UPTAKE AND REDUCTION OF OXIDIZED AND REDUCED ASCORBATE BY HUMAN LEUKOCYTES*

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Assayable derivatives of ascorbate in mammalian tissues include reduced ascorbate (AA),[1] dehydroascorbate (DHA), and diketogulonate (DKG). Only AA and DHA are antiscorbutic.

After intravenous administration to mammals, DHA is retained longer in whole blood than is AA; other tissues accumulate AA more avidly than DHA (1–3). With the exception of the findings in one study (4), it appears that DHA is taken up more rapidly than AA by human and guinea pig erythrocytes and leukocytes in vitro (5–7). It has been suggested that DHA, which is more lipid soluble than AA, is the form of ascorbate which penetrates cell membranes (8).

When DHA or AA are injected into mammals, both are concentrated in tissues as AA (2, 3, 8, 9). Most mammalian tissues incubated in vitro with AA or DHA also accumulate AA (6, 10, 11); however, guinea pig erythrocytes incubated with DHA accumulate predominantly DHA (5).

Three DHA-reducing activities are described in mammalian tissues: reduced glutathione-dependent activity in erythrocytes and liver of several species (12), NADPH-dependent activity in the granule fraction of human leukocytes (13) and NADH-dependent activity (reduced NAD(P): semidehydroascorbate oxidoreductase, EC 1.6.5.4.) in microsomes of several pig and rat tissues (14, 15). Reduction of DHA by each of these activities could directly or indirectly lead to oxidation of NADPH, and result in hexose monophosphate shunt (HMS) activation. This report describes studies of AA and DHA uptake, DHA reduction, and coincident HMS stimulation in human leukocytes.

Materials and Methods

Materials.—L-ascorbic acid, sodium salt, was obtained from Sigma Chemical Co., St. Louis, Mo. DHA was prepared from L-ascorbic acid by the technique of Pecherer (16) and purified by the method of Staudinger and Weis (17). These preparations of AA and DHA contained no contaminants detectable by thin-layer chromatography (5) or by the assay of Roe et al.

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[1] *Abbreviations used in this paper:* AA, reduced ascorbate; DHA, dehydroascorbate; DKG, diketogulonate; HMS, hexose monophosphate shunt; WBC, leukocytes.

(18), which quantitatively distinguishes AA, DHA, and DKG from each other, and from other organic compounds.

Leukocyte Separation.—All steps were performed at 25–28°C, using nonwettable containers. To each 10 ml of human venous blood, drawn into a plastic syringe containing 0.1 ml of heparin (beef lung, 1,000 U/ml; Upjohn Co., Kalamazoo, Mich.), 2 ml of 6% Dextran (mol wt 117,000; Sigma Chemical Co.) in phosphate-buffered saline, pH 7.4, were added. After sedimentation for 60–90 min, supernatant plasma was separated and diluted with 2 vol of 0.87% ammonium chloride to lyse the remaining erythrocytes, then centrifuged at 250 g for 5 min. Sedimented leukocytes were washed twice with calcium free Krebs-Ringer phosphate buffer, pH 7.4, containing glucose, 1 mg/ml, and resuspended in approximately 10 vol of the same medium. Calcium-free media were used here and in subsequent experiments to avoid phagocyte clumping. Cells were counted in a hemacytometer chamber. Leukocyte differential counts, determined from Wright's-stained smears, varied within 85–95% neutrophil granulocytes, 5–15% monocytes, 0–8% lymphocytes, and 0–5% eosinophil granulocytes.

Measurement of Ascorbate Uptake.—Leukocytes were incubated at 37°C in calcium-free Krebs-Ringer phosphate buffer containing 5.5 mM glucose (1 mg/ml) and either DHA or AA, for periods of time that are noted in the Results. Reaction mixtures were gassed with 95% O_2 and 5% CO_2 for aerobic experiments, and with 95% N_2 and 5% CO_2 for anaerobic experiments. Gas flow rate was 73 ml/min. Gases contained 5% CO_2 in order to assure that conditions for measuring ascorbate uptake and HMS activity were comparable. Leukocytes (WBC) tend to clump in Krebs-Ringer bicarbonate buffer, the standard medium for CO_2-incubated experiments, therefore Krebs-Ringer phosphate buffer was substituted. Using this buffer, reaction mixture pH was 7.10 after gassing with 5% CO_2 for 20 min, 7.05 after 30 min, and 6.95 after 60 min. Control experiments showed no effect of CO_2 gassing on DHA uptake and reduction, or on HMS activity measured by scintillation counting.

Paired flasks containing cells and medium were preincubated and gassed for 20 min. One flask of each pair was then removed for "zero time" control assay and, simultaneously, a freshly prepared solution of AA or DHA in pregassed Krebs-Ringed phosphate, final pH 7.1, was added to the remaining flask through a capillary tube. Ascorbate uptake was stopped by placing reaction tubes in an ice water bath. Cells were separated from medium by centrifugation at 250 g for 5 min at 4°C and washed twice with Krebs-Ringer phosphate buffer. AA and DHA contents of cells and medium were then measured by the method of Roe et al. (18). Using this method, DHA lost from medium is recovered as AA increase in cells with 98.5 ± 1.1% accuracy. DKG was not detectable when 20–40 × 10^6 cells were analyzed, either before or after incubation with AA or DHA.

Measurement of HMS Activity.—The rate of $^{14}CO_2$ production from ^{14}C-1 glucose (New England Nuclear, Boston, Mass.) was measured continuously, using the gas flow-ionization chamber method of Davidson and Tanaka (19). This technique converts charge accumulated in an ionization chamber to a millivolt signal by the high resistance leak method, using a Cary model 401 vibrating reed electrometer (Cary Instruments, Monrovia, Calif.). The signal was recorded continuously on a Sargent SRG recorder (Sargent Welch Co., Skokie, Ill.). A signal of 1.0 mV read at 10 min is equivalent to 44.8 × 10^{-5} μCi ^{14}C-1 glucose oxidized in 10 min, determined by parallel measurements of $^{14}CO_2$ evolution by liquid scintillation counting. Using this method, resting WBC oxidize 5.9–7.8 nmol of C-1 glucose/10^8 cells/10 min, and phagocytosing WBC oxidize 35.0–39.3 nmol/10^8 cells/10 min. These values are comparable to those reported using scintillation counting methods (13, 20).

1-ml reaction mixtures containing 20–40 × 10^6 WBC and 2.8 mM (0.5 mg/ml) of glucose which included 1.0 μCi of ^{14}C-1 glucose were incubated in 20-ml flat-bottomed glass vials in a Dubnoff shaking incubator (Precision Scientific Co., Chicago, Ill.) at 37°C, 80 oscillations/min. Gases and flow rates were as stated above. Depth of the reaction mixture was 2 mm and the surface area was 5 cm². CO_2 diffusion from the medium was inefficient when larger reac-

tion volumes with smaller surface area to volume ratios were used. Gas was not bubbled through the reaction mixture, since this caused clumping of and damage to WBC.

RESULTS

Cells Incubated with DHA.—Human WBC extract DHA efficiently from the surrounding medium, and promptly reduce it to AA. The amount of DHA taken up and reduced is proportional to the number of cells and to the concentration of DHA in the medium (Fig. 1). The figure does not reflect the DHA content of unincubated cells, which ranged from 0.012–0.028 μmol/10^8 WBC
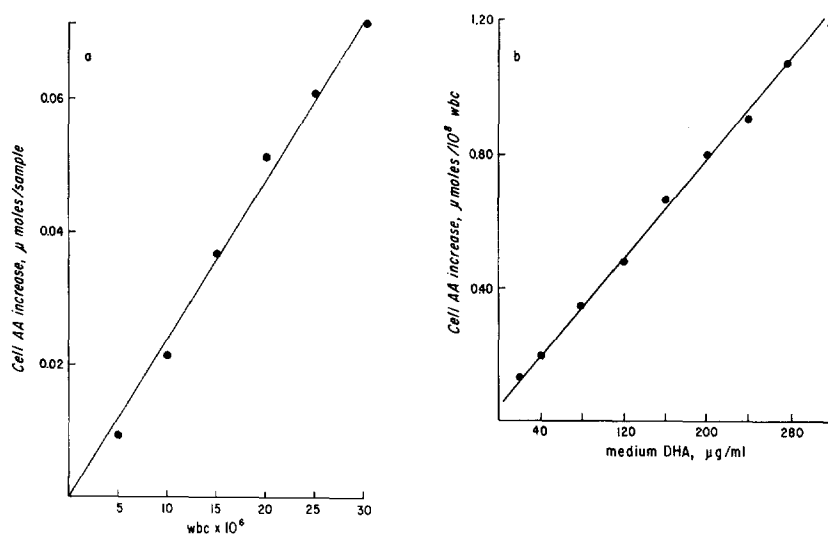

Fig. 1. Uptake and reduction of DHA by leukocytes. (*a*) Effect of numbers of WBC. 5-ml reaction mixtures containing DHA, 100 μg/ml, were incubated for 20 min. AA and DHA contents of unincubated cells were subtracted from observed values to obtain AA increase in cells. (*b*) Effect of medium DHA concentration. In a total volume of 10 ml, 40 × 10^6 WBC were incubated with DHA for 20 min. Cell AA increase was calculated as in Fig. 1 *a*.

and was unchanged after incubation of cells with DHA. The increase in cellular AA after incubation was equal to DHA loss from medium. The rate of DHA uptake and reduction by cells is dependent on the DHA concentration in the medium, is biphasic, and is the same under aerobic and anaerobic conditions (Fig. 2).

As cells take up and reduce DHA, glucose oxidation through the HMS is increased (Fig. 3). The enhancement of HMS activity induced by incubation with DHA is the same under aerobic and anaerobic conditions, and is dependent on DHA concentration in the medium. The difference in absolute values between samples incubated aerobically and anaerobically reflects oxygen-dependent HMS activity, independent of stimulation by DHA.
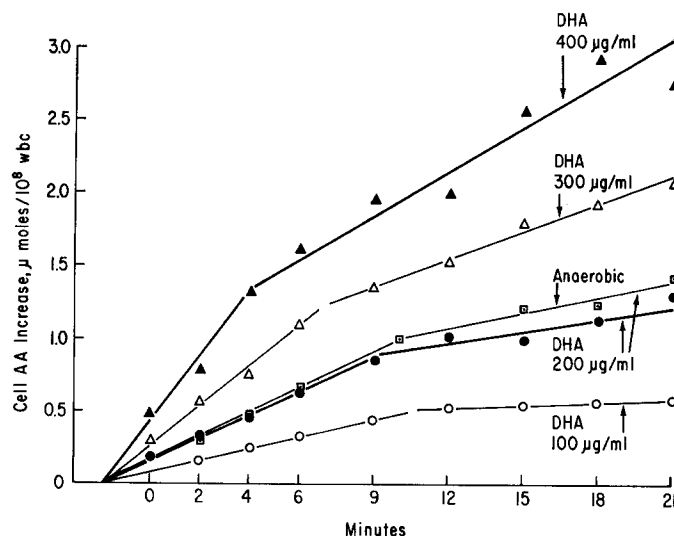
FIG. 2. Rate of DHA uptake and reduction by WBC; effects of medium DHA concentration and of oxygenation. In a total vol of 5 ml, 20 × 10^6 WBC were incubated with DHA. Incubation vials were gassed with 95% O_2 and 5% CO_2, except for the samples labeled Anaerobic which were gassed with 95% N_2 and 5% CO_2. AA and DHA contents of unincubated cells were subtracted from observed values to obtain AA increase in cells.
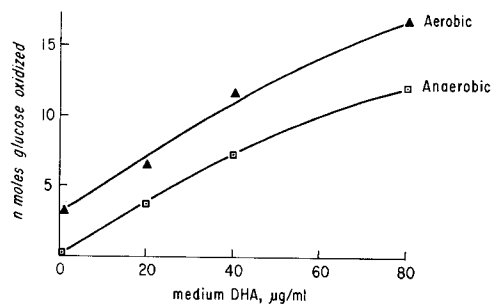

FIG. 3. Effect of DHA medium concentration on HMS activity. In a total vol of 0.9 ml, 30 × 10^6 WBC were incubated with 1.5 μCi of ^{14}C-1 glucose. C-1 glucose oxidation was calculated as described in Materials and Methods.

Cells enriched 20-fold with AA by previous incubation with DHA followed by washing had the same HMS activity as did control cells. HMS stimulation is thus related to DHA uptake and reduction, and is not related to AA content of cells.

Cells Incubated with AA.—When AA is used instead of DNA, remarkable differences are observed. AA added to the medium causes WBC AA content and HMS activity to increase only when the reaction mixture is oxygenated

(Table I). Much higher medium concentrations of AA than of DHA are required in order to produce comparable increases in cell AA content and HMS activity; with medium AA concentrations less than 100 μg/ml, no increase in cell AA content or HMS activity was detectable. The increase in WBC AA content and HMS activity observed during incubation with a given concentration of AA in the medium varied considerably among experiments using WBC from different donors; no such variation between donors was seen when cells were incubated with DHA.

The ratio of HMS increase to increase in cell AA content is the same when either AA or DHA is added to the medium (Table II). This quantitative relationship implies that NADPH oxidation is in some way involved in uptake and/or reduction of one or both ascorbate derivatives.

TABLE I

Effects of Anaerobic and Aerobic Incubation with AA on WBC AA Content and HMS Activity*

| | Cell AA‡ | | HMS activity§ | |
	Control	AA	Control	AA
Anaerobic¶	124.0	126.0	1.05	1.05
Aerobic¶	124.0	192.0	7.53	15.48

* Cell AA was measured after washing 90 × 10⁶ WBC that were incubated for 10 min in 3-ml reaction mixtures without or with 3 mg AA. HMS activity was measured 10 min after adding 0.1 ml of buffer without or with 1 mg AA through a capillary to 0.9 ml of reaction mixture containing 30 × 10⁶ WBC and 1.5 μCi ¹⁴C-1 glucose.

‡ nmol/10⁸ WBC.

§ nmol glucose oxidized/10⁸ WBC.

¶ Aerobic incubations were gassed with 95% O_2 and 5% CO_2; anerobic incubations were gassed with 95% N_2 and 5% CO_2.

TABLE II

Effects of Aerobic Incubation with DHA or AA on WBC AA Content and HMS Activity*

	Cell AA increase‡	HMS increase§	HMS increase/cell AA increase¶
Medium ascorbate			
DHA 20 μg/ml	100.0	12.00	0.12
DHA 40 μg/ml	200.0	24.05	0.12
AA 1000 μg/ml	68.0	7.94	0.12

* Conditions are described under Table I. Values for unincubated HMS activity and cell AA and DHA were subtracted from final values to obtain the increase in each measurement.

‡ nmol AA/10⁸ WBC.

§ nmol glucose oxidized/10⁸ WBC.

¶ nmol glucose oxidized/nmol AA.

DISCUSSION

These studies show that human WBC take up extracellular DHA and reduce it promptly to AA. The process of converting extracellular DHA to intracellular AA is accompanied by HMS activation. DHA uptake and reduction and HMS activation are dependent on DHA concentration in medium, and do not require oxygen. As cells take up and reduce 1.0 μmol of DNA, they oxidize 0.12 μmol of C-1 glucose, and therefore utilize only 0.24 μmol of NADPH. NADPH thus appears to contribute protons for DHA reduction either indirectly, or directly along with one or more other donors.

Human WBC do not take up AA from the medium in the absence of oxygen, but they do accumulate AA when oxygen is supplied. These observations are consistent with separate mechanisms for uptake of DHA and AA in leukocytes, that for DHA being anaerobic and that for AA being aerobic; alternatively, AA may require oxidation to DHA in the medium before uptake. It has been suggested that ascorbate-induced HMS stimulation in the human lens (21) and in human WBC (22) is a consequence of the metabolism of DHA produced during oxidation of AA. We observe that the ratio of HMS stimulation to WBC AA increase is the same, regardless whether cells are incubated with DHA or aerobically with AA. This suggests that shunt stimulation is a quantitative though not stoichiometric reflection of intracellular DHA reduction in both instances, and that in both instances DHA is the derivative of ascorbate taken up from the medium by WBC.

AA can be converted to DHA by cation-catalysed auto-oxidation, and by H_2O_2-dependent, peroxidase-catalysed oxidation (23). We observe that cells from different donors accumulate AA at different rates from media containing AA. This variation could result from variation in medium cation content contributed by cells or glassware. It could also result from variation in release of myeloperoxidase from damaged WBC into the medium which contains H_2O_2 produced by the WBC. The latter possibility is unlikely, since Cooper et al. (22) have shown that WBC HMS stimulation by AA is independent of WBC capacity to produce H_2O_2. It is also possible that some of the variation may reflect cellular control of AA uptake. Mechanisms for control of cellular ascorbate levels have not been described. Possibly, regulation of the AA content of WBC is mediated in part through control of AA oxidation to DHA in the extracellular fluid or at the cell membrane.

In erythrocytes incubated aerobically with AA, HMS stimulation may be caused by glutathione peroxidase-catalysed oxidation of the H_2O_2 produced during auto-oxidation of AA (23, 24). It is not likely that this mechanism contributes to HMS stimulation in our studies of WBC incubated with AA. If H_2O_2 derived from AA oxidation had added to shunt activity, the HMS increase to cell AA increase ratio should have been higher when cells were incubated with AA than when they were incubated with DHA, since H_2O_2 is not produced

during DHA oxidation. It may be that WBC catalase reduces the H_2O_2 which diffuses into cells, and/or that the H_2O_2 produced in the medium oxidizes another molecule of AA to DHA, as proposed by de Chalelet et al. (25).

Membrane lipids of phagocytosed bacteria are peroxidated (26). Since diffusble H_2O_2 and activated oxygen are produced by active phagocytes (27–29), membrane lipids and other constituents of the phagocytosing cell might also suffer critical oxidative denaturation unless the cell is able to repair such damage. Green and O'Brien (30) demonstrated that ascorbate enhances NADH-dependent reduction of a lipid peroxide. They found this activity in rat liver mitochondria and microsomes, and proposed that semidehydroascorbate reductase participates in the reaction. During 10 minutes of phagocytosis, 10^8 WBC consume 1,000 μmol of oxygen (31). In the same time, as shown in Fig. 1, 10^8 WBC can reduce more than 2,000 μmol of DHA to AA. Thus, human WBC have considerable capacity to regenerate AA promptly after oxidation. This DHA-reducing system may function along with AA as a significant part of the cell's mechanism for prevention of oxidative damage to cell constituents.

SUMMARY

Incubation of human leukocytes with dehydroascorbate (DHA) results in an increase in their reduced ascorbate (AA) content and hexose monophosphate shunt (HMS) activity, independent of oxygen supply. Incubation with AA induces these changes only in the presence of oxygen. The increase in HMS activity observed as cell AA increases by 1 μmol is the same during incubation with either DHA or AA.

We propose that human leukocytes take up ascorbate as DHA (AA after oxidation to DHA) and reduce it promptly to AA, and that HMS stimulation upon incubation with either AA or DHA is a result of DHA reduction.

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