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Assessing the reductive capacity of cells by measuring the recycling of ascorbic and lipoic acids

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Abstract

Most mammalian cells cannot synthesize vitamin C, or ascorbic acid, and thus must have efficient mechanisms for its intracellular recycling. Ascorbate can be recycled from both its oxidized forms using electrons from several intracellular reducing co-factors, including GSH and the reduced pyridine nucleotides. Methods have been developed to assess the ability of intact cells to recycle ascorbate, which include assay of extracellular ferricyanide reduction and measurement of the ability of the cells to reduce dehydroascorbic acid to ascorbate. Lipoic acid, a disulfide containing medium chain fatty acid, is also taken up by cells and reduced to dihydrolipoic acid, which can be measured upon efflux from the cells using Ellman's reagent. Together, these assays provide an estimate of the ability of different cell types to recycle ascorbate, and to generate intracellular reducing equivalents to required to maintain the redox status of the cells.

Keywords

ascorbic acid; lipoic acid; ferricyanide; Ellman's reagent; intracellular redox environment; oxidant stress

1. Introduction

1.1. Ascorbic acid recycling

Vitamin C, or ascorbic acid, is required for collagen synthesis, serves as a cofactor for various dioxygenase enzymes, and also contributes to the antioxidant defenses of plasma and cells. Regarding the latter, ascorbate is a sensitive marker of oxidant stress in plasma (1) and cells (2). Ascorbate is synthesized *de novo* in the liver in mammals and not at all in primates and humans (3). To maintain levels of the vitamin, there are efficient mechanisms for recycling it from its oxidized forms. In contrast to thiols and pyridine nucleotides, which typically donate two electrons, ascorbate functions primarily as a one-electron donor (4), generating the ascorbate free radical (AFR) (Fig. 1). Even though the AFR is more strongly reducing than ascorbate (5), it is relatively stable, sharing the unpaired electron across three vicinal oxygen molecules. Rather than lose another electron to become the two-electron-oxidized form of ascorbate, dehydroascorbic acid (DHA), the AFR dismutates to form one molecule each of ascorbate and dehydroascorbate (6). The AFR can also be reduced to ascorbate by NAD(P)H-dependent reductases within cells (7–10). These enzymes have affinity for the AFR in the low micromolar range. Although their activity can be measured in cell lysates as NAD(P)H-dependent AFR reduction, assays specific for AFR reductases are not feasible in intact cells. In contrast to the AFR, DHA can undergo two-electron

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reduction back to ascorbate by a variety of mechanisms (Fig. 1). These include direct reduction by GSH (11) or by GSH-dependent enzymes, such as glutaredoxin or thiol-disulfide isomerase (12,13). DHA is also reduced by NADPH-dependent enzymes, including a 3α-hydroxysteroid dehydrogenase in liver (14) and thioredoxin reductase (15). Most cells appear to use multiple mechanisms to recycle ascorbate from its oxidized forms, although they may vary in the extent to which they depend on a specific mechanism. For example, despite the presence of GSH in all cells, neither HL-60 cells (16) nor human skin keratinocytes (17) require GSH for DHA reduction.

Since the ability of cells to recycle ascorbate is likely to contribute to their ability to maintain intracellular ascorbate and to withstand oxidant stress, it is useful to assess this capacity, especially in intact cells. Two approaches have been used to do this in either suspended cells or cells in monolayer culture. It was found several decades ago that human erythrocytes reduce ferricyanide to ferrocyanide in an ascorbate-dependent manner (18). Since ferricyanide does not enter cells due to its size and charge (19), and since ascorbate is also trapped within cells, it was postulated that electrons from ascorbate are transferred across the plasma membrane by an oxidoreductase activity (20,21). Although this putative enzyme or enzyme complex has not been identified, all cells tested thus far can reduce ferricyanide, and this reduction is enhanced by intracellular ascorbate. For example, in freshly prepared human erythrocytes, endogenous ascorbate contributes about 2/3rds of basal ferricyanide reductase activity (21). When cells are loaded with increasing ascorbate concentrations, ferricyanide reduction increases in a saturable manner that is limited in part by the ability of the cells to regenerate ascorbate from its oxidized forms (22). Ferricyanide is a one-electron oxidant, and has been shown to generate the AFR in human erythrocytes (23,24). However, because the AFR undergoes rapid dismutation to ascorbate and DHA (6) (Fig. 1), rates of ferricyanide reduction likely measure the capacity of cells to reduce both the AFR and DHA. Indeed, ferricyanide reduction is dependent on the GSH status of human erythrocytes (25) and other cells (26,27), implying that GSH-dependent mechanisms serve to recycle ascorbate from DHA. Rates of ferricyanide reduction also depend on rates of trans-plasma membrane electron transport (28–31) and the extent to which electrons are derived from other intracellular donors, such as pyridine nucleotides. Despite these caveats, assay of ascorbate-dependent ferricyanide reduction provides at least an indirect measure of cellular ascorbate recycling capacity from both the AFR and DHA. To the extent that ascorbate recycling depends upon both the reserve of both pyridine nucleotides and cellular thiols, it may reflect the redox capacity of the cells in general. The assay, originally developed by Avron and Shavit (32) is easy to perform using aliquots of medium from either suspended cells or monolayer cultures, is non-destructive to the cells, and requires only a spectrophotometer.

A second approach to measuring ascorbate recycling depends on their ability to take up and reduce DHA to ascorbate. DHA enters cells on the ubiquitous glucose transporter (33), and is then rapidly reduced to ascorbate once inside the cells (25,34). Ascorbate is not a substrate for transport by these GLUT-type glucose transporters (33). DHA is considered to form an hydrated hemiketal in solution (35) that resembles glucose in its structure and thus is taken up by cells in competition with glucose. In fact, the term “dehydroascorbic acid” is a misnomer, since it is not an acid in solution (35). Once DHA enters cells, it is rapidly reduced to ascorbate by the mechanisms outlined above. The ascorbate content of cells is measured after cell lysis. Although various methods have been developed to measure ascorbate, the most sensitive and specific is by HPLC, either using electrochemical or UV detection (36). Since reduction of DHA by GSH- and pyridine nucleotide-dependent mechanisms does not involve the AFR (25), this assay specifically measures DHA reduction to ascorbate and not AFR reduction. Depending on the source of donor electrons, it can also

provide an estimate of the ability of cells to recycle GSH and of their redox metabolism in general.

1.2. Lipoic acid recycling

Lipoic or thioctic acid is a medium chain saturated fatty acid that contains a thiolane ring comprised of an internal disulfide between carbons 6 and 8 (Fig. 2). Lipoic acid is a natural constituent of α-keto reductases, where it contributes to decarboxylation reactions. It has received increasing attention over the last few years for its pharmacologic property as an antioxidant precursor. Although lipoic acid itself can chelate transition metals, it becomes a potent antioxidant when reduced to the dithiol form, dihydrolipoic acid (DHLA). In contrast to ascorbic acid, both lipoic and dihydrolipoic acid can rapidly enter cells by several mechanisms. In some cells it appears to use a multivitamin transporter (37), in most cells it uses a medium chain fatty acid transporter (38), and there is also likely to be simple diffusion of the protonated forms across the plasma membrane. Once inside the cells, lipoic acid is rapidly reduced to dihydrolipoic acid by any of several pyridine nucleotide-dependent oxidoreductases. Because its midpoint reduction potential of −0.34 mV is less than that of GSH (−0.29 mV) (39), it cannot be reduced directly by thiols in the cell unless they form the active site of an enzyme. Lipoic acid reduction has been described only for NADH-dependent lipoamide dehydrogenase in mitochondria (the enzyme responsible for reduction of lipoamide bound to a-keto dehydrogenases), glutathione reductase, and thioredoxin reductase (40,41). The latter two enzymes are NADPH-dependent, so the ability of a cell to reduce lipoic acid or its derivatives reflects both the activities of these enzymes, and NAD(P)H availability. In this regard, it also differs somewhat from recycling of ascorbate from DHA, which may involve reduction by cellular thiols. An important feature of the enzyme-dependent reduction of lipoic acid is that the different enzymes have different rates of reduction for the R- and S- forms of lipoic acid (40,41), which could provide clues as to the enzymes involved.

In contrast to ascorbic acid, which is trapped in cells, dihydrolipoic acid readily exits cells into the interstitium or culture medium (42,43). However, because of its high reducing capacity, dihydrolipoic acid is readily oxidized, especially in oxygenated buffer or culture medium. Although lipoic acid recycling by cells can be measured directly as dihydrolipoic acid present in cells or medium, because of the short half-life of dihydrolipoic acid, assays have been developed (44,45) to measure its appearance in cell buffers by its ability to reduce 5,5’-(dithiobis)-2-nitrobenzoic acid (DTNB), also known as Ellman’s reagent. This disulfide has two negative charges due to carboxylic acid groups and does not enter cells. Thus, it detects only lipoic acid that has been released into the incubation medium, and does so as soon as this release occurs, thus decreasing the chance for oxidation of dihydrolipoic acid. Reaction of DTNB with a thiol releases the 5-thio-2-nitrobenzoic acid anion, which is a bright yellow, strongly absorbing light at 410 nm, so that it can be detected at visible wavelengths in a spectrophotometer.

This chapter describes approaches for measuring ascorbic and lipoic acid recycling, which can be used to provide time-dependent and non-destructive estimates of the redox capacity of cells in suspension and culture.

2. Materials

2.1. Assay of ascorbate-dependent ferricyanide reduction

1. Ferricyanide: analytical grade potassium ferricyanide (potassium hexacyanoferrate (III)) is dissolved in deionized water or appropriate buffer to a concentration of 100

mM (32.9 mg/ml) for dilution into the assay. Stable in the dark in the refrigerator for several months.

2. Sodium acetate buffer: prepare to 3M by dissolving 40.8 g of sodium acetate trihydrate in 100 ml of deionized water and adjusting the pH to between 6.0–6.5. Stable in the refrigerator for several months.

3. Citric acid solution: prepare to 0.2 M by dissolving 3.84 g of citric acid in 100 ml of deionized water. Stable several months in the refrigerator.

4. Ferric chloride solution: prepare to 3.3 mM by dissolving 53.5 mg of ferric chloride in 100 ml of 0.1 M acetic acid (5.75 ml glacial acetic acid brought to 1 liter with deionized water). This must be made fresh every few days.

5. Phenanthroline derivatives (see Note 1): The disodium salt of 4,7-diphenyl-1,10-phenanthroline disulfonate (bathophenanthroline disulfonate) is prepared by dissolving 100 mg in 30 ml of deionized water. If 1,10-phenanthroline is to be used, it is prepared by dissolving 100 mg in 10 ml of ethanol. Stable in the refrigerator for several weeks.

2.2. Assay of DHA reduction capacity

1. DHA solution: prepare a 10 mM DHA solution by dissolving 1.74 mg of DHA in the appropriate cell buffer just for use (see Note 2).

2. *Meta*-phosporic acid: prepare 25% *meta*-phosphoric acid (w/v) by adding 25 g of *meta*-phosphoric acid to 75 ml of deionized water. After it is dissolved, bring the final volume to 100 ml with deionized water. This is stable indefinitely at room temperature.

3. Phosphate-EDTA buffer: prepare 100 mM phosphate-EDTA buffer containing 0.05 mM EDTA by dissolving 1.42 g of disodium phosphate (Na_2HPO_4) in 80 ml of deionized water, adding 1.86 mg disodium EDTA, bringing to total volume to 100 ml with deionized water, and adjusting the pH to 8.0.

4. Sodium acetate solution: prepare to 1 M by combining 11.3 g anhydrous sodium acetate with 20.9 ml of glacial acetic acid and bringing the total volume to 500 ml with deionized water and store at 3 °C.

5. Ion-pair reagent: dissolve 3.79 g of tetrapentylammonium bromide in 100 ml of methanol and store at 3 °C (see Note 3).

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[1]Ferricyanide has an absorption maximum at 420 nm, so its disappearance from the incubation medium could be simply followed with time. However, this approach is only about 5–10% as sensitive as assay of the complex formed between ferrocyanide and phenanthroline derivatives developed by Avron and Shavit (32). Although use of bathophenanthroline disulfonate increases sensitivity of the assay about 2-fold, this is usually not a problem and we typically use 1,10-phenanthroline.

[2]Whereas commercial DHA (e.g., Sigma-Aldrich # 261556) is adequate for most determinations, it is contaminated with about 0.5% ascorbate, which will directly react with ferricyanide outside the cells. Although we have not found it necessary for this type of assay, DHA may be generated directly from ascorbate using the bromine oxidation method. Dissolve ascorbic acid to a concentration of 50 mM in deionized water (8.8 mg/ml) in a 12 × 75 mm glass culture tube. To this add about 2 μl of liquid bromine in the hood (the amount is not critical, it is in large excess). Vortex vigorously for 1–2 min to allow the bromine to dissolve. The solution should turn yellow. Then pass a stream of nitrogen gas over the surface of the solution for several minutes until the solution loses its color, but not so long as to cause evaporation of the water. The final solution is diluted just before use into the appropriate buffer and added to the cell assay This method results in an acidic solution of DHA that is relatively stable. On the other hand, care must be taken that the medium used for cell incubations has adequate buffering capacity to neutralize the residual hydrobromic acid after dilution of into cells. This can be checked directly with a pH meter.

[3]Any of several ion pair reagents work well. We have also used tridecylamine and tetraoctylammonium bromide. These reagents bind the hydrophobic column material because of their short-chain fatty acids, and bind the ascorbate anion due to their positive quaternary ammonium charge.

6. HPLC mobile phase: mix 80 ml of the sodium acetate solution, 10 ml of the tetrapentylammonium bromide solution, 300 ml of HPLC grade methanol, and 610 ml of HPLC grade water.

7. Standard solutions of ascorbic acid: prepare by dissolving 1.76 mg of ascorbic acid in 1 ml of HPLC mobile phase to give an ascorbate concentration of 10 mM. The concentration of his solution can be verified by measuring the absorbance at 265 nm of an aliquot that has been diluted to 0.1 mM ascorbate. The optical density of this solution should be 0.33, based on a molar extinction coefficient of 3300 $M^{-1}cm^{-1}$ at this wavelength. Standards of 0.5 to 4 nmol/ml are prepared by serial dilution of this stock solution with mobile phase. The ascorbate stock and standards should be kept on ice and prepared daily.

2.3. Assay of lipoic acid recycling

1. Krebs-Ringer Hepes buffer (KRH): prepare a solution in deionized water of 20 mM *N*-2-hydroxyethylpiperazine-NN-2-ethanesulfonic acid (Hepes), 128 mM NaCl, 5.2 mM KCl, 1 mM NaH_2PO_4, 1.4 mM $MgSO_4$, and 1.4 mM $CaCl_2$, pH 7.4.

2. Lipoic acid solution: dissolve 0.41 mg of R,S-α-lipoic acid in 1 ml of KRH, resulting in a stock solution of 2 mM.

3. 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) solution: prepare a 2 mM solution by dissolving 0.8 mg of DTNB in 0.8 ml of KRH and adjusting the pH to 7.4 by carefully adding 5 μl aliquots of 1 M NaOH. The total volume is adjusted to 1 ml with KRH after the pH is neutralized (see Note 4).

3. Methods

3.1. Assay of ascorbate-dependent ferricyanide reduction

1. Incubation of cells with ferricyanide. Reduction of ferricyanide can be assessed either in suspended cells (e.g., erythrocytes), or in monolayer cell cultures. It is necessary only that ferricyanide have access to all cells in the incubation. After treatments as required, the cells may be rinsed before addition of ferricyanide (see Note 5). Ferricyanide is added by dilution from the stock solution and the incubations continued at 37 °C. At the end of the incubation (see Note 6), cell-free aliquots of the incubation medium are sampled for assay of ferrocyanide. If suspended cells are used, they are pelleted by centrifugation and aliquots of the supernatant used for assay.

2. To start the assay of ferrocyanide, dilute 100 μl of a sample containing 1–100 nmol of ferrocyanide with 0.6 ml of water in a plastic disposable spectrophotometer cuvette.

3. Just before use, prepare a reaction mixture of the following: 10 ml of the sodium acetate buffer, 10 ml of the citric acid solution, 5 ml of the ferric chloride solution,

[4]DTNB is only sparingly soluble in water. As it is neutralized, it will gradually dissolve. However, overshooting the pH will result in hydrolysis of the DTNB and a bright yellow color, so the additions must be done slowly. This solution must be made just before use, since the intensity of the yellow color will increase with time.

[5]Although ferricyanide is a weak oxidant, it will react with ascorbate and thiols if present outside the cells. It is important to remove such interfering substances if they are present by rinsing the cells before adding ferricyanide, or to test for their presence by comparing results in rinsed and non-rinsed cells, which should have similar rates of reduction.

[6]If a single time point (typically 30 min) is to be used, a time course of ferricyanide reduction by cells must be determined to document that the reaction is linear. The main reasons for lack of a linear response are depletion of extracellular ferricyanide below 50% of the starting concentration, or exhaustion of intracellular reducing equivalents. The former can be avoided by using extracellular ferricyanide concentrations as high as 5 mM, or by decreasing cell number. The latter may be the outcome of interest, but can be minimized by shorter times of incubation with ferricyanide.

and 5 ml of the solution containing the phenanthroline derivative. This is a volume adequate to assay 100 samples.

4. Add 0.3 ml of the reaction mixture from #3 to each cuvette, mix, and allow 5 min to allow full development of color (see Note 7).

5. Read the optical density at 510 nm if 1,10-phenanthroline is used, and at 535 nm if bathophenanthroline disulfonate is used.

6. Calculate the concentration of ferrocyanide in the cuvette based on the molar extinction coefficient of the phenanthroline derivative used for color development (32). For 1,10-phenanthroline this is 10,500 $M^{-1}cm^{-1}$, and for bathophenanthroline disulfonate it is 20,500 $M^{-1}cm^{-1}$. Accordingly, an optical density of 1.0 will be generated by a solution of 95.2 μM ferrocyanide in the cuvette using 1,10-phenanthroline; for bathophenanthroline disulfonate, an optical density of 1.0 will be generated by a ferrocyanide concentration of 48.8 μM in the cuvette (see Note 8).

7. After correcting for the 10-fold dilution of the sample into the cuvette, the amount of ferrocyanide generated over the time of exposure to the cells (see Note 9) can be normalized to cell number, cell protein, or intracellular water space (see Note 10).

8. Correction for a reagent blank is unnecessary, but color in the cell medium may require correction using a sample not treated with ferricyanide that is carried through the assay and subtracted before the calculation (see Note 11). Additional correction may be required for extracellular ascorbate, if present (see Note 12)

3.2. Assay of DHA reduction capacity

1. Cells in culture or in a defined physiologic medium are incubated with freshly prepared DHA. The optimal concentration of DHA must be determined empirically, but is usually in the range of 0.1–1 mM. DHA is very unstable at physiologic pH and must be added to the cells quickly after it is dissolved. Uptake and reduction of DHA is usually complete after 15–20 min, and the cells can be taken for assay of intracellular ascorbate. The extent to which intracellular ascorbate increases in different cell types or under different conditions provides an estimate of the ability of the cells to recycle ascorbate from DHA (see Note 13).

2. Intracellular ascorbate is determined by lysis of the cells and assay of the ascorbate content in the lysate by HPLC. The method of cell lysis varies with the cell type. For cells not containing hemoglobin, after rinsing or centrifugation to remove the incubation buffer, the cell pellet or monolayer of cells (approximately 50–100 μl in

[7]The color of the reaction mixture is stable at room temperature for up to 2 h if the cuvettes containing the reaction mixtures are stored in the dark. As long as the pH range of the reaction mixture is between 2.5 and 6.5, the color of the reaction remains unchanged (32).
[8]The assays are linear with ferrocyanide up to 100 μM ferrocyanide for bathophenanthroline disulfonate as the color indicator, and up to 200 μM ferricyanide for 1,10-phenanthroline as the color indicator (32).
[9]The assay is typically linear for at least 30 min of treating cells with ferricyanide, but this should be determined for each cell type. Using the linear phase, results can be expressed as a rate, or mol ferrocyanide generated per min.
[10]For erythrocytes, which vary in volume and surface area from different species, rates of ferricyanide reduction have been typically expressed as a function of the intracellular water space, which is about 70% of the packed cell volume of human erythrocytes (18). For cultured cells, the rate of ferricyanide reduction is usually expressed per mg cell protein (50).
[11]Examples of colored media requiring correction might be a small amount of hemoglobin resulting from erythrocyte lysis or colored cell culture medium. In general, conditions should be adjusted so that such a blank is less than 5% of the basal reading.
[12]In some experiments evaluating ascorbate-dependent ferricyanide reduction, cells may be loaded with ascorbate using DHA. If commercial DHA used, its content of ascorbate (~0.5%) will raise the apparent “background” by reducing ferricyanide directly. In most instances is a small effect that can be corrected for in by using a blank containing the loading DHA concentration.
[13]Conditions that have been used stress or modify the ability of the cells to reduce DHA include removing glucose from the medium, or depleting the cells of endogenous GSH by various agents.

volume) is lysed according the method of Hissin and Hilf (46), which also allows assay of GSH as another peak in the chromatogram. Cells are treated with 0.1 ml of 25% meta-phosphoric acid, mixed or vortexed, and then partially neutralized with 0.35 ml of phosphate-EDTA buffer. The extract is centrifuged for 5 min in the cold to pellet cell debris, and aliquots of the supernatant are stored at −80 °C or taken for assay immediately (see Note 14). For erythrocytes or samples that contain substantial amounts of iron bound in hemoglobin, an alternative method of lysis is required (see Note 15).

3. The ascorbate content of cell lysates or ultrafiltrates is determined by HPLC according to the ion-pairing electrochemical method of Pachla and Kissinger (47). Although many HPLC systems are adequate to detect ascorbate (48), including UV detection (see Note 16), we have found the following method to provide excellent sensitivity and reliability. Samples and standards are prepared in mobile phase and injected in 100 µl volumes. Mobile phase is pumped by an ESA Model 582 Solvent Delivery system (ESA Biosciences, Chelmsford, MA), followed by an in-line pulse dampener to decrease background fluctuations, and by an ESA Guard cell set at 0.5 V to decrease background in the mobile phase. Samples are separated on a 10×1 cm Waters RadialPak C18 column (BondaPak, 5 µm particle size), although similar results are obtained with other C18 columns, such as an Alltech Absorbosphere C18 column (4.6 × 100 mm, 5 µm particle size). The main column is preceded by a 4 mm guard column of the same packing material. Detection is accomplished with either an ESA Model 5100A or a Coulochem II detector using an ESA Model 5010 analytical cell with the first electrode set at 0.4 volts. Peaks are analyzed using either an ESA 501 data analysis package, or a Shimadzu C-R5A Chromatopac integrator. The sensitivity of detection for ascorbate is 10 pmol/sample with this system (2).

3.3. Assay of lipoic acid recycling

1. Cultured cells (see Note 17) in 6-well plates (see Note 18) are subjected to pretreatments in culture and rinsed 3 times in 2 ml of KRH buffer and incubated at 37 °C with gentle mixing in 2 ml of KRH containing additives as desired (e.g., 5 mM D-glucose), 0.1 mM lipoic acid, and 0.2 mM DTNB (see Note 19).
2. After 30 min, a 1 ml aliquot of the supernatant is transferred to a 1 ml disposable plastic cuvette and the absorbance at 412 nm is determined in spectrophotometer.

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[14]Cells can also be lysed using 5–10% (w/v) *meta*-phosphoric acid alone, followed by the centrifugation step. The acid precipitation method has the advantage that ascorbate is stable at a low pH and can be stored at −80 °C for analysis at a later date. It has the disadvantage that the low pH may eventually damage the HPLC column. Alternatively, cells in suspension or monolayer culture may also be lysed in 60–90% methanol (v/v) containing saturating amounts of EDTA (51,52) and incubated for 10 min on ice. After centrifugation to pellet cell debris, aliquots of the supernatant are taken for assay of ascorbate.

[15]In erythrocytes or cells that contain heme proteins, acidic or methanolic lysis denatures hemoglobin and releases ferric iron that immediately oxidizes ascorbate in the sample. To avoid this problem, the ultrafiltration method of Iheanacho, et al. (53) for hemoglobin-containing plasma was modified for use with lysed cells (2). In this method, erythrocytes at a 25% packed cell volume are frozen in a mixture of dry ice and acetone and allowed to thaw on ice. The hemolysate is transferred to a Centricon-YM10 filter apparatus (Catalog #4241, Millipore Corporation) and centrifuged at 3–4 °C for 30 min at 5000 ×*g*. This results in a clear ultrafiltrate that can be diluted as needed with mobile phase for assay of ascorbate.

[16]UV detection of ascorbate at 265 nm, although slightly less sensitive than coulometric detection, may be useful if there are overlapping peaks on the chromatogram.

[17]Suspended cells such as erythrocytes can also be used in this assay. Erythrocytes are suspended in 1 ml of phosphate-buffered saline (140 mM NaCl, 12.5 mM NaH_2PO_4, pH 7.4) to a packed cell volume of 1%. All other incubation conditions are the same as described for cultured cells, except that erythrocytes are pelleted by centrifugation at the end of the assay, and the results are expressed per ml of packed cells.

[18]Twelve-well plates can also be used by decreasing all volumes by 50%.

[19]The concentrations of lipoic acid and DTNB are determined empirically, but for cultured cells and erythrocytes, 0.1 mM lipoic acid and 0.2 mM DTNB are useful starting points.

3. The concentration of 2-nitro-5-thiobenozate anion of DTNB is calculated based on a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$ (49). Results are corrected for absorbance of a blank containing the amounts of lipoic acid and DTNB used in the experiment without cells. This blank is typically near zero, indicating that the LA preparations used do not contain contaminants that can reduce DTNB.
4. Results are expressed relative to the cell protein present in each well.

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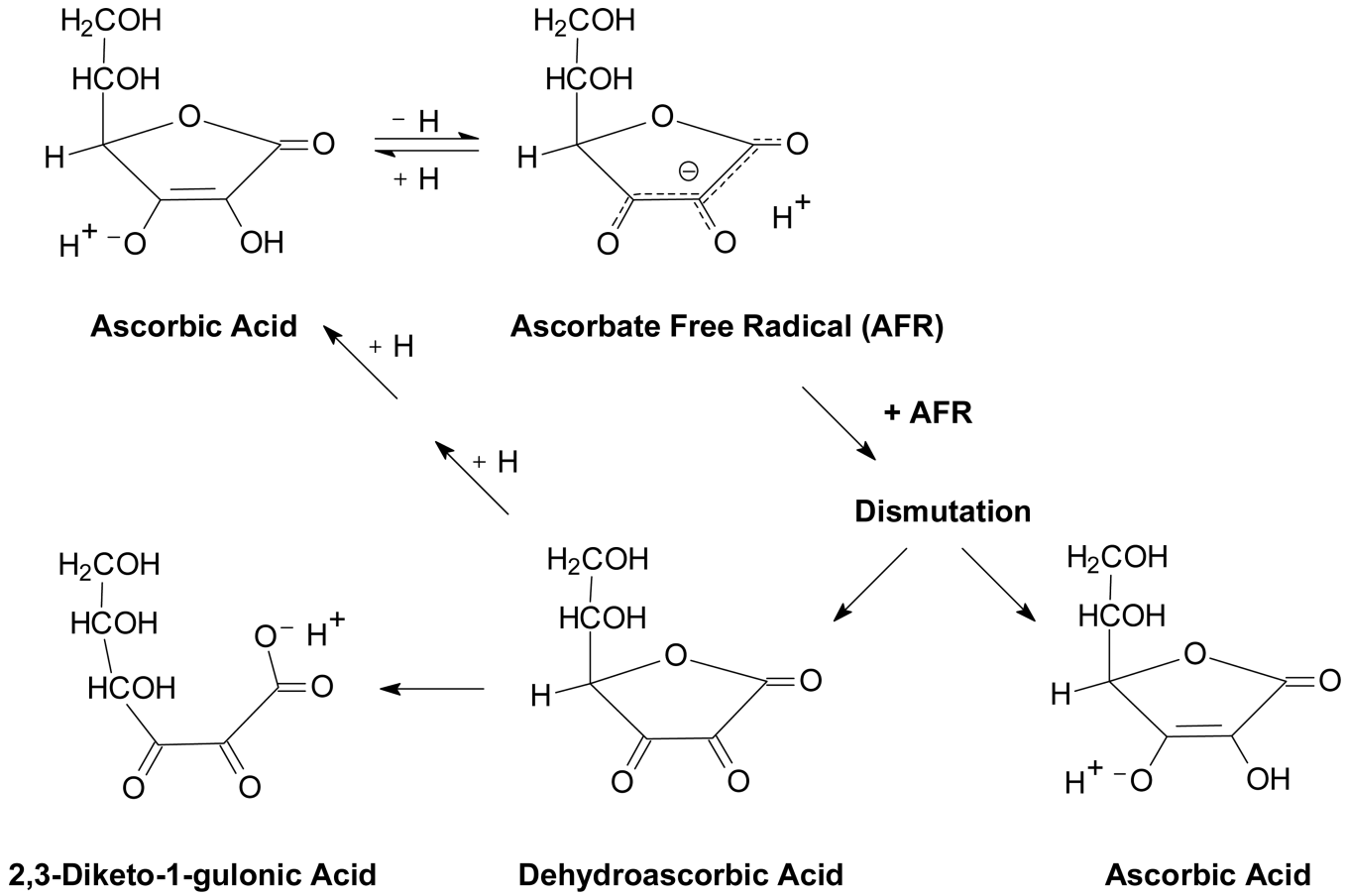

Figure 1.
Ascorbic acid recycling.

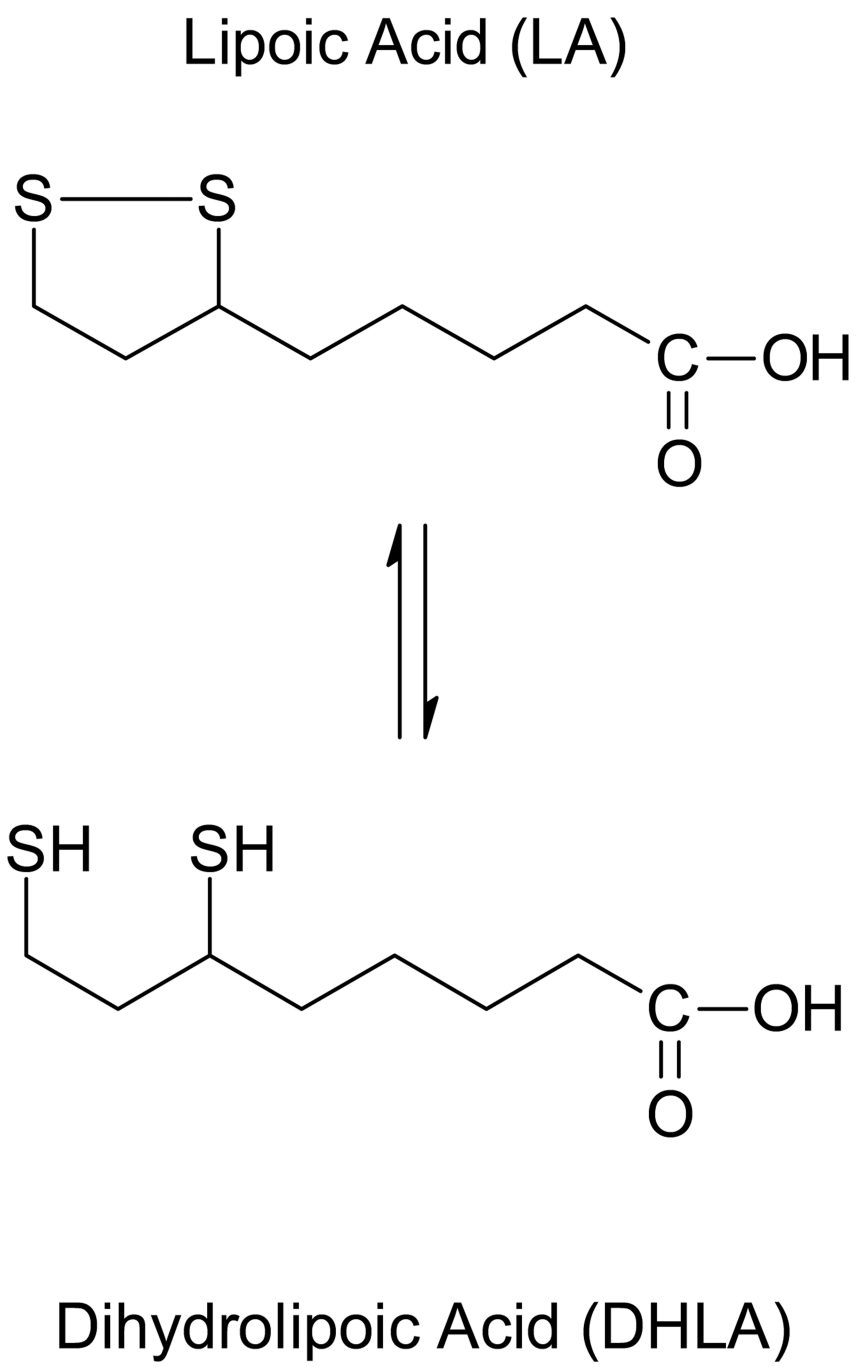

Figure 2.
Lipoic acid recycling.