Regeneration of Ascorbic Acid by Rat Colon (42874)

JEE-LIM CHOI AND RICHARD C. ROSE[1]

Intercollege Nutrition Graduate Program, The Pennsylvania State University, University Park, Pennsylvania 16802, and the Department of Physiology, School of Medicine, University of North Dakota, Grand Forks, North Dakota 58202

Abstract. **Plants and animals alike use ascorbic acid in a variety of reactions that result in net generation of dehydro-L-ascorbic acid. The ability to reduce dehydro-L-ascorbic acid back to ascorbic acid would conserve "total ascorbate" and would help to maintain the toxic oxidized form of the molecule at a low level. This study evaluated the rate of dehydro-L-ascorbic acid reduction either by following the rate of NADPH consumption or by analysis of the amount of ^{14}C-labeled dehydro-L-ascorbic acid converted to ascorbic acid. A large percentage of the NADPH consumed by a semipurified preparation of rat colonic mucosa *in vitro* was dependent on the presence of dehydro-L-ascorbic acid. The tissue factor active in regenerating ascorbic acid is intermediate in size between cytochrome c and blue dextran. The present results indicate that the mucosa reduced dehydro-L-ascorbic acid by a cytosolic enzyme that uses NADPH as a hydrogen donor. Subsequent to precipitation by ammonium sulfate, the 55–70% fraction contains most of the reductase activity while consisting of only 17% of the cellular soluble protein.**

[P.S.E.B.M. 1989, Vol 190]

Dehydro-L-ascorbic acid is formed in animals from ascorbic acid by auto-oxidation, by participation in reactions that produce collagen and catecholamines, and by scavenging free radical products that are formed from dietary carcinogens, oxidative metabolism, radiation, and phagocytic infiltration. Some of these events proceed through single-electron transfer from ascorbate, with the initial product being the ascorbate-free radical. Pairs of ascorbate-free radicals that are not immediately reduced (1, 2) rapidly disproportionate, with net dehydro-L-ascorbic acid formation.

The enzyme responsible for regeneration of ascorbic acid has been given the name dehydro-L-ascorbic acid-reductase (glutathione: dehydro-L-ascorbate oxidoreductase, EC 1.8.5.1; also, glutathione dehydrogenase). We wished to consider the possibility that reduced NADPH is the preferred cofactor for enzymatic dehydro-L-ascorbic acid reduction and that earlier studies on animal tissues (3–7) that indicated the reaction was GSH dependent were conducted under incompletely defined experimental conditions. This study makes use of tissue extracts processed through ammonium sulfate fractionation, dialysis, and centrifugation to allow for more precise interpretation. The possibility must be considered that multiple enzymatic pathways exist for maintaining an optimal ascorbate redox potential. The present study focuses on rat colonic mucosa. The colon of every animal species is exposed to a variety of dietary carcinogens, contains extensive bactericidal defenses (e.g., NADPH oxidase) against microorganisms present in the lumen, and is subject to the possibility of mucosal ischemia. In each case damage is considered to proceed through interaction of free radical species with tissue components (8, 9); ascorbic acid might have a protective role by scavenging free radicals with consequent generation of dehydro-L-ascorbic acid. The present results indicate that dehydro-L-ascorbic acid is reduced by an enzyme that has properties different from EC 1.8.5.1.

Materials and Methods

Tissue Preparations. Male Sprague-Dawley rats of 250–300 g were killed by an overdose of pentobarbital. The colon was removed, opened along the mesenteric border, and rinsed in buffer at 4°C. The serosal musculature was removed with the use of glass slides. The mucosa was processed with 10 strokes of a Teflon homogenizer in buffer of the following composition: 20

[1] Address requests for reprints to: Richard C. Rose, PhD, Department of Physiology, School of Medicine, University of North Dakota, Grand Forks, ND 58202.

Received June 10, 1988. [P.S.E.B.M. 1989, Vol 190]
Accepted December 14, 1988.

0037-9727/89/1904-0369$2.00/0

m*M* NaCl, 120 m*M* KCl, 62 m*M* sucrose, 5 m*M* 3-(*N*-morpholino)propanesulfonic acid, and 0.1 m*M* EDTA at pH 7.2. The homogenate was centrifuged for 10 min at 8000 rpm (5000*g*) in a Beckman J-21 centrifuge with a JA-21 rotor. The supernatant was used in some studies subsequent to overnight dialysis in M_r 12,000 tubing (Spectrum Medical) against 30 volumes of buffer at 4°C. Most often, the supernatant was fractionated by ammonium sulfate precipitation with five consecutive fractions (I–V) containing the 0–40%, 40–55%, 55–70%, 70–90%, or >90% $(NH_4)_2SO_4$. Fraction III was used in most studies. Fractions I–IV were resuspended in a volume of buffer one-eighth the original supernatant volume. All fractions were dialyzed overnight. We determined that Fraction III could be stored at 4°C for at least 3 days without significant loss of activity. Each preparation (experimental unit) was made from the pooled colonic mucosa of five to six rats; mean values and SEM are calculated on at least three preparations.

Enzyme Assay Conditions. Nonlabeled or ^{14}C-labeled dehydro-L-ascorbic acid was made from nonlabeled or ^{14}C-labeled ascorbic acid (17 mCi/mmol; Amersham). The reaction mixture (250 μl) contained 1 m*M* thiourea to slow spontaneous oxidation of ascorbic acid. Dehydro-L-ascorbic acid reduction was routinely monitored at 23°C by including ^{14}C-dehydro-L-ascorbic acid, freshly prepared from ^{14}C-ascorbic acid by bromination. Bromine was removed by blowing N_2 through the buffer. Following incubation under conditions specified below, samples of the reaction mixture (200 μl) were added to 10% metaphosphoric acid (85 μl) and frozen until the time of analysis. Assay of dehydro-L-ascorbic acid, ascorbic acid, and the product of dehydro-L-ascorbic acid degradation, diketogulonic acid, were measured according to the high pressure liquid chromatography method of Bianchi and Rose (10). An indirect evaluation of dehydro-L-ascorbic acid reduction was obtained in one study by following the rate of NADPH disappearance, as monitored by absorbance at 340 nm. Protein content was measured according to the method of Lowry *et al.* (11).

Molecular Weight Determination. The molecular weight of rat colon dehydro-L-ascorbic acid reductase was estimated by exclusion chromatography on Sephadex G-100 (Sigma). The column (19 cm × 0.41 cm) was preequilibrated at 23°C with the buffer used for sample preparation. Sample Fraction III was prepared in a volume of buffer one-twentieth the original supernatant volume containing 0.1 m*M* EGTA and 0.2 m*M* phenylmethyl sulfonylfluoride, and 250 μl was applied to the column. An elution flow rate of 6.6 ml/hr was used and 0.44-ml fractions were collected at 4-min intervals. Protein amount and enzyme activity were determined for each fraction. Ascorbic acid formation is expressed as the amount of dehydro-L-ascorbic acid reduction (pmole) per mg of protein eluted in 1 min.

Results

The Supernatant. The supernatant of colonic mucosa was dialyzed overnight to remove low molecular weight-reducing agents. Samples were then incubated in the presence of exogenous GSH, NADPH, NADH, and cysteine to assess the amount of ^{14}C-dehydro-L-ascorbic acid reduced. NADPH at 30 μ*M* is seen (Table I) to be at least as effective as GSH at 600 μ*M*. Cysteine was effective only at a concentration much higher than that found in animal tissues. NADH was least effective. Preliminary studies showed that ultracentrifugation (100,000*g*) did not alter the activity of the dialyzed supernatant (data not presented).

Partial Purification. The properties of dehydro-L-ascorbic acid reductase were evaluated subsequent to fractionation of the supernatant by ammonium sulfate precipitation. The primary NADPH-dependent activity is shown in Figure 1 to be in the 55–70% ammonium sulfate fraction (Fraction III). Only 17% of the protein content of the supernatant was present in Fraction III.

An assessment was made of the cofactor requirement of dehydro-L-ascorbic acid reductase activity in Fraction III for comparison with the supernatant activity presented in Table I. With ^{14}C-dehydro-L-ascorbic acid present initially at 10 μ*M*, Fraction III brought about dehydro-L-ascorbic acid reduction most completely in the presence of NADPH (Table II).

Because the various tissues and extracellular fluids of the body differ widely in their GSH content, the effectiveness of GSH was further evaluated on Fraction III over the range 30–2000 μ*M*. The amount of ascorbic acid formed by GSH was evaluated in the presence and absence of Fraction III. As seen in Figure 2, a maximally effective reaction rate was reached at a GSH concentration of 600–2000 μ*M*. GSH was therefore used at 600 μ*M* in subsequent studies. The nonenzymatically cata-

Table I. Reduction of Dehydro-L-Ascorbic Acid by the Dialyzed Supernatant of Rat Colonic Mucosa or by Buffer[a]

Preparation	Reducing factor (μ*M*)	Ascorbic acid formed (nmol)
Supernatant	None	0.12 ± 0.02
Supernatant	NADPH (30)	0.53 ± 0.01
Supernatant	NADH (30)	0.30 ± 0.04
Supernatant	GSH (600)	0.44 ± 0.02
Supernatant	Cysteine (500)	0.46 ± 0.03
Supernatant	NADPH (30) + GSH (600)	0.94 ± 0.06
Supernatant	NADPH (30) + cysteine (500)	0.62 ± 0.05
Buffer	NADPH (30)	0.13 ± 0.04
Buffer	NADPH (30) + GSH (600)	0.16 ± 0.04

[a] Values are mean (± SEM) of evaluations on three preparations. Dehydro-L-ascorbic acid was present initially at 10 μ*M*. Incubation time, 15 min.

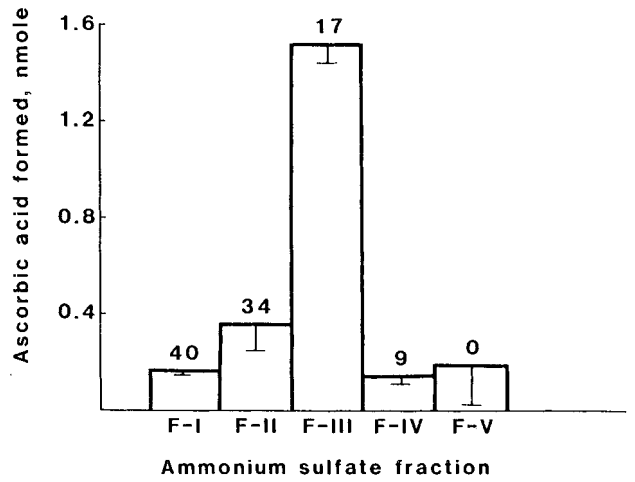

Figure 1. Dehydroascorbic acid reductase activity of colonic mucosa subsequent to fractionation by ammonium sulfate precipitation. Bars are means (±SEM) of three evaluations. ^{14}C-Dehydro-L-ascorbic acid was present initially at 10 μM. Reaction mixture contained thiourea (1 mM) and NADPH (30 μM). Incubation was 23°C for 15 min. Percentage of distribution of protein in each fraction is given above bars.

Table II. Reduction of Dehydro-L-Ascorbic Acid by Fraction III of Rat Colonic Mucosa or by Bovine Serum Albumin[a]

Preparation	Reducing factor (μM)	Ascorbic acid formed (nmol)
Fraction III	NADPH (30)	1.27 ± 0.18
Fraction III	GSH (600)	0.16 ± 0.03
Fraction III	NADPH (30) + GSH (600)	1.16 ± 0.25
BSA (4 mg/ml)	NADPH (30) + GSH (600)	0.20 ± 0.01

[a] Values are mean (± SEM) of evaluations on three preparations. Dehydro-L-ascorbic acid was present initially at 10 μM. Incubation time, 15 min.

lyzed reduction of dehydro-L-ascorbic acid brought about by GSH (in buffer) was low.

The time-dependent reduction of dehydro-L-ascorbic acid was evaluated in the presence of NADPH concentrations from 0 to 100 μM. As seen in Figure 3, reduction was greater at 15 min than at 4 min when NADPH was present at concentrations above 30 μM. The maximal rate of dehydro-L-ascorbic acid reduction appears to be reached at a NADPH concentration of 30 μM, which is considered a physiologic level.

The rate of NADPH consumption was evaluated to assess the stoichiometric relation with ascorbic acid formation. Studies were performed with NADPH present initially at 100 μM. In buffer with GSH present at 600 μM, the rate was 1.47 nmol/min (Fig. 4); in Fraction III without GSH the rate was 1.25 nmol/min. Dehydro-L-ascorbic acid (20 μM, final) was added to each reaction mixture at 27.5 min. The increased rate of NADPH consumption during the subsequent 1.5 min is considered to be associated with dehydro-L-ascorbic acid reduction. Addition of dehydro-L-ascorbic acid immediately enhanced the NADPH consumption rate in Fraction III to 8.07 nmol/min in the presence of GSH or 6.41 nmol/min in the absence of GSH. Between 35 and 55 min of incubation the rate of NADPH consumption returned to the original value, presumably because the dehydro-L-ascorbic acid had been either reduced to ascorbic acid or spontaneously degraded to diketogulonic acid. Parallel samples were incubated with the same preparation of Fraction III to establish the amount of ^{14}C-dehydro-L-ascorbic acid reduced during the 27.5- to 29-min period. The amount

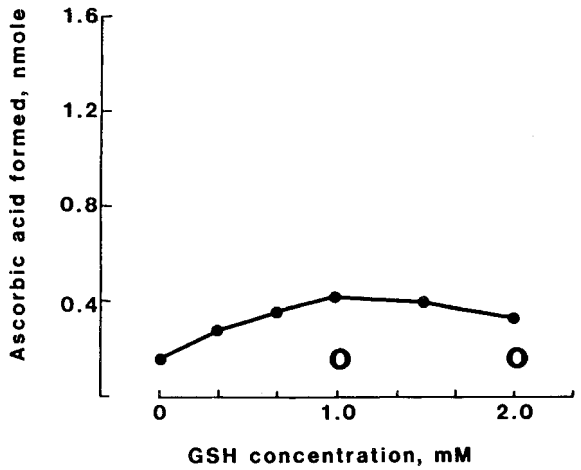

Figure 2. Effect of GSH concentration on dehydro-L-ascorbic acid reductase activity. Fraction III (200 μl) was incubated in the presence of thiourea (1 mM) and ^{14}C-dehydro-L-ascorbic acid (10 μM) for 15 min. In parallel samples on the same tissue preparation, NADPH (30 μM) brought about formation of 1.59 nmol of ascorbic acid. The amount of ascorbic acid formed by the presence of GSH in the absence of Fraction III is shown in open circles. Data presented are from a representative preparation of tissue pooled from five rats.

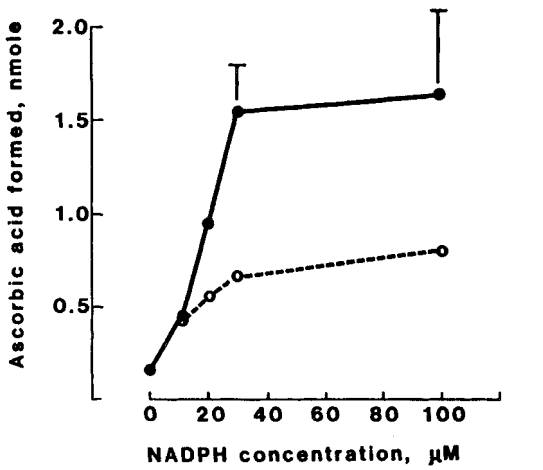

Figure 3. Effect of NADPH concentration on dehydro-L-ascorbic acid reductase activity. Fraction III was incubated in the presence of thiourea (1 mM) and ^{14}C-dehydro-L-ascorbic acid (10 μM) for 4 (○) or 15 (●) min. Values are mean of data from three preparations. Bars are SEM and where not shown are smaller than the symbol.

of ascorbic acid formed in the presence and absence of GSH was 3.92 and 3.22 nmol/min, respectively. It is therefore calculated that the molar consumption of NADPH (corrected for dehydro-L-ascorbic acid-independent consumption) relative to the production of ascorbic acid is 1.68 in the presence of GSH and 1.60 in the absence of GSH.

It was of interest to evaluate further by gel chromatography whether the tissue factor responsible for reducing dehydro-L-ascorbic acid is of high or low molecular weight. As seen in Figure 5 the reductase activity has an elution volume between that of cytochrome *c* and blue dextran. The participating factor appears to be the size of a protein.

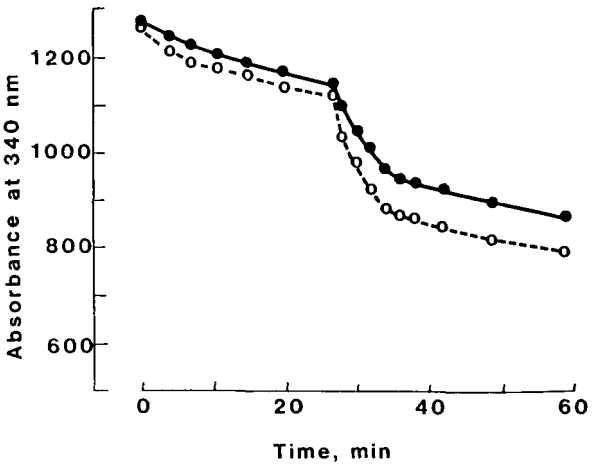

Figure 4. Consumption of NADPH by Fraction III is indicated by a decrease in absorbance at 340 nm. Fraction III was incubated in the presence of 100 μM NADPH and in the presence (○) or absence (●) of GSH at 600 μM. Dehydro-L-ascorbic acid (20 μM) was added to each preparation at 27.5 min. Total volume was 1 ml.

Discussion

Dehydro-L-ascorbic acid is a cytotoxic compound; it hemolyzes erythrocytes and leukocytes (12, 13), disrupts vesicles made from cell membranes (12), and increases the permeability of pancreatic islets *in vitro* (14), resulting in cellular uptake of compounds the size of mannitol and loss of insulin. *In vivo*, dehydro-L-ascorbic acid is diabetogenic (15).

Dehydro-L-ascorbic acid is unstable; it delactonizes in a biologically irreversible reaction to the open chain diketoglulonic acid. Thus, conservation of "total ascorbate" by reduction of dehydro-L-ascorbic acid is as important to health as is intestinal absorption (16) and renal reabsorption (17, 18) of ascorbic acid and dehydro-L-ascorbic acid. Although Szent-Gyorgyi (19) reported in 1928 that animal tissues contain a factor that reduces the oxidized form of "hexuronic acid," little has been learned about the primary tissue sites and mechanisms of dehydro-L-ascorbic acid reduction.

The preference for GSH as a reducing equivalent by dehydro-L-ascorbic acid reductase was originally documented in plant studies (20). Early investigators of ascorbic acid metabolism in animal tissues recognized that GSH might reduce dehydro-L-ascorbic acid but did not identify a specific cellular enzyme. The current concept that animal tissues reduce dehydro-L-ascorbic acid by a GSH-dependent enzyme is frequently attributed to a series of short articles published in the 1960s (3–5). The tissue preparations had been homogenized and dialyzed free of endogenous reducing equivalents; although GSH promoted reduction of dehydro-L-ascorbic acid, no comparative evaluation of the effectiveness of NADPH was made. Thus, the primary reducing factor that participates in maintenance of the ascorbate

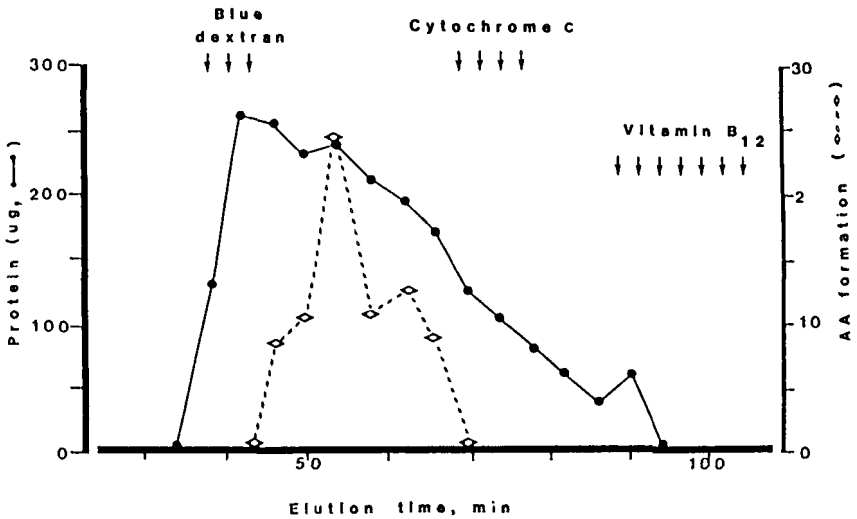

Figure 5. Sephadex chromatography of rat colonic dehydro-L-ascorbic acid reductase activity. Fraction III from ammonium sulfate precipitation was eluted from a 19- × 0.4-cm Sephadex G-100 column. Fractions of 0.45 ml were collected at a flow rate of 6.7 ml/hr and assayed for 45 min in the presence of ^{14}C-dehydro-L-ascorbic acid (10 μM), NADPH (400 μM), GSH (600 μM), and thiourea (1 mM). Ascorbic acid formation in pmol/mg protein/min.

redox potential in animal tissues is not clearly identified.

More recent studies are divided between those that did not demonstrate enzymatic reduction of dehydro-L-ascorbic acid (21–23), those that could not conclude whether or not enzymatic reduction occurs (24), and those that claim a dehydro-L-ascorbic acid reductase is operative (6, 7, 25). Each of these studies had used either intact tissue or crude homogenates and therefore little additional information on the enzymatic mechanism of dehydro-L-ascorbic acid reduction has been generated.

The data presented herein begin to describe the mechanism by which rat colonic mucosa reduces dehydro-L-ascorbic acid. A factor extracted from the cytosol has been partially purified by ammonium sulfate fractionation with primary activity in the 55–70% fraction. It is retained by M_r 12,000 dialysis tubing and indicates a size intermediate between cytochrome *c* (M_r = 12327) and blue dextran ($M_r = 2 \times 10^6$) on gel chromatography. NADPH is the preferred reducing agent; GSH alone is ineffective as a reducing agent.

Properties of the dehydro-L-ascorbic acid-reducing factor from colonic mucosa differ from previous descriptions of dehydro-L-ascorbic acid reductase in that it requires NADPH rather than GSH as a hydrogen donor. Thus, we question the existing concept that animal tissues are similar to plant tissues (20, 26) in using exclusively GSH as a reductant of dehydro-L-ascorbic acid. In-depth studies on erythrocytes and liver are necessary to reevaluate the concepts discussed in references 17–20. It is possible that multiple enzymes participate in the reduction of dehydro-L-ascorbic acid and that the present factor should be considered a distinct enzyme from EC 1.8.5.1. The presence of a colonic glutathione-dependent reductase is suggested in Table I; it might be found in one of the ammonium sulfate fractions not investigated extensively in the present study.

In rat colonic mucosa, we propose that dehydro-L-ascorbic acid reduction is catalyzed by a soluble enzyme that transfers hydrogen from NADPH. Dehydro-L-ascorbic acid might result from the scavenging action of ascorbic acid on any of a variety of free radicals ($\dot{R}$). The most highly reactive and potentially damaging $\dot{R}$ is the hydroxyl radical ($OH\cdot$). Although $OH\cdot$ have been shown to be scavenged *in vitro* by dimethyl sulfoxide, 2-keto-4-thiomethylbutyric acid, *t*-butanol, benzoate, ethanol, urea, mannitol, and bilirubin (9, 27, 28), none of these compounds would appear to be sensitive to damage at a particular site in the body, nor are there known enzymes to recycle or otherwise process the immediate products of their oxidation. The immediate products of a reaction between $\dot{R}$ and ascorbic acid are a detoxified RH and the ascorbate-free radical. An enzymatic mechanism for reducing the ascorbate-free radical has been demonstrated only in select cellular membranes and appears not to exist in the cytosol (1, 2). Therefore, rapid disproportionation of pairs of the ascorbate-free radical is likely, with net production of dehydro-L-ascorbic acid. The present work has focused on a dehydro-L-ascorbic acid reductase that uses NADPH as an electron donor in maintaining ascorbic acid in the reduced state; additional dehydro-L-ascorbic acid reductase enzymes with different cofactor preferences might exist. By promoting a favorable ascorbate redox potential, and indirectly conserving other antioxidants such as α-tocopherol, dehydro-L-ascorbic acid reductase assumes a prominent role in the defense mechanisms of the body. In particular this enzyme is prominent in intestinal mucosa, which has been referred to as a "free radical time bomb" because of the quantity and diversity of reactive radical species (9) that may be scavenged by ascorbate.

It should be noted that the nomenclature "dehydro-L-ascorbic acid reductase" used in this text differs from that recommended by the International Union of Biochemistry (i.e., glutathione: dehydroascorbate oxidoreductase) in two important respects: (i) the present factor relies on NADPH rather than on glutathione and (ii) the term dehydroascorbate has not been used. The latter choice is in response to the recognition that dehydro-L-ascorbic acid lacks the hydroxyl groups at the carbon 2 and 3 positions that allow the parent compound to ionize. Thus, the term dehydro-L-ascorbic acid is not chemically relevant and should be discarded.

This work was supported by Grant AM19119 from the National Institutes of Health.

1. Diliberto EJ, Dean G, Carter C, Allen PL. Tissue, subcellular, and submitochondrial distributions of semidehydroascorbate reductase: Possible role of semidehydroascorbate reductase in cofactor regeneration. J Neurochem **39**:563–568, 1982.
2. Khatami M, Roel LE, Li W, Rockey JH. Ascorbate regeneration in bovine ocular tissues by NADPH-dependent semidehydroascorbate reductase. Exp Eye Res **43**:167–175, 1986.
3. Hughes RE, Kilpatrick GS. The reduction of dehydroascorbic acid by haemolysates of pernicious anaemia erythrocytes. Clin Chim Acta **9**:241–244, 1964.
4. Hughes RE. Reduction of dehydroascorbic acid by animal tissues. Nature **203**:1068–1069, 1964.
5. Grimble RF, Hughes RE. A "dehydroascorbic acid reductase" factor in guinea-pig tissues. Experientia **23**:362, 1967.
6. Bigley R, Stankova L, Roos D, Loos J. Glutathione-dependent dehydroascorbate reduction: A determinant of dehydroascorbate uptake by human polymorphonuclear leukocytes. Enzyme **25**:200–204, 1980.
7. Bigley R, Riddle M, Layman D, Stankova L. Human cell dehydroascorbate reductase kinetic and functional properties. Biochim Biophys Acta **659**:15–22, 1981.
8. Ames BN. Dietary carcinogens and anticarcinogens. Science **221**:1256–1264, 1963.
9. McCord JM. Radical explanations for old observations. Gastroenterology **92**:2026–2028, 1987.

10. Bianchi J, Rose RC. The chromatographic separation and radiochemical quantification of ascorbic acid, dehydroascorbic acid and diketogulonic acid. J Micronutr Anal **1**:3–11, 1985.
11. Lowry OH, Rosebrough NJ, Farr AL, Randell RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
12. Bianchi J, Rose RC. Dehydroascorbic acid and cell membranes: Possible disruptive effects. Toxicology **40**:75–82, 1986.
13. Raghoebar M, Huisman JAM, van den Berg WB, van Ginneken CAM. Characteristics of the transport of ascorbic acid into leucocytes. Life Sci **40**:499–510, 1987.
14. Pillsbury S, Watkins D, Cooperstein SJ. Effect of dehydroascorbic acid on permeability of pancreatic islet tissue *in vitro*. J Pharmacol Exp Ther **185**:713–718, 1973.
15. Patterson JW. Notes and comments: The diabetogenic effect of dehydroascorbic acid. Endocrinology **45**:344, 1949.
16. Rose RC. Intestinal absorption of water-soluble vitamins. In: Johnson LR, Ed. Physiology of the Gastrointestinal Tract. New York: Raven Press, pp 1581–1596, 1986.
17. Bianchi J, Rose RC. Na^+-independent dehydro-L-ascorbic acid uptake in renal brush-border membrane vesicles. Biochim Biophys Acta **819**:75–82, 1985.
18. Rose RC. Ascorbic acid transport in mammalian kidney. Am J Physiol **250**:F627–F632, 1986.
19. Szent-Gyorgyi A. Observations on the function of perdoxidase systems and the chemistry of the adrenal cortex: Description of a new carbohydrate derivative. Biochem J **22**:1387–1409, 1928.
20. Crook EM. The system dehydroascorbic acid-glutathione. Biochem J **35**:226–236, 1941.
21. Hughes RE, Maton SC. The passage of vitamin C across the erythrocyte membrane. Br J Haematol **14**:247–253, 1968.
22. Basu S, Som S, Deb S, Mukherjee D, Chatterjee IB. Dehydroascorbic acid reduction in human erythrocytes. Biochem Biophys Res Commun **90**:1335–1340, 1979.
23. Hornig D, Weber F, Will O. Uptake and release of [I-^{14}C]ascorbic acid and [I-^{14}C]dehydroascorbic acid by erythrocytes of guinea pigs. Clin Chim Acta **31**:25–35, 1971.
24. Hornig D, Weber F, Will O. Studies on the uptake of [I-^{14}C] ascorbic acid [I-^{14}C]dehydroascorbic acid by platelets of guinea pigs. Clin Chim Acta **33**:187–196, 1971.
25. Stankova L, Rigas DA, Bigley RH. Dehydroascorbate uptake and reduction by human blood neutrophils, erythrocytes, and lymphocytes. Ann NY Acad Sci **258**:238–242, 1975.
26. Stahl RL, Liebes LF, Silver R. [2]glutathione dehydrogenase (ascorbate). Methods Enzymol **122**:10–12, 1986.
27. Cederbaum AI, Cohen G. Microsomal oxidation of hydroxyl radical scavenging agents. In: Greenwald RA, Ed. CRC Handbook of Methods for Oxygen Radical Research. Boca Raton, FL: CRC Press, pp 81–84, 1985.
28. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN. Bilirubin is an antioxidant of possible physiological importance. Science **235**:1043, 1987.