent of ferricyanide reduction should be proportional to the intracellular ascorbate concentration. Accordingly, we found that ascorbate loading increased and ascorbate depletion decreased the capacity of the cells for ferricyanide reduction (Figure 5). Further, ascorbate-dependent ferricyanide reduction was increased under conditions in which either ascorbate or DHA was retained within cells. Thus, a concentrated cell suspension had a greater capacity for ferricyanide reduction than the same number of cells in dilute suspension, and the capacity to reduce ferricyanide was enhanced by preventing exit of

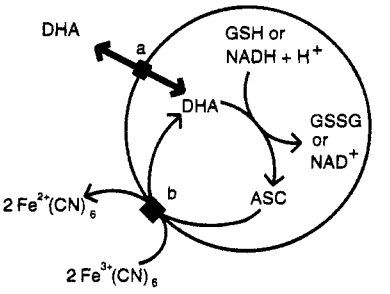

FIGURE 7: Hypothetical model of ascorbate recycling in erythrocytes. DHA enters and leaves cells on the glucose transporter ("a"). Intracellular DHA is converted to ascorbate ("ASC"), via GSH- or NADH-dependent reduction. Extracellular ferricyanide ["Fe^{3+}-$(CN)_6$"] oxidizes intracellular ascorbate through the action of a transmembrane oxidoreductase ("b").

DHA on the glucose transporter with cytochalasin B (Table 2).

The manner in which ascorbate electrons are transferred to ferricyanide across the erythrocyte membrane remains to be explained at the molecular level. One possibility is that the transmembrane transfer is mediated by a monodehydroascorbate reductase (Schipfer et al., 1985; Schweinzer & Goldenberg, 1992, 1993). This enzyme is thought to use intracellular reducing equivalents to reduce very low concentrations of extracellular monodehydroascorbyl radical back to ascorbate, which then donates a single electron to a molecule of ferricyanide (Schweinzer & Goldenberg, 1993). The ultimate source of reducing equivalents remains intracellular, presumably donated to the transmembrane enzyme by ascorbate or NADH. A monodehydroascorbyl free radical reductase has been described in erythrocyte membranes (Goldenberg et al., 1983). However, this system does not account for our observations that ferricyanide reduction was proportional to the intracellular ascorbate concentration, and that the extracellular ascorbate concentration should have been very low around cells loaded with [^{14}C]DHA and washed several times to remove residual extracellular radiolabel.

It is more likely that intracellular ascorbate reduces extracellular ferricyanide via a transmembrane oxidoreductase (Zamudio & Canessa, 1966; Zamudio et al., 1969), as depicted in Figure 7. Such an enzyme has been purified (Wang & Alaupovic, 1978), and its kinetic features have been studied with NADH as a substrate (Wang, 1980). NADH has been considered the natural substrate for this enzyme (Zamudio et al., 1969; Agutter et al., 1980; Schipfer et al., 1985). However, our results suggest that ascorbate may be the major electron donor, especially when erythrocyte ascorbate concentrations are increased by loading the cells with ascorbate. For example, selective depletion of erythrocyte ascorbate decreased the capacity for ferricyanide reduction to about one-third that of untreated cells, and subsequent DHA-loading increased this capacity to more than two-fold that of untreated cells (Figure 5).

Although erythrocytes carry only about 30% of the ascorbic acid present in whole blood (Evans et al., 1982), their ability to regenerate the vitamin in an energy-dependent manner substantially enhances the ascorbate-related antioxidant capacity of blood. In contrast to other cells that accumulate ascorbate against a concentration gradient (Rose, 1988), ascorbate regenerated within erythrocytes will gradually equilibrate with plasma ascorbate. By using the rate of

ferricyanide reduction in freshly prepared cells as a measure of intracellular ascorbate recycling [≈40 nmol·(mL of cell water)$^{-1}$·min^{-1}], it can be calculated that 45% hematocrit erythrocytes can regenerate the ascorbate present in a milliliter of blood once every 3 min at a plasma ascorbate concentration of 40 μM. At higher blood ascorbate concentrations (100−200 μM), such as those present in ascorbate-supplemented individuals (Dhariwal et al., 1991; Evans et al., 1982), this capacity will increase proportionately. It remains to be seen whether the presumed transmembrane oxidoreductase is involved in the erythrocyte response to an extracellular oxidant stress. Nonetheless, the capacity of erythrocytes to regenerate ascorbate, measured indirectly as the rate of ascorbate-dependent ferricyanide reduction, may constitute a substantial antioxidant reserve *in vivo*.

REFERENCES

Agar, N. S., Sadrzadeh, S. M. H., Hallaway, P. E., & Eaton, J. W. (1986) *J. Clin. Invest. 77*, 319−321.

Agutter, P. S., Dallas, J. M., Newman, P., & Kadlubowski, M. (1980) *Biochem. Soc. Trans. 8*, 326.

Ames, B. N., Magaw, R., & Gold, L. S. (1987) *Science 236*, 271−280.

Avron, M., & Shavit, N. (1963) *Anal. Biochem. 6*, 549−554.

Balmukhanov, B. S., Zamula, S. V., & Ataullakhanov, F. I. (1980) *Biokhimiia 45*, 945−949.

Bianchi, J., & Rose, R. C. (1986) *Proc. Soc. Exp. Biol. Med. 181*, 333−337.

Christine, L., Thomson, G., Iggo, B., Brownie, A. C., & Stewart, C. P. (1956) *Clin. Chim. Acta 1*, 557−569.

Crane, F. L., Löw, H., & Clark, M. G. (1985) in *The Enzymes of Biological Membranes* (Martonosi, A. N., Ed.) pp 465−510, Plenum Press, New York.

Dhariwal, K. R., Hartzell, W. O., & Levine, M. (1991) *Am. J. Clin. Nutr. 54*, 712−716.

Evans, R. M., Currie, L., & Campbell, A. (1982) *Br. J. Nutr. 47*, 473−482.

Finn, F. M., & Johns, P. A. (1980) *Endocrinology 106*, 811−817.

Frei, B., England, L., & Ames, B. N. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 6377−6381.

Goldenberg, H., Grebing, C., & Löw, H. (1983) *Biochem. Int. 6*, 1−9.

Grebing, C., Crane, F. L., Löw, H., & Hall, K. (1884) *J. Bioenerg. Biomembr. 16*, 517−533.

Hissin, P. J., & Hilf, R. (1976) *Anal. Biochem. 74*, 214−226.

Hornig, D., Weber, F., & Wiss, O. (1971) *Clin. Chim. Acta 31*, 25−35.

Hughes, R. E., & Maton, S. C. (1968) *Br. J. Haematol. 14*, 247−253.

Iheanacho, E. N., Stocker, R., & Hunt, N. H. (1993) *Biochim. Biophys. Acta 1182*, 15−21.

Iheanacho, E. N., Hunt, N. H., & Stocker, R. (1995) *Free Radicals Biol. Med. 18*, 543−552.

Jacob, R. A., Skala, J. H., & Omaye, S. T. (1987) *Am. J. Clin. Nutr. 46*, 818−826.

Jennings, P. E., Chirico, S., Jones, A. F., Lunec, J., & Barnett, A. H. (1987) *Diabetes Res. 6*, 151−154.

Loh, H. S., & Wilson, C. W. M. (1971) *Br. Med. J. 3*, 733−735.

McGown, E. L., Lyons, M. F., Marini, M. A., & Zegna, A. (1990) *Biochim. Biophys. Acta 1036*, 202−206.

Mehlhorn, R. J. (1991) *J. Biol. Chem. 266*, 2724−2731.

Meister, A. (1994) *J. Biol. Chem. 269*, 9397−9400.

Mishra, R. K., & Passow, H. (1969) *J. Membr. Biol. 1*, 214−224.

Okamura, M. (1979) *J. Nutr. Sci. Vitaminol. 25*, 269−279.

Orringer, E. P., & Roer, M. E. (1979) *J. Clin. Invest. 63*, 53−58.

Pachla, L. A., & Kissinger, P. T. (1979) *Methods Enzymol. 62*, 15−24.

Robertson, J. D., Maughan, R. J., Duthie, G. G., & Morrice, P. C. (1991) *Clin. Sci. 80*, 611−618.

Rose, R. C. (1988) *Biochim. Biophys. Acta 947*, 335−366.

Schipfer, W., Neophytou, B., Trobisch, R., Groiss, O., & Goldenberg, H. (1985) *Int. J. Biochem. 17*, 819−823.

Schweinzer, E., & Goldenberg, H. (1992) *Eur. J. Biochem. 206*, 807−812.

Schweinzer, E., & Goldenberg, H. (1993) *Eur. J. Biochem. 218*, 1057−1062.

Sinclair, A. J., Girling, A. J., Gray, L., LeGuen, C., Lunec, J., & Barnett, A. H. (1991) *Diabetologia 34*, 171−175.

Stankova, L., Riddle, M., Larned, J., Burry, K., Menashe, D., Hart, J., & Bigley, R. (1984) *Metabolism 33*, 347−353.

Székely, M., Mányai, S., & Straub, F. B. (1952) *Acta Physiol. Acad. Sci. Hung. 3*, 571−583.

Taverna, R. D., & Langdon, R. G. (1973) *Biochim. Biophys. Acta 323*, 207−219.

Tomoda, A., Ida, Y., Yoneyama, S., Kitajima, S., & Minakami, S. (1980) *Experientia 36*, 1345−1347.

Tsybyshev, V. P., & Kuznetsov, A. N. (1988) *Izv. Akad. Nauk, Ser. Biol. 1*, 64−74.

Vera, J. C., Rivas, C. I., Fischbarg, J., & Golde, D. W. (1993) *Nature 364*, 79−82.

Vinson, J. A., Staretz, M. E., Bose, P., Kassm, H. M., & Basalyga, B. S. (1989) *Diabetes 38*, 1036−1041.

Wagner, E. S., White, W., Jennings, M., & Bennett, K. (1987) *Biochim. Biophys. Acta 902*, 133−136.

Wang, C.-S. (1980) *Biochim. Biophys. Acta 616*, 22−29.

Wang, C.-S., & Alaupovic, P. (1978) *J. Supramol. Struct. 9*, 1−14.

Washko, P. W., Wang, Y., & Levine, M. (1993) *J. Biol. Chem. 268*, 15531−15535.

Winkler, B. S. (1987) *Biochim. Biophys. Acta 925*, 258−264.

Winkler, B. S., Orselli, S. M., & Rex, T. S. (1994) *Free Radicals Biol. Med. 17*, 333−349.

Winterbourn, C. C., & Stern, A. (1987) *J. Clin. Invest. 80*, 1486−1491.

Zamudio, I., & Canessa, M. (1966) *Biochim. Biophys. Acta 120*, 165−169.

Zamudio, I., Cellino, M., & Canessa-Fischer, M. (1969) *Arch. Biochem. Biophys. 129*, 336−345.

Zerez, C. R., Lee, S. J., & Tanaka, K. R. (1987) *Anal. Biochem. 164*, 367−373.

BI9506738