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Ascorbate is the major electron donor for a transmembrane oxidoreductase of human erythrocytes

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Abstract

Ascorbic acid is an important antioxidant in human blood. Erythrocytes contribute to the antioxidant capacity of blood by regenerating ascorbate and possibly by exporting ascorbate-derived reducing equivalents through a transmembrane oxidoreductase. The role of ascorbate as an electron donor to the latter enzyme was tested in human erythrocytes and ghosts using nitroblue tetrazolium as an electron acceptor. Although nitroblue tetrazolium was not directly reduced by ascorbate, erythrocyte ghosts facilitated reduction of nitroblue tetrazolium in the presence of ascorbate and ascorbate derivatives containing a reducing double bond. The resulting blue monoformazan product was deposited directly in ghost membranes. Ascorbate-induced monoformazan deposition showed several features of an enzyme-mediated process, including hyperbolic dependence on substrate and acceptor concentrations, as well as sensitivity to enzyme proteolysis, detergent solubilization, and sulfhydryl reagents. Incubation of intact erythrocytes with nitroblue tetrazolium caused deposition of the monoformazan in ghost membranes prepared from the cells. This deposition reflected the intracellular ascorbate content and was inhibited by extracellular ferricyanide, a known electron acceptor for the transmembrane oxidoreductase. Although nitroblue tetrazolium did not cross the cell membrane, like the cell-impermeant ferricyanide, it oxidized intracellular [^{14}C]ascorbate to [^{14}C]dehydroascorbate, which then exited the cells. In resealed ghosts, both monoformazan deposition and ferricyanide reduction were proportional to the intravesicular ascorbate concentration. NADH was only about half as effective as a donor for the enzyme as ascorbate in both open and resealed ghosts. These results suggest that not only can ascorbate donate electrons to a transmembrane oxidoreductase, but that it may be the major donor in intact erythrocytes.

Keywords: Ascorbic acid; Ascorbate recycling; Nitroblue tetrazolium; Transmembrane oxidoreductase; Erythrocyte; (Human)

1. Introduction

Ascorbic acid, or vitamin C, is considered one of the most important antioxidants in human serum [1,2]. About 30% of ascorbate in whole blood is carried in erythrocytes [3]. In contrast to other cell types, in which ascorbate concentrations may be many-fold higher than in plasma [4], intracellular erythrocyte ascorbate concentrations are the same as those in plasma [3,5]. However, an intracellular recycling mechanism may provide many more ascorbate reducing equivalents for the antioxidant reserve of whole blood than suggested by the ascorbate concentration alone.

The concept of ascorbate recycling by erythrocytes [6] received experimental support from the work of Orringer and Roer [7], who showed that intracellular ascorbate concentrations correlated with the ability of human erythrocytes to reduce extracellular ferricyanide. Ferricyanide is a mild oxidant that does not cross the erythrocyte plasma membrane [8,9]. In the original hypothesis [7], a molecule of ascorbate released from cells directly reduces two molecules of ferricyanide. The resulting dehydroascorbate reenters the cells and undergoes reduction back to ascorbate via an NADH- [7] or GSH-dependent [10,11] process. In support of this model, dehydroascorbate is rapidly taken up by erythrocytes, either on the glucose [4,12] or another transporter [13]. Further, when added to an erythrocyte suspension, dehydroascorbate enhances extracellular ferricyanide reduction [7,14]. Against the model is the fact that exit of ascorbate from cells is very slow

Abbreviations: NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline.

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[15,16], at least in the absence of an extracellular oxidant. Further, Schipfer et al. [14] found no evidence for ferricyanide-induced release of ascorbate from intact erythrocytes. Therefore, a mechanism other than transport of ascorbate itself must be considered to explain the transfer of ascorbate reducing equivalents out of erythrocytes.

Erythrocytes possess a transmembrane oxidoreductase activity (E.C. 1.6.99.3) that can use extracellular ferricyanide as an acceptor for electrons derived from NADH [17,18]. Such an enzyme might mediate the ascorbate-dependent ferricyanide reduction observed in intact erythrocytes [7,14]. To account for ascorbate-mediated electron transfer, the enzyme must be able to use ascorbate as well as NADH. However, testing directly whether ascorbate can serve as an electron donor to ferricyanide is not possible in isolated membranes or in purified enzyme preparations. This is because ascorbate itself quantitatively reduces ferricyanide in solution [7,14]. A different electron acceptor must be used to test the hypothesis that ascorbate is a substrate for the transmembrane oxidoreductase. Zamudio et al. [17] showed that nitroblue tetrazolium (NBT) is reduced by NADH in the presence of erythrocyte membranes. They also found that the precipitate of the monoformazan was deposited in the membranes, leading them to suggest that the enzyme responsible was located within the membrane. We reasoned that NBT, which is typically used as an electron acceptor from superoxide [19], might serve as a suitable acceptor for ascorbate electrons in this system. In this work we show that erythrocyte membranes do accelerate reduction of NBT in the presence of ascorbate, and that the monoformazan is deposited in the erythrocyte membrane. The monoformazan of NBT is also deposited in the plasma membranes of intact erythrocytes and resealed ghosts in a way that is dependent on the intracellular ascorbate concentration and that is inhibited by ferricyanide. These results suggest that one or more transmembrane oxidoreductases mediate the transfer of electrons from intracellular ascorbate to extracellular oxidants.

2. Experimental procedures

2.1. Materials

Solid [1-^{14}C]ascorbic acid (4.7 Ci/mol) was obtained from New England Nuclear. Ascorbate oxidase, superoxide dismutase, dehydroascorbate, ascorbate 6-palmitate, NBT, and D-isoascorbate were obtained from Sigma, St. Louis, MO. NBT was stored as a 58 mM solution in dimethylsulfoxide at 4°C. A working solution of NBT was prepared just before each assay by dilution into phosphate-buffered saline (PBS), which consisted of deionized, distilled water, containing 140 mM NaCl, 12.5 mM Na_2PO_4, pH 7.4. The amount of dimethylsulfoxide in the assay depended on the NBT concentration. At a final NBT concentration of 116 μM in the assay, the concentration of dimethylsulfoxide was 0.2% (v/v).

2.2. Preparation of human erythrocytes, ghosts, and membranes

Human erythrocytes were prepared from heparinized blood drawn from normal volunteers. The cells were washed three times in ten volumes of PBS, with careful removal of the buffy coat. Sometimes cells were stored up to 48 h in autologous serum at 4°C before use. Erythrocyte ghosts that had been depleted of cytoskeletal proteins were prepared according to the method described by Gorga and Lienhard [20]. Resealed impermeable erythrocyte ghosts were prepared from intact cells as described by Steck and Kant [21]. Reconstituted erythrocyte band 4.5 (nomenclature of Fairbanks et al. [22]) was prepared by chromatography over diethylaminoethyl cellulose according to the method of Baldwin et al. [23], as previously described [24]. This method removes the band 3 anion transporter and allows reconstitution of the remaining intrinsic membrane proteins into endogenous erythrocyte lipids. The predominant protein in these vesicles is the band 4.5 glucose transporter, GLUT1 [23]. Protein content of erythrocyte ghosts and reconstituted band 4.5 was determined by the BCA method (Pierce).

2.3. Measurement of NBT reduction in erythrocyte ghosts and membranes

In a typical assay, 50 μl of erythrocyte ghosts (60–200 μg) or purified band 4.5 (25–60 μg) were incubated for 60 min at 37°C in a total volume of 0.5 ml of PBS containing 58–116 μM NBT and other additives as indicated. The samples were mixed by swirling every few minutes. At the end of the assay, the samples were diluted to 1 ml with PBS and the absorbance at 560 nm was read in a Gilford Model 250 spectrophotometer. The absorbance reading of the sample was corrected for absorbance of a membrane sample incubated with NBT under identical conditions, but without ascorbate. In the absence of erythrocyte membranes, incubation of 100 μM ascorbate or NADH with 100 μM NBT for 1 h at 37°C resulted in an absorbance that was less than 10% of that observed in the presence of membranes. Essentially all of the detectible color was in the ghost membranes, since removal of the ghosts by centrifugation resulted in a supernatant with less than 2% absorbance compared to a membrane suspension (data not shown). The two-electron reduced monoformazan is considered the major reduction product of NBT under the conditions used [25], and it has a molar extinction coefficient of 10800 M^{-1} cm^{-1} [26]. Thus in a typical experiment with 100 μg of ghost protein incubated in the presence of 100 μM ascorbate and 58 μM NBT, 4.6 μM monoformazan or about 10% of the NBT was deposited in the membrane after 1 h of incubation. However, the data

have not been presented as molar values, due to the confounding effects of deposition of the product in the lipid bilayer.

2.4. Assay of erythrocyte ferricyanide reduction

Resealed erythrocyte ghosts containing the indicated agent were washed three times in 10 volumes of PBS containing 5 mM D-glucose. For measurement of ferricyanide reduction, packed ghosts (0.1 ml) were diluted to 1 ml with PBS containing 5 mM D-glucose and 100 μM ferricyanide. The ghost suspension was incubated at 37°C in a shaking water bath. At the indicated times, 0.2 ml aliquots of ghosts 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 [27], using an assay volume of 1 ml. The amount of ferrocyanide was determined from its optical density at 510 nm, using an extinction coefficient of 10500 M^{-1} cm^{-1} for the 1,10-phenanthroline complex [27]. Following correction for absorbance of the ghost-conditioned PBS at 510 nm, the amount of ferrocyanide generated was expressed relative to the aqueous resealed ghost space [7].

2.5. Measurement of radiolabeled ascorbate efflux from erythrocytes

Efflux of [^{14}C]dehydroascorbate from erythrocytes was measured as follows. Erythrocytes at a 40% hematocrit in PBS containing 5 mM D-glucose were loaded with 100–200 μM [^{14}C]dehydroascorbate for 10 min at room temperature. Ascorbate oxidase (2 units/ml) was used to generate [^{14}C]dehydroascorbate from [^{14}C]ascorbate added to the cell suspension. In the absence of cells, this concentration of ascorbate oxidase completely converts 200 μM [^{14}C]ascorbate to [^{14}C]dehydroascorbate within 5 min (data not shown). Following the loading step, extracellular radioactivity was removed by washing the cells three times by centrifugation in ten volumes of PBS containing 5 mM D-glucose. Over 90% of the radioactivity was retained by the cells under these conditions. It has been demonstrated previously by infrared spectroscopy that the retained form of ascorbate is reduced ascorbate and not dehydroascorbate [16]. Packed cells were pipetted into microfuge tubes and efflux was initiated by vigorously diluting the cells with 22 volumes of the same buffer at 37°C. The assay was terminated by another 5-fold dilution with ice-cold PBS containing 0.1 mM phloretin. The cells were pelleted in a microfuge and an aliquot of the medium was counted for radioactivity released from the cells.

2.6. HPLC measurement and identification of ascorbate and derivatives

Aliquots of the efflux medium from cells containing ascorbate or ascorbate metabolites labeled with carbon-14 were chromatographed by HPLC using the reverse-phase ion-pair HPLC method of Pachla and Kissinger [28]. An aliquot of 0.2 ml of the efflux medium in a 1.4 ml microfuge tube was treated with 0.4 ml of ice-cold 15% trichloroacetic acid, vortexed vigorously, and allowed to sit on ice for 10 min. The solution was microfuged for 1 min in a Beckman Model B microfuge and the supernatant was extracted five times with 0.4 ml of diethyl ether to remove the trichloroacetic acid. Residual traces of ether were removed under a stream of nitrogen and the sample was placed on ice until measurement within 3 h. A 100 μl aliquot of the extract was injected onto a Waters DeltaPak C_{18} column (300 μm, 5 μm). This mobile phase consisted of 70 mM ammonium acetate, 1 mM tridecylamine, and 15% methanol, pH 5.2. Labeled fractions were collected over 30 s intervals and counted for radioactivity. At a flow rate of 1 ml/min, labeled or unlabeled ascorbate eluted at 5.8–6.2 min. The sensitivity for unlabeled ascorbate was 200 pmol using UV detection at 254 nm. Elution of [^{14}C]dehydroascorbate occurred just after the void volume, at 1.7 min, whereas the presumed breakdown product of dehydroascorbate, 2,3-diketogulonic acid [29], eluted at 7.9–8.3 min.

Attempts to directly measure the ascorbate content of intact erythrocytes by the above method were unsuccessful, due to oxidation of intracellular ascorbate to dehydroascorbate during cell lysis and extraction. Use of other protein precipitants (90% methanol or 50 mM perchloric acid), addition of chelating agents (e.g., 1 mM EDTA) [30], and displacement of oxygen from hemoglobin by carbon monoxide before lysis [31] showed similar results. Erythrocyte ascorbate has typically been estimated as the dinitrophenyl hydrazones of dehydroascorbate and 2,3-diketogulonic acid [3,32], perhaps for this reason.

2.7. Data analysis

Except where noted, results are expressed as mean ± standard error for the indicated number of experiments, in which duplicate or triplicate determinations were made. Statistical comparisons were made using the Student's paired *t*-test. Data fitting was carried out using the nonlinear curve-fitter in the graphics analysis package Axum (TriMetrix, Seattle, WA).

3. Results

3.1. Reduction of NBT and deposition of the monoformazan in erythrocyte membranes

Incubation of increasing amounts of erythrocyte ghosts with 58 μM NBT and 100 μM ascorbate for 1 h at 37°C resulted in deposition of the monoformazan reduction product of NBT in the ghost membranes (Fig. 1). This was detected as an increase in UV absorbance a 560 nm.

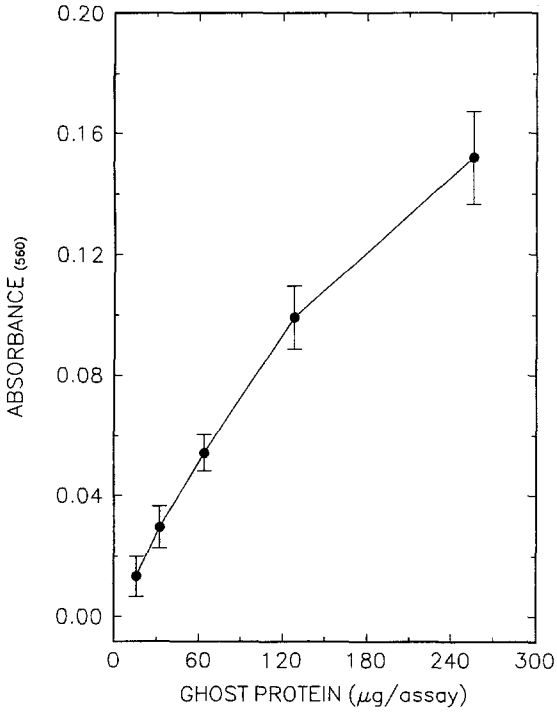

Fig. 1. Deposition of the monoformazan of NBT with increasing amounts of ghost membranes. Erythrocyte ghost membranes were incubated with 58 μM NBT and 100 μM ascorbate for 1 h at 37°C and the amount of monoformazan deposited in the membrane was determined as described under Experimental procedures. The data are from four experiments.

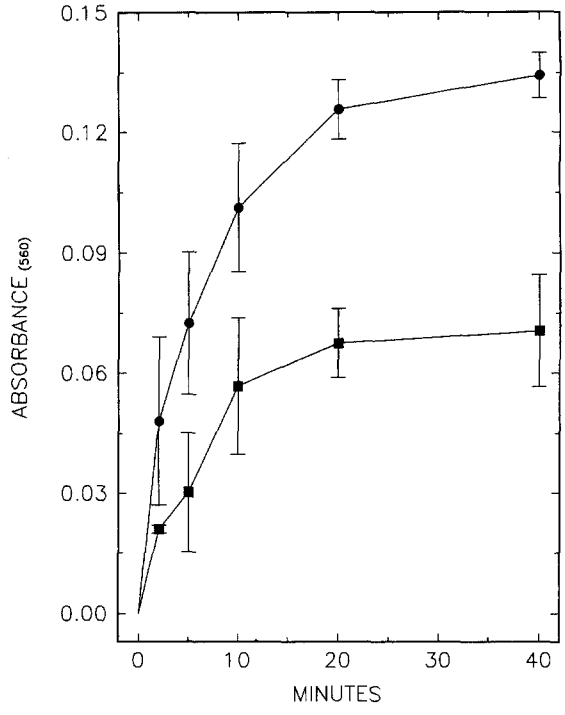

Fig. 2. Effects of temperature on the time-course of monoformazan deposition in ghost membranes. Ghost membranes (200 μg/assay) were incubated with 58 μM NBT and 100 μM ascorbate in 5 mM phosphate buffer, pH 8.0, either at 23°C (squares) or at 37°C (circles). At the indicated times, the amount of monoformazan deposited was measured as described under Experimental procedures. The results shown are from four experiments.

Deposition of the monoformazan increased with increasing concentrations of ghosts up to about 140 μg of ghost protein, beyond which the rate of deposition decreased. At higher ghost concentrations, the amounts of ascorbate or NBT may have become limiting. The effects of incubation at 23°C or 37°C on monoformazan deposition in ghost membranes are shown in the Fig. 2. At both temperatures the rate of monoformazan deposition was rapid over the first 10 min, reaching a plateau after about 15–20 min. Incubation at 23°C lowered the initial rate of monoformazan deposition by half that seen at 37°C. All subsequent assays were performed at 37°C.

The amount of monoformazan deposited in ghost membranes showed an hyperbolic dependence on the concentration of ascorbate, NADH and NBT (data not shown). Kinetic parameters for the two donors and the acceptor are provided in Table 1. The apparent K_m values for NADH and ascorbate were similar, although the maximal amount of monoformazan deposited with ascorbate was almost twice that seen with NADH as the donor. With ascorbate as the electron donor, the apparent affinity of NBT of 41 μM was lower than for the electron donors (Table 1).

The specificity of NBT reduction was evaluated by assessing the effects of ascorbate derivatives, enzyme treatments, detergent solubilization, and sulfhydryl reagents on monoformazan deposition (Table 2). The stereoisomer of L-ascorbate, D-isoascorbate, enhanced monoformazan deposition to the same extent as ascorbate. Neither ascorbate 2-sulfate nor dehydroascorbate caused an appreciable change in membrane color over that due to NBT alone. These results indicate that the reducing double bond structure of ascorbate is necessary for the observed effect, but that stereospecificity is not required. Monoformazan deposition with ascorbate 6-palmitate as the electron donor was

Table 1
Kinetic parameters of the transmembrane oxidoreductase

Experimental variable	K_m (μM)	V_{max}
Ascorbate (7)	21±7	1.7±0.3
NADH (4)	13±4	0.9±0.2
NBT (6)	41±7	1.7±0.4

Rates of monoformazan deposition in erythrocyte ghost membranes were measured at 10 min as described under Experimental procedures. When ascorbate and NADH were substrates, the concentration of NBT was 116 μM. Measurement of parameters for NBT was performed at an ascorbate concentration of 100 μM. Results are shown for the number of experiments indicated in parentheses. The V_{max} values are given as the change in absorbance per 10 min per mg of ghost protein. With increasing concentrations of both ascorbate and NADH there was a small amount of NBT converted to the monoformazan in the absence of ghosts, and this was corrected for with the use of reagent blanks in each assay.

greater than observed with ascorbate. This lipophilic ascorbate derivative had a higher background of reduction of NBT in the absence of membranes (about 30% of the total), but even when correction was made for this effect, monoformazan deposition was clearly increased with this derivative.

The effects of several enzymes on ascorbate-mediated NBT reduction are also shown in Table 2. Superoxide dismutase did not modify the extent of monoformazan deposition, indicating that superoxide was not the mediator of the effect. Treatment of erythrocyte ghosts with either trypsin or chymotrypsin decreased the amount of monoformazan deposited in the ghost membranes by 40% of that observed in controls. Incubation of intact erythrocytes with 40 μg/ml trypsin or chymotrypsin for 30 min at 37°C had no effect on monoformazan deposition in erythrocyte ghosts prepared from these cells (data not shown). These results suggest that at least part of the electron transfer activity is due to a protein exposed on the cytoplasmic face of the membrane.

Monoformazan deposition in ghost membranes in the presence of ascorbate was sensitive to detergent solubilization of the membranes. Solubilitization of ghosts in either octyl-ß-D-glucopyranoside or Triton X-100 increased absorbance at 560 nm several-fold, as shown in Table 2. On the other hand, SDS essentially abolished NBT reduction. Similar results were found with NADH as the electron donor (data not shown). When freshly prepared, neither octyl-ß-D-glucopyranoside nor Triton X-100 reduced NBT in the presence of ascorbate alone (data not shown).

Table 2
Formazan deposition in ghost membranes: effects of substrates, enzymes, and detergents

Additive	Fraction of control	n
Ascorbate 2-sulfate (100 μM)	0.16 ± 0.06 *	3
D-Isoascorbate (100 μM)	1.22 ± 0.2	3
Ascorbate 6-palmitate (100 μM)	3.3 ± 0.3 *	3
Dehydroascorbate (100 μM)	0.08 ± 0.0 *	3
Superoxide dismutase (100 units/ml)	0.95 ± 0.03	3
Trypsin (40 μg/ml)	0.6 ± 0.06 *	4
Chymotrypsin (40 μg/ml)	0.6 ± 0.1 *	4
Octyl-ß-D-glucopyranoside (38 mM)	4.2 ± 0.5 *	5
Triton X-100 (0.3%)	2.7 ± 0.3 *	6
SDS (0.05%)	0.1 ± 0.1 *	2
N-Ethylmaleimide (2 mM)	0.59 ± 0.04 *	4
p-CMB (20 μM)	0.53 ± 0.09 *	3
p-CMBS (20 μM)	0.61 ± 0.1 *	3

Where added, ascorbate was 100 μM, NBT was 58 or 116 μM, and there was 100–200 μg of ghost protein in each assay. Incubations with dehydroascorbate also contained two units of ascorbate oxidase to ensure absence of contaminating ascorbate. Proteinases and thiol reagents were incubated with ghost membranes for 30 min at 37°C, followed by two centrifugation washes in 1 ml of fresh 5 mM sodium phosphate buffer, pH 8.0, and assay of monoformazan deposition. The mercurials were *p*-chloromercuribenzoate (*p*-CMB) and its sulfonate (*p*-CMBS). Data are expressed as a fraction of an ascorbate-treated control performed in the same experiment. An '*' indicates $P < 0.05$ versus incubation with ascorbate. *n* = number of experiments.

Table 3
Comparison of NBT reduction by ascorbate and NADH in protein-depleted ghosts and in purified band 4.5

Membrane preparation	Ascorbate (100 μM)	NADH (100 μM)
Ghosts	0.06 ± 0.001	0.03 ± 0.001 *
Band 4.5	0.28 ± 0.02	0.16 ± 0.01 *

Erythrocyte ghosts and purified band 4.5 were incubated at the same protein concentration (60 μg/ml) with 116 μM NBT and 100 μM ascorbate or 100 μM NADH1 h at 37°C in PBS, followed by measurement of monoformazan deposition as described under Experimental procedures. Data are shown from three experiments, with an asterisk ('*') indicating $P < 0.01$ compared to the ascorbate-treated preparation.

Treatment of ghost membranes with any of several sulfhydryl reagents irreversibly inhibited monoformazan deposition (Table 2). The inhibition shown for each sulfhydryl reagent is the maximal observed. The observed inhibitions suggest that the protein-mediated electron transfer requires one or more sulfhydryls for full activity. Lack of a more extensive inhibition by the sulfhydryl reagents indicates that the process involved is only partially sensitive to sulfhydryl blockade, or that more than one reducing process is involved.

To further assess the hydrophobicity and exposure of the oxidoreductase, erythrocyte ghosts were solubilized in 44 mM octyl-glucoside and passed over a small column of diethylaminoethyl cellulose. This treatment results in a preparation depleted of proteins with regions of exposed negative charge, such as the band 3 anion transporter [23]. Reconstitution of the unbound proteins in endogenous erythrocyte lipids and removal of octyl-ß-D-glucoside by dialysis resulted in a preparation that had almost fivefold greater NBT reducing activity than observed for erythrocyte ghosts at the same protein concentration (Table 3). Further, monoformazan deposition with NADH was only about half that observed with ascorbate in either ghosts or in reconstituted band 4.5. Thus, the higher maximal effect of ascorbate compared to NADH persists in a reconstituted preparation. These results also suggest either that the specific activity of the enzyme(s) involved was increased in the band 4.5 preparation, or that the activity was more accessible in the reconstituted state.

The results thus far indicate that ascorbate-associated monoformazan deposition in erythrocyte membranes is mediated by one or more enzymes that are cryptic in the membrane. However, they do not show the sidedness of the process with respect to the membrane bilayer. This was investigated by measuring monoformazan deposition in intact erythrocytes and resealed ghosts containing varying amounts of ascorbate.

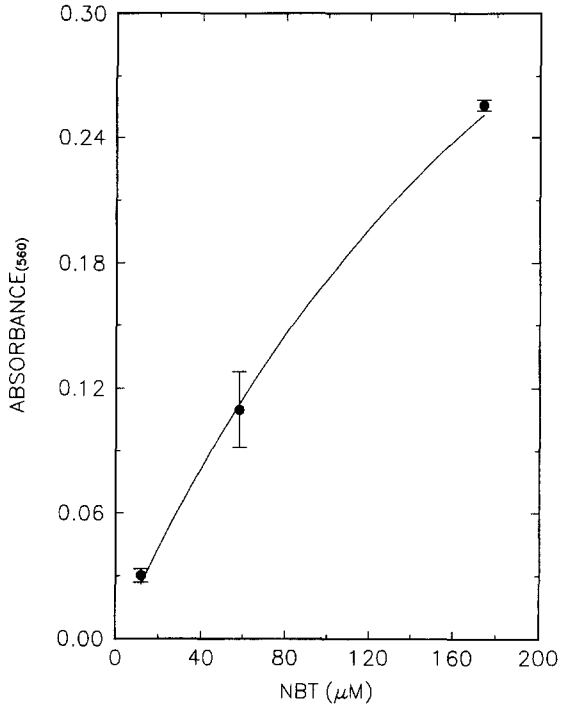

Fig. 3. NBT reduction by intact erythrocytes. Erythrocytes at a 20% hematocrit were incubated in PBS containing 5 mM D-glucose and the indicated concentration of NBT for 1 h at 37°C with mixing. The cells were pelleted, white erythrocyte ghosts were prepared, and resuspended in 1 ml of 5 mM sodium phosphate buffer (pH 8.0) for measurement of monoformazan deposition. No hemolysis was observed in the first or subsequent washes of the cells. Although deposition of the monoformazan in the membrane prevented accurate measurement of ghost protein, no differences were observed in the sizes of ghost membrane pellets. Data are shown from two experiments.

3.2. Reduction of NBT by intact erythrocytes

The results of several different types of experiments suggest that extracellular NBT uses intracellular ascorbate as an electron donor. First, addition of increasing amounts of extracellular NBT to freshly prepared erythrocytes containing endogenous ascorbate resulted in monoformazan deposition in ghost membranes that were subsequently prepared from these cells (Fig. 3). No monoformazan was detected in dilutions of the initial cellular lysate, nor was methemoglobin formation observed (not shown). These results suggest that NBT does not appreciably enter the cytoplasm under these conditions.

Second, a 10 min incubation with 100 μM dehydroascorbate in the presence of 5 mM D-glucose increased subsequent monoformazan deposition by 1.5 ± 0.2-fold over that observed in untreated cells ($n = 6$ experiments, $P < 0.01$). As noted for [^{14}C]dehydroascorbate under Experimental procedures and previously by others [13,33] dehydroascorbate is rapidly transported across the erythrocyte membrane and converted intracellularly to ascorbate [13,33]. Enhanced NBT reduction in such ascorbate-preloaded cells strongly suggests that at least part of the NBT reduction requires ascorbate as an electron donor.

Third, in cells preloaded with [^{14}C]ascorbate, NBT added to the extracellular space caused efflux of radiolabel (Fig. 4). Control cells lost only about 8% of intracellular [^{14}C]ascorbate following a 30 min incubation at 37°C. At a concentration of 100 μM, ferricyanide caused a rapid loss of radioactivity from the cells over the first 5 min of incubation. The same concentration of NBT induced markedly slower release of radiolabel. At 400 μM NBT, the release of radioactivity was greater, but with a distinct time lag. In other experiments, HPLC analysis of the radioactivity that had effluxed into the extracellular medium revealed only [^{14}C]dehydroascorbate; no [^{14}C]ascorbate or 2,3-[^{14}C]diketogulonic acid was detected from control or oxidant-treated cells. These results suggest that both ferricyanide and NBT induce an oxidant stress across the erythrocyte membrane, although the effect of NBT is smaller than with ferricyanide. Since ferricyanide is a stronger oxidant than NBT, it is not surprising that ferricyanide caused a much greater loss of [^{14}C]dehydroascorbate than did NBT.

In the experiment of Fig. 4, it is likely that [^{14}C]dehydroascorbate left the cells primarily on the glucose transporter, since 10 μM cytochalasin B inhibited the oxidant-

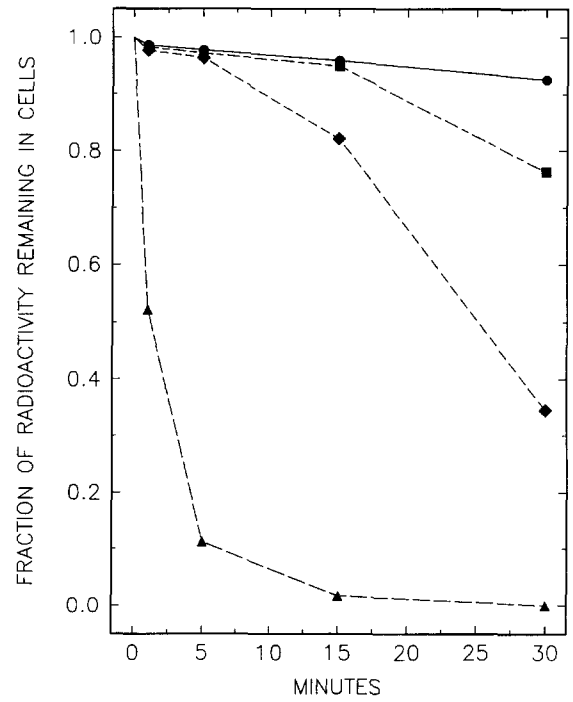

Fig. 4. Release of [^{14}C]dehydroascorbate from intact cells induced by NBT and ferricyanide. Erythrocytes preloaded with [^{14}C]ascorbate were incubated at 37°C in PBS without additions (circles), with 100 μM NBT (squares), with 400 μM NBT (diamonds), or with 100 μM ferricyanide (triangles). At the indicated times, aliquots of cells were taken for measurement of radiolabel efflux as described under Experimental procedures. The calculated [^{14}C]ascorbate concentration inside the cells at the start of the efflux assay in this experiment was 166 μM. The results shown are from one of two such experiments performed.

induced loss of radiolabel from ascorbate-loaded erythrocytes by more than 80% (not shown). It should be noted that during the efflux phase of this experiment, cells were incubated without D-glucose. When 5 mM D-glucose was present, no efflux of radiolabel was evident at even the highest NBT concentration, and the effect of ferricyanide was also attenuated (see below). In the presence of an energy source, intracellular recycling of dehydroascorbate to ascorbate appears to be adequate to maintain low dehydroascorbate concentrations, so that little [^{14}C]dehydroascorbate is released.

The possibility that NBT and ferricyanide reduction are mediated by the same mechanism was tested as follows. Intact erythrocytes were incubated in the presence of NBT and increasing concentrations of ferricyanide, followed by preparation of ghost membranes and measurement of NBT reduction. It is apparent from Fig. 5 that extracellular ferricyanide progressively inhibited monoformazan deposition in ghost membranes, with a half-maximal effect observed at 200 μM ferricyanide. Only part of this effect could be due to ferricyanide-induced oxidation of ascorbate within the cells, since in the presence of 5 mM D-glucose, only about 30% of radiolabeled ascorbate is lost from cells in dilute solution (results not shown). Accordingly, most of the ferricyanide-induced inhibition of NBT reduction in intact cells appears due to direct competition between NBT and ferricyanide for the transmembrane enzyme.

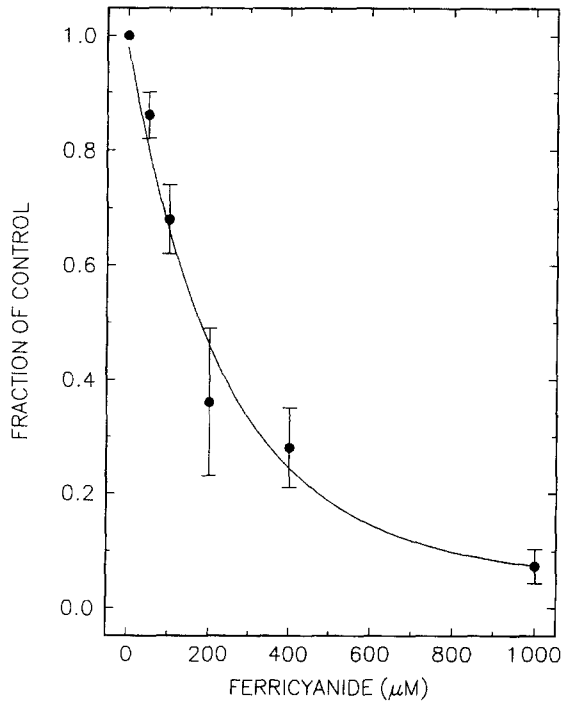

Fig. 5. Inhibition of monoformazan deposition in intact cells by treatment with ferricyanide. Erythrocytes were incubated at a 25% hematocrit in PBS containing 5 mM D-glucose, 100 μM NBT, and the indicated concentration of ferricyanide for one hour at 37°C. The cells were pelleted by centrifugation and white erythrocyte ghosts were prepared. No hemolysis was evident following the incubations. Results from two or more experiments at each ferricyanide concentration are expressed as a fraction of the amount of monoformazan deposited in cells treated with NBT alone.

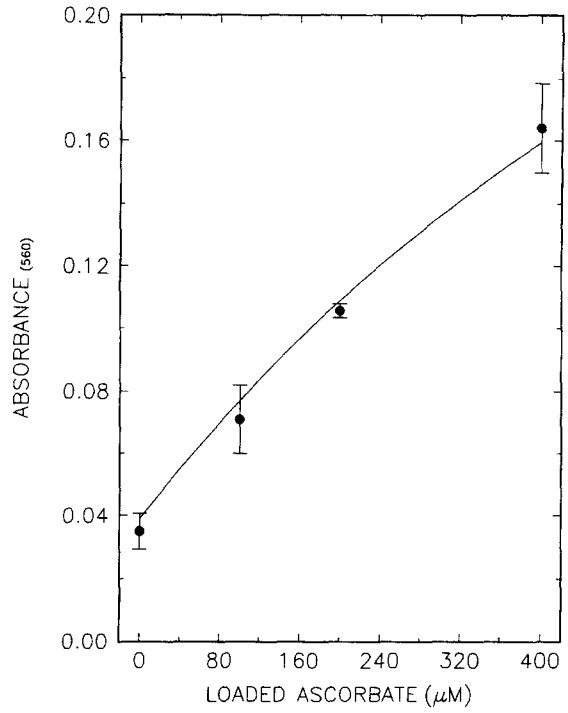

Fig. 6. NBT reduction by resealed erythrocyte ghosts loaded with ascorbate. An 0.2 ml aliquot of packed, washed erythrocytes was lysed with the addition of 40 volumes of ice-cold 5 mM phosphate buffer (pH 8.0) containing 1 mM $MgSO_4$ and the indicated concentration of ascorbic acid. Resealing of the ghosts was accomplished by addition of NaCl to a final concentration of 150 mM and warming the cells at 37°C for 1 h. The resealed ghosts were washed three times at 4°C by centrifugation at 15000× g in 40 ml of PBS, and incubated with 116 μM NBT for 30 min at 37°C. The extent of monoformazan deposition in the ghosts was measured as described under Experimental procedures. The reading for each sample treated with NBT was corrected for absorbance of the ghost preparation alone. The results of three experiments are shown, with the solid line depicting the results of curve-fitting to a hyperbolic model.

3.3. Effects of loading resealed erythrocyte ghosts with ascorbate or NADH

The role of ascorbate in mediating the deposition of the monoformazan in the cell membrane of intact erythrocytes was tested directly in the experiments shown in Fig. 6, in which resealed erythrocyte ghosts were loaded with varying amounts of ascorbate. Incubation of the resealed 'pink' ghosts with 116 μM NBT resulted in progressively increasing amounts of the monoformazan deposited in the ghost membranes. Even without exogenous ascorbate, the monoformazan was deposited in the membranes of resealed ghosts. This background was likely due to endogenous ascorbate or NADH trapped within the ghosts during resealing. A kinetic analysis of such data is not possible, since the ascorbate concentration inside the ghosts will be

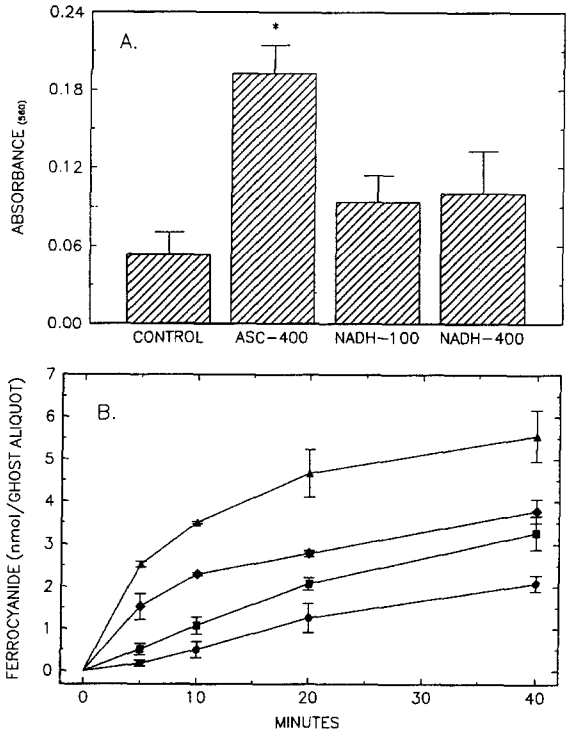

Fig. 7. Comparison of NBT and ferricyanide reduction in resealed ghosts preloaded with ascorbate or with NADH. Experimental procedures and conditions were those described in the legend to Fig. 6. In Panel A, ghosts were resealed to contain no additions ('CONTROL'), 400 μM ascorbate ('ASC-400'), 100 μM NADH ('NADH-100'), and 400 μM NADH ('NADH-400'). The amount of monoformazan deposition in resealed ghost membranes from three experiments is shown for each treatment. An asterisk ('*') indicates $P < 0.05$ compared to control ghosts. In Panel B, resealed ghosts were loaded in the same manner as in Panel A and incubated with 100 μM ferricyanide for the indicated times before sampling of the suspension for ferrocyanide production as described under Experimental procedures. The circles represent control ghosts, the squares represent ghosts resealed with 100 μM NADH, the diamonds represent ghosts resealed with 400 μM NADH, and the triangles represent ghosts resealed with 400 μM ascorbate. Results from three experiments are shown.

constantly changing during the assay. However, these results are in accord with the notion that intravesicular ascorbate can donate electrons to NBT, resulting in deposition of its monoformazan derivative in the erythrocyte membrane.

We then compared the abilities of NADH and ascorbate trapped in resealed ghosts to reduce extracellular NBT and ferricyanide. Ghosts resealed in the presence of 400 μM ascorbate had a highly significant increase in monoformazan deposition, whereas ghosts resealed with the same concentration of NADH contained less than half the monoformazan observed with ascorbate (Fig. 7A). Even 400 at μM NADH, the increase in monoformazan deposition failed to reach statistical significance in the three experiments performed. Similar experiments were performed with ascorbate- or NADH-containing resealed ghosts, using extracellular ferricyanide as the electron acceptor. In Fig. 7B it is evident that the same relationship was observed, with 400 μM ascorbate showing a greater effect than the same concentration of NADH. These results suggest that ascorbate is a natural donor for the transmembrane oxidoreductase, and that it may be the preferred donor to NADH.

4. Discussion

In this work we provide evidence that ascorbate is a donor for a transmembrane oxidoreductase activity in human erythrocytes. This activity has usually been studied with NADH as the electron donor and ferricyanide as the electron acceptor, and has been termed NADH:ferricyanide oxidoreductase [18,34,35]. It is differentiated from NADH:cytochrome b_5 reductase activity by its kinetic features and by its transmembrane, as opposed to cytosolic, orientation [35,36]. The NADH:ferricyanide oxidoreductase has been purified from erythrocyte membranes and shown to migrate in SDS-polyacrylamide gel electrophoresis with an $M_r = 40\,000$. The enzyme has a K_m for NADH of 14 μM [34], and is inhibited by exogenous proteinases and sulfhydryl reagents [37]. In open ghosts the enzyme was found to be cryptic, since its activity was increased many-fold by dissolution of the membranes in nonionic detergents [17,38]. Because of its transmembrane orientation and ability to donate electrons to ferricyanide, it is likely that this enzyme mediates the extracellular reduction of ferricyanide by intact erythrocytes [7,9,14]. Since the extent of ferricyanide reduction in intact erythrocytes has been shown to parallel the intracellular ascorbate concentration [7,14], it is reasonable to consider whether ascorbate may be a natural or even the most important electron donor for this enzyme.

To approach this question, we needed to study the enzyme activity in membrane preparations where concentrations of electron donors and acceptors could be better controlled than in the intact cell. Ferricyanide rapidly oxidizes ascorbate in solution [7,14], so its reduction could not be used to follow ascorbate-dependent enzyme activity in open membranes. On the other hand, we found that ascorbate does not reduce NBT to an appreciable extent at physiologic pH. Zamudio et al. [17] had previously used NBT to document the location of the NADH-dependent oxidoreductase in erythrocyte membranes. NBT is a redox dye used extensively in the detection of superoxide release in biologic systems [19]. The complete reduction of NBT involves transfer of four electrons to produce the diformazan. However, when the NBT concentration is much greater than that of the ultimate electron acceptor, molecular oxygen, the final product of NBT reduction is the monoformazan [25]. This compound has accepted two electrons and retains one of the two positive charges of nitroblue tetrazolium. It is poorly soluble in aqueous solutions. However, as noted by Zamudio et al. [17], the monoformazan is preferentially deposited in the lipid bi-

layer of erythrocyte membranes. This feature allows easy visual or spectrophotometric detection of the reaction, but complicates quantitation of the reaction rate. Despite our inability to calculate reaction rates, use of the NBT-based assay in ghost membranes showed that ascorbate served as an electron donor for an oxidoreductase with several features expected of the transmembrane NADH:ferricyanide oxidoreductase (Table 1 and Table 2). These include similar substrate affinities, inhibition by sulfhydryl reagents and proteolytic enzymes, and enhancement following detergent solubilization.

Additional evidence that ascorbate is a donor for the transmembrane oxidoreductase was obtained in studies using intact erythrocytes and resealed erythrocyte ghosts. In such preparations, it is critical to document that NBT does not cross the cell membrane and enter the cytoplasm, where it could be reduced by an ascorbate- or NADH-dependent cytosolic enzyme, such as cytochrome-b_5 reductase. In neutrophils the reduced monoformazan of NBT has been shown in electron microscopic studies to be deposited as a thin layer on the extracellular face of the plasma membrane, or within phagocytic granules, but not within the cytoplasm [39,40]. We were unable to detect either NBT or the reduced monoformazan in lysates of erythrocytes incubated with NBT. These considerations, along with the size and positive charge of NBT, make it highly unlikely that NBT crossed the cell membrane and was reduced inside cells in these studies.

The monoformazan of NBT was deposited in the cell membranes of intact erythrocytes incubated with NBT (Fig. 3), and this effect was enhanced by loading the cells with ascorbate. Both NBT and ferricyanide caused efflux of radiolabel as [^{14}C]dehydroascorbate from cells loaded with [^{14}C]ascorbate (Fig. 4). Together, these data suggest that extracellular NBT oxidizes intracellular ascorbate via a transmembrane process. Since ferricyanide inhibited NBT reduction in intact cells (Fig. 5), the extracellular oxidants may work through similar mechanisms, with the observed inhibition resulting from competition for intracellular reducing equivalents derived from ascorbate or from NADH. Alternatively, since ferricyanide inhibited NBT reduction with a K_i similar to its own apparent K_m of 120 μM for reduction by erythrocytes [14], both acceptors may be competing for the same transmembrane enzyme.

Our data also support the notion that ascorbate may be the major electron donor to the transmembrane oxidoreductase. At a concentration within the physiologic range (100 μM), ascorbate consistently produced about twice as much monoformazan deposition in ghosts or reconstituted band 4.5 as the same concentration of NADH (Table 1 and Table 3). Also, when supraphysiologic concentrations of ascorbate and NADH were resealed inside ghosts, both ferricyanide reduction and deposition of the monoformazan of NBT were about twice as large for ascorbate as observed for NADH (Fig. 7). The apparent K_m for ascorbate-induced monoformazan deposition of 21 μM is in the lower range of the observed intracellular ascorbate concentrations within erythrocytes in unsupplemented individuals, usually considered to be 25–60 μM [3]. A similar relationship exists for NADH, which has an apparent K_m for ferricyanide reduction of 13 μM [34], and an intracellular concentration in human erythrocytes of 14–40 μM [41]. These considerations, and the fact that dietary ascorbate supplementation may raise plasma and erythrocyte ascorbate concentrations above 100 μM [3,42], suggest that ascorbate may be the major donor for the transmembrane oxidoreductase.

The physiologic acceptor for the ascorbate- or NADH-dependent transmembrane oxidoreductase is unknown. It is possible that the enzyme serves to donate electrons to extracellular ascorbate free radical to regenerate ascorbate, as suggested by Schipfer et al. [14]. Alternatively, the transmembrane oxidoreductase may facilitate conversion of the Fe^{3+} in diferric transferrin to Fe^{2+}, which is released from transferrin [43], and may be taken up and used by cells [44,45]. Since most cells take up transferrin through receptor-mediated endocytosis [46], a more likely possibility is that the transmembrane oxidoreductase functions in the endocytotic vesicle to reduce ferric to ferrous iron. Another possible acceptor of electrons from the transmembrane oxidoreductase is lipid hydroperoxide in the lipid bilayer of the plasma membrane. Agutter and colleagues [37,47] have suggested that an enzyme distinct from the NADH:ferricyanide oxidoreductase in erythrocyte membranes can donate electrons from NADH to exogenous lipid hydroperoxides incorporated into the erythrocyte membrane bilayer. Although speculative, it is possible that such an enzyme could transfer electrons from intracellular ascorbate or NADH to membrane-resident antioxidants, such as oxidized α-tocopherol [48,49] or ubiquinone-10 [50].

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References

[1] Frei, B., Stocker, R. and Ames, B.N. (1988) Proc. Natl. Acad. Sci. USA 85, 9748–9752.
[2] Frei, B., England, L. and Ames, B.N. (1989) Proc. Natl. Acad. Sci. USA 86, 6377–6381.
[3] Evans, R.M., Currie, L. and Campbell, A. (1982) Br. J. Nutr. 47, 473–482.
[4] Rose, R.C. (1988) Biochim. Biophys. Acta 947, 335–366.
[5] Jacob, R.A., Skala, J.H. and Omaye, S.T. (1987) Am. J. Clin. Nutr. 46, 818–826.
[6] Hughes, R.E. (1964) Nature 203, 1068–1069.
[7] Orringer, E.P. and Roer, M.E. (1979) J. Clin. Invest. 63, 53–58.

[8] Székely, M., Mányai, S. and Straub, F.B. (1952) Acta Physiol. Acad. Sci. Hung. 3, 571–583.
[9] Mishra, R.K. and Passow, H. (1969) J. Membr. Biol. 1, 214–224.
[10] Basu, S., Som, S., Deb, S., Mukherjee, D. and Chatterjee, I.B. (1979) Biochem. Biophys. Res. Commun. 90, 1335–1340.
[11] Winkler, B.S., Orselli, S.M. and Rex, T.S. (1994) Free Radic. Biol. Med. 17, 333–349.
[12] Vera, J.C., Rivas, C.I., Fischbarg, J. and Golde, D.W. (1993) Nature 364, 79–82.
[13] Bianchi, J. and Rose, R.C. (1986) Proc. Soc. Exp. Biol. Med. 181, 333–337.
[14] Schipfer, W., Neophytou, B., Trobisch, R., Groiss, O. and Goldenberg, H. (1985) Int. J. Biochem. 17, 819–823.
[15] Hughes, R.E. and Maton, S.C. (1968) Br. J. Haematol. 14, 247–253.
[16] Wagner, E.S., White, W., Jennings, M. and Bennett, K. (1987) Biochim. Biophys. Acta 902, 133–136.
[17] Zamudio, I., Cellino, M. and Canessa-Fischer, M. (1969) Arch. Biochem. Biophys. 129, 336–345.
[18] Zamudio, I. and Canessa, M. (1966) Biochim. Biophys. Acta 120, 165–169.
[19] Babior, B.M., Kipnes, R.S. and Curnutte, J.T. (1973) J. Clin. Invest. 52, 741–744.
[20] Gorga, F.R. and Lienhard, G.E. (1981) Biochemistry 20, 5108–5113.
[21] Steck, T.L. and Kant, J.A. (1974) Methods Enzymol. 31, 172–180.
[22] Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617.
[23] Baldwin, S.A., Baldwin, J.M. and Lienhard, G.E. (1982) Biochemistry 21, 3836–3842.
[24] May, J.M. (1988) Biochem. J. 254, 329–336.
[25] Goldstein, S., Michel, C., Bors, W., Saran, M. and Czapski, G. (1988) Free Radic. Biol. Med. 4, 295–303.
[26] Bielski, B.H., Shiue, G.G., and Bajuk, S. (1980) J. Phys. Chem. 84, 830–833.
[27] Avron, M. and Shavit, N. (1963) Anal. Biochem. 6, 549–554.
[28] Pachla, L.A. and Kissinger, P.T. (1979) Methods Enzymol. 62, 15–24.
[29] Levine, M. (1986) N. Engl. J. Med. 314, 892–902.
[30] Washko, P., Rotrosen, D. and Levine, M. (1990) FEBS Lett. 260, 101–104.
[31] Fung, L.W.-M. and Zhang, Y. (1990) Free Radic. Biol. Med. 9, 289–298.
[32] Omaye, S.T., Turnbull, J.D. and Sauberlich, H.E. (1979) Methods Enzymol. 62, 3–11.
[33] Mann, G.V. and Newton, P. (1975) Ann. NY Acad. Sci. 258, 243–252.
[34] Wang, C.-S. (1980) Biochim. Biophys. Acta 616, 22–29.
[35] Crane, F.L., Löw, H. and Clark, M.G. (1985) in The Enzymes of Biological Membranes (Martonosi, A.N., ed.), pp. 465–510, Plenum Press, New York.
[36] Choury, D., Leroux, A. and Kaplan, J.-C. (1981) J. Clin. Invest. 67, 149–155.
[37] Agutter, P.S., Dallas, J.M., Newman, P. and Kadlubowski, M. (1980) Biochem. Soc. Trans. 8, 326.
[38] Stewart, C.A., Agutter, P.S. and Kadlubowski, M. (1980) Biochem. Soc. Trans. 8, 325.
[39] Segal, A.W. and Levi, A.J. (1973) Clin. Sci. Mol. Med. 45, 817–826.
[40] Briggs, R.T., Drath, D.B., Karnovsky, M.L. and Karnovsky, M.J. (1975) J. Cell Biol. 67, 566–586.
[41] Busch, D. and Boie, K. (1969) Klin. Wochschr. 47, 1172–1174.
[42] Ginter, E., Cerna, O., Budlovosky, J., Baláz, V., Hrubá, F., Roch, V. and Sasko, E. (1977) Int. J. Vit. Nutr. Res. 47, 123–134.
[43] Kojima, N. and Bates, G.W. (1979) J. Biol. Chem. 254, 8847–8854.
[44] Navas, P., Sun, I.L., Morre, D.J. and Crane, F.L. (1986) Biochem. Biophys. Res. Commun. 135, 110–115.
[45] Toole-Simms, W., Sun, I.L., Faulk, W.P., Löw, H., Lindgren, A., Crane, F.L. and Morre, D.J. (1991) Biochem. Biophys. Res. Commun. 176, 1437–1442.
[46] Ciechanover, A., Schwartz, A.L. and Lodish, H.F. (1983) J. Cell. Biochem. 23, 107–130.
[47] Agutter, P.S., Sherriff, R.V. and Kadlubowski, M. (1979) Biochem. Soc. Trans. 7, 710–711.
[48] Packer, L., Maguire, J.J., Mehlhorn, R.J., Serbinova, E. and Kagan, V.E. (1989) Biochem. Biophys. Res. Commun. 159, 229–235.
[49] Packer, J.E., Slater, T.F. and Willson, R.L. (1979) Nature 278, 737–738.
[50] Sun, I.L., Sun, E.E., Crane, F.L., Morré, D.J., Lindgren, A. and Löw, H. (1992) Proc. Natl. Acad. Sci. USA 89, 11126–11130.