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# Relationship of the Metabolism of Vitamins C and E in Cultured Hepatocytes Treated with *tert*-Butyl Hydroperoxide

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## SUMMARY

The relationship between the metabolism of α-tocopherol (α-T) (vitamin E) and that of ascorbic acid (vitamin C) was examined in cultured hepatocytes intoxicated with *tert*-butyl hydroperoxide (TBHP). Unlike vitamin E, the cellular content of vitamin C did not decline after overnight culturing of freshly prepared hepatocytes. In addition, this basal vitamin C content was not affected by the presence of α-T phosphate in the overnight culture medium. Supplementation of the overnight culture medium with vitamin C (10 μM to 10 mM) increased the cellular content of vitamin C by >1 order of magnitude. Increasing the cellular content of ascorbate increased the protection against TBHP toxicity, with or without the presence of a physiological content of vitamin E. In vitamin E-supplemented cells, a loss of α-T occurred within 15 min of exposure to TBHP and before the decrease in cellular ascorbate content. The vitamin C content declined in parallel with the loss of cell viability. Supplementation of the overnight culture medium with increasing concentrations of ascorbate progressively spared the depletion of α-T while preventing the cell killing. Pretreatment with the ferric iron chelator deferoxamine or the antioxidant *N,N'*-diphenyl-1,4-phenylenediamine prevented the loss of ascorbate and the cell killing by TBHP in hepatocytes either sufficient or deficient in α-T. However, neither α-T nor ascorbate prevented the accumulation of DNA single-strand breaks caused by TBHP, indicating that these vitamins do not effectively scavenge the TBHP-derived radicals responsible for DNA damage. The data in the present study indicate that vitamins E and C act as independent antioxidants and that ascorbate does not regenerate α-T in cultured rat hepatocytes.

Vitamins E and C are important antioxidant defenses of the cell. Vitamin E (α-T) is the major, if not the only, antioxidant found in membranes, whereas vitamin C is one of the major water-soluble antioxidants. α-T donates a hydrogen from the 6-position on its chromanol ring to free radicals (1, 2), thereby neutralizing the radical and forming the tocopheroxyl radical. Compared with the hydroxyl radical, which has a half-life of approximately 1 nsec (3), the tocopheroxyl radical is relatively persistent, with a half-life of milliseconds (4). Ascorbate provides its reducing equivalents by donating a hydrogen from the 3-position to produce the ascorbate anion radical ($t_{½}$ = 50 sec for 100 nM), before oxidizing to form DHAA (3, 5). DHAA has a half-life of approximately 6 min at physiological pH (5) and can be reduced back to ascorbate by an excess of reducing equivalents such as those provided by homocysteine or dithiothreitol.

Because cellular α-T is present in concentrations of picomoles/milligram of protein, is obtained solely through the diet, and has a persistent radical, it has been speculated that a mechanism exists to regenerate α-T. Two cellular donors of reducing equivalents present in millimolar concentrations, namely vitamin C and GSH, are thought to be involved in this regeneration.

Numerous *in vitro* experiments utilizing homogeneous solutions (6–8), liposomes (9–13), plasma (14–17), and cellular organelles (12, 13, 18–22) suggest that vitamin C reduces the tocopheroxyl radical to α-T. In brief, a lag occurs between the decline of α-T and the initiation of lipid peroxidation. Addition of ascorbate prolongs this lag, suggesting that there is an interaction between vitamin E and vitamin C. The tocopheroxyl radical appears upon the depletion of ascorbate concentrations or the decay of the ascorbate anion radical. Other studies demonstrate the disappearance of the tocopheroxyl radical after the addition of ascorbate.

Intact cell preparations and whole-animal studies yield conflicting results with regard to the ability of vitamin C to regenerate vitamin E (23–29). For example, ODS rats are mutant Wistar rats that lack the enzyme gulonolactone oxidase and thus cannot synthesize ascorbate. When fed diets enriched in ascorbate, ODS rats had greater tissue concen-

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**ABBREVIATIONS:** α-T, nonesterified α-tocopherol; α-TP, α-tocopherol phosphate; CDTA, cyclohexanediaminetetraacetate; DHAA, dehydroascorbic acid; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid; DPPD, *N,N'*-diphenyl-1,4-phenylenediamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TBHP, *tert*-butyl hydroperoxide; GSH, reduced glutathione; GSSG, oxidized glutathione.

trations of α-T (27). In contrast, the turnover of deuterated α-T in guinea pig tissues was not different among animals receiving diets with either high or low α-T concentrations, supplemented with high, normal, or low levels of ascorbate. It was concluded that ascorbate had little effect on the sparing of α-T (28).

We have previously shown that hepatocytes cultured for 16–18 hr lose 85% of their endogenous α-T (30). The Williams' E medium in which the hepatocytes are incubated does not contain sufficient vitamin E to maintain physiological concentrations of α-T. Overnight supplementation with 1 μM α-T, or one of its esters, is needed to maintain normal cellular concentrations of vitamin E. To address the question of the interaction of vitamin E and vitamin C, we have examined the cellular disposition of α-T and ascorbate during an oxidative stress induced by TBHP in α-T-sufficient and -deficient hepatocytes. The resulting data support the hypothesis that ascorbate does not regenerate α-T in intact hepatocytes under conditions that initiate lipid peroxidation.

## Materials and Methods

**Hepatocytes.** Male Sprague-Dawley rats (150–200 g) obtained from Charles River (Wilmington, MA) were housed for at least 1 week in quarters approved by the American Association of Laboratory Animals, allowed food (Purina Rodent Laboratory Chow 5001) and water *ad libitum*, and fasted overnight before use. Cultured hepatocytes were prepared by the method of Seglen (31), as described previously (30).

After a 2-hr incubation at 37° in an atmosphere of 5% $CO_2$/95% room air, the cultures were rinsed twice with 3 ml of warm HEPES buffer (142 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, 10.1 mM HEPES, pH 7.4, 37°) to remove any unattached or dead cells. Fresh complete Williams' E medium (5 ml) was then added, and the hepatocytes were incubated overnight.

Depending upon the experiment, a final concentration of 1 μM DL-α-TP disodium (Sigma Chemical Co., St. Louis, MO) was added (at 1% volume in water) to or omitted from flasks during overnight incubation. In experiments involving vitamin C, sodium ascorbate (Sigma) was added (at 1% volume in water) to flasks after the initial washing, at the concentrations indicated in the Results section and figure legends, and cells were then incubated overnight as described above.

Overnight (16–18 hr) cultures were washed twice with 3 ml of warm HEPES buffer. Five milliliters of Williams' E medium without fetal calf serum (serum-free Williams' E medium) were added to cultures. It should be emphasized that the additional α-TP and ascorbate added to the overnight cultures were omitted from the medium during all experiments. Cells were incubated under 95% air/5% $CO_2$ at 37°. TBHP (Sigma) was diluted in serum-free medium and added to the cultures (1% volume). The concentrations of TBHP and times of sampling are indicated in the Results section and figure legends.

In a separate series of experiments, cells were cultured overnight in the presence or absence of 1 μM α-TP and were pretreated the next day with a final concentration of 10 mM deferoxamine mesylate (Ciba Pharmaceutical Co., Summit, NJ) for 1 hr before washing with HEPES buffer. Deferoxamine was dissolved in deionized water and added to the cultures in a volume of 3%. After the cells were washed twice with 3 ml of warm HEPES buffer (37°), 5 ml of serum-free medium (37°) without deferoxamine were added to the flasks, and the cells were treated with 400 μM TBHP for 60 min.

In another series of experiments, hepatocytes were cultured overnight with or without 1 μM α-TP. After 16–18 hr, cells were washed twice with 3 ml of warm HEPES buffer and 5 ml of serum-free Williams' E medium were added. DPPD (Aldrich Chemical Co., Milwaukee, WI), dissolved and diluted in dimethylsulfoxide (Sigma), was added to the cultures at a final concentration of 1 μM (0.5% volume). Dimethylsulfoxide alone at this concentration has no effect on cultured cells. Cultures were then treated with 400 μM TBHP for 60 min.

**Ascorbate determinations.** Ascorbic acid (reduced form) was determined by a modification of the method of Behrens and Madere (32). Briefly, the flasks were washed twice with 3 ml of warm HEPES buffer. Two milliliters of 0.85% *m*-phosphoric acid containing 25 μg/ml L-tyrosine (internal standard) (33) were added to the flasks. The cells were scraped from the plates and kept on ice until sonication, also on ice, for 10–15 sec. An aliquot was removed for protein determination and the remaining sample was placed in a microfuge tube. The microfuge tube was centrifuged at 12,000 × *g* for 6 min to pellet the protein. Approximately 600 μl of the acid supernatant were removed with a 1-ml syringe and filtered through a 0.5-μm filter (Millipore Millex-$LCR_4$, 4 mm) into an autosampler vial. Ten microliters were injected onto a high performance liquid chromatography system (Perkin-Elmer LC-410 solvent delivery system with an ISS 100 autoinjector) with a UV/visible spectrophotometer (264 nm; Perkin-Elmer LC-95). The remaining acid supernatant was used for thiol determinations as described below.

Either a 4.6- × 150-mm Supelcosil LC-ABZ $C_{18}$ column (5-μm packing) with a LC-ABZ $C_{18}$ precolumn or a 4.6- × 150-mm Supelcosil LC-18 $C_{18}$ column (3-μm packing) with a LC-18 $C_{18}$ precolumn (Supelcǫ, Bellefonte, PA) was used. The conditions for analysis employed a mobile phase of 0.34% glacial acetic acid (60 mM), 0.0825% octylamine (5 mM), and 10% (v/v) methanol. The mobile phase was adjusted to a final pH of 4.8 with 10 N sodium hydroxide, filtered (0.45-μm filter), and purged with helium before use. The flow rate was 1 ml/min for sample determination (approximately 6 min), after which the flow rate was increased to 1.6 ml/min for 7 min to elute acid-extractable peaks that appear after tyrosine and ascorbate. The retention times for tyrosine and ascorbate with the LC-ABZ column were approximately 2.7 min and 3.3 min, respectively. The retention times for tyrosine and ascorbate with the LC-18 column were approximately 3.1 min and 4.7 min, respectively. Ascorbate was quantified with a Nelson series 900 interface and 2100 PC integrator (version 5.1) software, comparing the peak height of unknown ascorbate with the peak height of tyrosine. The standard curve was based on the ratio of the peak height of known ascorbate to the peak height of tyrosine. Endogenous tyrosine from cultured hepatocytes was not detectable under experimental conditions.

The total ascorbate content of cultured hepatocytes was determined by the complete reduction of samples with 1 mM homocysteine. DHAA content was determined indirectly by the difference between the total ascorbate content and the reduced ascorbate content (32).

**Tocopherol determinations.** Cellular α-T and the internal standard δ-tocopherol (Sigma) were determined by a modification of the methods of Burton *et al.* (34) and Liebler *et al.* (10), as described previously (30). High performance liquid chromatography conditions and procedures for tocopherol quantification were also described previously (30).

**Thiol determinations.** The nonprotein sulfhydryl content of hepatocytes was determined spectrophotometrically, using DTNB, by a modification of the methods of Riddles *et al.* (35) and Jocelyn (36). Briefly, 400 μl of *m*-phosphoric acid supernatant, prepared as described for ascorbate determination, were added to 1 ml of a 600 mM potassium phosphate/1.5 mM EDTA buffer, pH 7.2. To this were added 100 μl of a 3 mM solution of DTNB dissolved in the phosphate/EDTA buffer. After 2 min, the absorbance was read at 412 nm.

The protein thiol content of cultured hepatocytes was determined spectrophotometrically at 412 nm, using DTNB, according to the method of Sedlak and Lindsay (37), as modified by Albano *et al.* (38). GSH was used for both the protein and nonprotein thiol standard curves.

**DNA single-strand break determinations.** DNA single-strand breaks were determined fluorometrically, using the rate of DNA unwinding, by a modification of the method of Birnboim and Jevcak (39) used for cultured hepatocytes (40). An increase in DNA single-strand breaks increased the rate of DNA unwinding, thus reflecting the decrease in the content of double-stranded DNA. Autoclaved water from a Milli-Q system was used for solution preparation. Cells were washed twice with warm HEPES buffer and then scraped into 3 ml of cold HEPES-CDTA buffer (142 mM NaCl, 6.7 mM KCl, 10.1 mM HEPES, 2.5 mM CDTA). The cells were centrifuged at 3000 × *g* for 4 min and resuspended in 670 μl of a solution containing 250 mM *myo*-inositol, 10 mM sodium phosphate, and 2.5 mM CDTA, pH 7.2. Aliquots (200 μl) of the cells were distributed into three tubes in an ice bath (T tubes measured the total double-stranded DNA, R tubes measured the rate of unwinding of the DNA in alkali, and B tubes measured the background fluorescence). Two hundred microliters of a solution containing 9 M urea and 0.1% sodium lauryl sulfate were added to each tube. Tubes were incubated for 10 min at 0°. After addition of the urea solution, it was important for the tubes to be kept in the dark (as dark as experimentally possible) and vibration-free. Four hundred microliters of a solution containing 1 M glucose and 7 mM dithiothreitol were added to the T tubes to prevent unwinding. Then, 200 μl of a solution containing 0.45 volumes of the urea solution in a final concentration of 0.2 N NaOH were carefully added to each tube. The DNA was allowed to unwind for 10 min at 0° in the dark. Before the end of the 10 min, the B tubes were sonicated to completely disrupt the DNA. The unwinding of the DNA in the R and B tubes was arrested by gentle addition of 400 μl of the glucose/dithiothreitol solution. Two milliliters of a 1 μg/ml ethidium bromide solution in 13.3 mM NaOH were added to all tubes. The tubes were gently vortex-mixed and placed in a water bath at 37°. The fluorescence of the samples was measured with a Perkin-Elmer LS-5 spectrofluorometer (excitation, 520 nm; emission, 590 nm; slit width, 10 nm; fixed factor, 10). The ratio of double-stranded DNA fluorescence (T tubes) to background fluorescence arising from other cellular sources (B tubes) was between 2.3 and 2.7. The percentage of double-stranded DNA was calculated as $[(R - B)/(T - B)] \times 100$.

**Other assays.** Measurement of lipid peroxidation, as determined by the accumulation of thiobarbituric acid-reactive products released into the medium, was a modification of the method of Ohkawa *et al.* (41) and was described previously (30). Cell killing was determined by release of lactate dehydrogenase into the medium (30). Protein was determined for each sample by either the method of Smith *et al.* (42) (using bicinchoninic acid) or that of Lowry *et al.* (43), using bovine serum albumin as the standard.

**Statistical analysis.** Data from duplicate or triplicate flasks for each data point were averaged to obtain a single value for each point in each experiment. The number of times the experiment was repeated is indicated in the figure legends. Data from flasks within a given experiment were considered paired, because all hepatocytes for each experiment came from a single rat. One- and two-way analyses of variance, using a repeated-measures design and Student-Neuman-Keuls *post hoc* tests, were performed on data using the PC version of the SAS statistical package (version 6.04) (44). When unequal variance was encountered, the logarithmically transformed data were analyzed (45). Data represent mean ± standard deviation.

## Results

**Vitamin C concentrations in cultured hepatocytes.** Table 1 shows the vitamin C concentrations of freshly isolated hepatocytes and hepatocytes cultured overnight. There is no difference between isolated and cultured cells. Supplementation of hepatocytes with 1 μM α-TP does not affect the ascorbate concentration. Freshly prepared Williams' E medium contains 11 μM ascorbate. However, when the medium is stored for >2 days before use there is no measurable ascorbate present, presumably as a consequence of its autooxidation.

TABLE 1

**Ascorbate concentrations under different conditions**

Cultured hepatocytes were incubated overnight in 5 ml of complete Williams' E medium in 95% air/5% $CO_2$ at 37°. Williams' E medium contains 18 nM α-TP disodium and 11 μM ascorbate. Complete Williams' E medium has 10 IU/ml penicillin, 10 μg/ml streptomycin, 0.05 mg/ml gentamicin, 0.02 unit/ml insulin, and 10% (final concentration) heat-inactivated (55° for 15 min) fetal calf serum. High performance liquid chromatographic analysis of Williams' E medium indicated that ascorbate was below detection limits. Data are the mean ± standard deviation of *n* experiments.

| | Ascorbate |
|---|---|
| | *nmol/mg of protein* |
| Isolated hepatocytes (*n* = 13) | 4.62 ± 0.92[a] |
| Cultured hepatocytes (16–18 hr) (*n* = 13) | 4.87 ± 1.42 |
| Cultured hepatocytes incubated overnight with 1 μM α-TP (*n* = 11) | 4.94 ± 1.67[b] |
| Cultured hepatocytes incubated overnight with 1 mM homocysteine (*n* = 5) | 6.12 ± 1.66 |
| Cultured hepatocyte lysate + 1 mM homocysteine for 1 hr (*n* = 4) | 6.03 ± 1.05 |
| Cultured hepatocyte lysate + 1 mM homocysteine for 2 hr (*n* = 5) | 6.05 ± 0.93 |

[a] Not significantly different from cultured hepatocytes, by paired *t* test (13 experiments).

[b] Not significantly different from cultured hepatocytes, by paired *t* test (11 experiments).

Homocysteine reduces DHAA to ascorbate and thus is used to quantify the proportion of ascorbate in the oxidized and reduced states (32). Homocysteine was added to cells by one of two methods. Hepatocytes were either cultured overnight with 1 mM homocysteine or cultured overnight, scraped from the flasks, sonicated, and then incubated with 1 mM homocysteine for 1 or 2 hr. After incubation with homocysteine, the ascorbate concentrations increase (Table 1), indicating that 10–20% of total ascorbate in the cultured hepatocytes is in the oxidized state. A 1-hr incubation with homocysteine is sufficient to reduce any oxidized ascorbate in the cell lysates and produces total ascorbate levels similar to those seen in cells cultured overnight with homocysteine.

**Antioxidant action of vitamin C.** Hepatocytes were cultured overnight with increasing concentrations of vitamin C in the presence or absence of 1 μM α-TP. The next day, the cells were washed, placed in fresh medium without either vitamin, and exposed to 750 μM TBHP for 90 min. Fig. 1A details the cellular content of ascorbate immediately before treatment with TBHP. Medium ascorbate concentrations of >100 μM significantly raise the cellular content of vitamin C. Addition of 10 mM ascorbate produces a 10-fold increase in the vitamin C concentrations of the hepatocytes.

Fig. 1B shows that hepatocytes enriched with ascorbate are increasingly resistant to oxidative cell killing, whether or not they have a normal content of vitamin E. In both groups, the protection against TBHP increases with increasing concentrations of ascorbate. Nevertheless, cells also supplemented with vitamin E show less cell killing than unsupplemented hepatocytes, except when incubated overnight with ascorbate concentrations of >1 mM. In other words, overnight ascorbate concentrations of >1 mM, whether in the presence or in the absence of supplemental vitamin E, protect the cells against TBHP to the same extent.

**Vitamin C content after exposure to TBHP.** Hepatocytes cultured overnight in the presence or absence of 1 μM α-TP were washed and placed in serum-free medium without

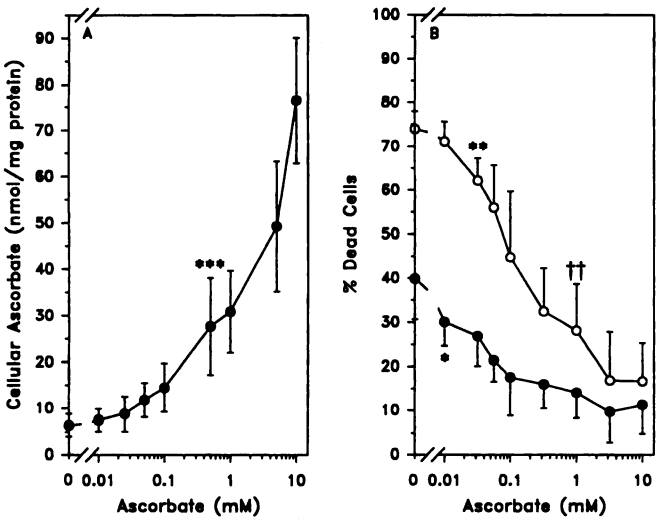


**Fig. 1.** Effect of overnight ascorbate supplementation on initial cellular ascorbate concentrations in hepatocytes (A) and cell killing after a 90-min exposure to 750 μM TBHP (B). Hepatocytes were prepared and incubated overnight with (●) or without (○) 1 μM α-TP and 0.01–10 mM ascorbate. After 16–18 hr, cells were either washed and assayed for ascorbate or washed, placed in Williams' E medium without serum, additional α-TP, or additional ascorbate, and then incubated with 750 μM TBHP for 90 min. Values are the mean ± standard deviation for the results of four experiments. Because the presence of α-T did not affect ascorbate concentrations (Table 1), α-T-deficient cells were not assayed for ascorbate. *, $p < 0.05$, compared with the respective control group; **, $p < 0.01$, compared with the respective control group; ***, $p < 0.005$, compared with the respective control group; ††, $p < 0.01$, compared with the same concentration in the α-TP-treated group.

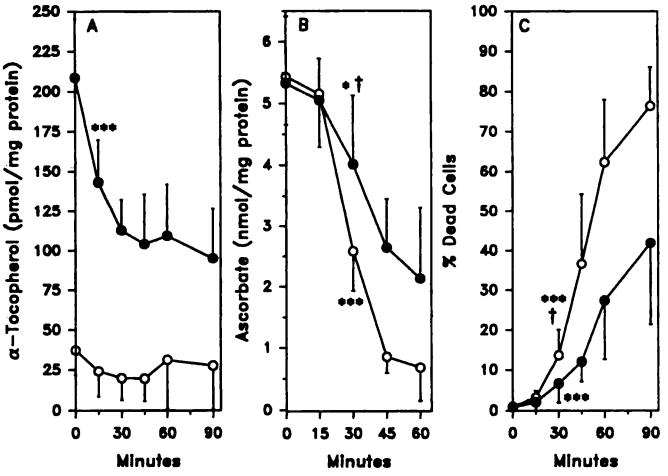


**Fig. 2.** Effect of 750 μM TBHP on α-T concentrations (A), ascorbate concentrations (B), and cell killing (C) in hepatocytes cultured overnight with (●) or without (○) 1 μM α-TP. Hepatocytes were prepared and incubated overnight with or without 1 μM α-TP. After 16–18 hr, cells were washed, placed in Williams' E medium without serum or additional α-TP, and then incubated with 750 μM TBHP. Values are the mean ± standard deviation for the results of three or four experiments. Control cells not treated with TBHP showed no significant change in the α-T or ascorbate concentration over the course of 90 min (data not shown). *, $p < 0.05$, compared with respective time 0; ***, $p < 0.001$, compared with respective time 0; †, $p < 0.05$, compared with same time in unsupplemented cells.

any vitamin supplementation. Fig. 2 shows the time course of the changes in the contents of vitamin E and vitamin C and the viability of the hepatocytes after treatment with 750 μM TBHP. In cells cultured overnight with α-TP, cellular α-T declines by 45% in the first 15 min and then remains constant between 30 and 90 min (Fig. 2A). Hepatocytes cultured without α-TP contain approximately 15% of the initial α-T concentrations and show no change in this vitamin over the 90-min course of the experiment.

Cellular ascorbate concentrations in α-TP-supplemented hepatocytes do not differ from those in unsupplemented hepatocytes during the first 15 min after exposure to TBHP (Fig. 2B). There is no significant decline in the ascorbate content in either group within 15 min. After 15 min, the ascorbate declines in both groups, although at a faster rate in the unsupplemented hepatocytes. Within 60 min, the ascorbate content of the hepatocytes has decreased by 60% for vitamin E-supplemented cells and by 90% for unsupplemented cells.

The decline in ascorbate parallels the increase in cell killing (Fig. 2C). Although there is no loss of viability in either group within the first 15 min, cell killing is significant in both groups by 30 min. In the absence of vitamin E supplementation, the cell killing is significantly greater at 30 min and at all times thereafter.

Fig. 3 details the changes in cellular GSH and protein thiols in the experiment illustrated in Fig. 2. GSH decreases within 15 min after exposure to 750 μM TBHP, with or without supplementation with vitamin E, and continues to decline in both groups over the 60-min course of the experiment. However, after 15 min GSH decreases to a greater extent in the absence of vitamin E supplementation. Thus, cells supplemented with α-TP have higher GSH concentrations at 30, 45, and 60 min.

The initial protein thiol concentrations are also unaffected by vitamin E supplementation. Within 15 min of exposure to TBHP, however, protein thiols decrease significantly in both groups. After 45 and 60 min, cells supplemented with α-TP

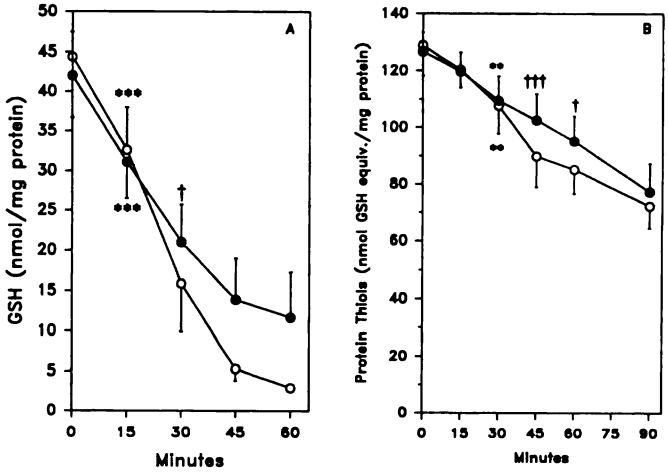


**Fig. 3.** Effect of 750 μM TBHP on the GSH content (A) and protein thiol content (B) of hepatocytes treated overnight with (●) or without (○) 1 μM α-TP. Hepatocytes were prepared and incubated overnight with or without 1 μM α-TP. After 16–18 hr, cells were washed, placed in Williams' E medium without serum or additional α-TP, and then incubated with 750 μM TBHP. Values are the mean ± standard deviation for the results of three or four experiments. Control cells not treated with TBHP showed no change in GSH concentration over the course of 90 min (data not shown). **, $p < 0.005$, compared with respective time 0; ***, $p < 0.001$, compared with respective time 0; †, $p < 0.05$, compared with the same time in unsupplemented cells; †††, $p < 0.005$, compared with the same time in unsupplemented cells.

maintain a small but significantly higher protein thiol content, compared with the unsupplemented cells. Lipid peroxidation occurs in both groups but is significantly less in the vitamin E-supplemented groups (30).

**Effect of ascorbate on the α-T content in TBHP-intoxicated hepatocytes.** Hepatocytes were again cultured overnight with 1 μM α-TP and concentrations of ascorbate ranging from 10 μM to 10 mM, washed, and placed in fresh medium without supplementation with either vitamin. As seen in Fig. 4, the cells were then exposed to 500 μM TBHP for 60 min, at which time the concentration of α-T and the extent of cell killing were determined. TBHP decreases cellular α-T by 28% in cells not supplemented with ascorbate; the α-T concentration in untreated control cells is 158 ± 23 pmol/mg of protein. TBHP-treated hepatocytes that receive >0.1 mM ascorbate have significantly higher α-T concentrations than do cells not receiving ascorbate. At the same time, cultures receiving ≥0.1 mM ascorbate show less cell killing than do those receiving no ascorbate supplementation. Thus, only very large and clearly pharmacological supplements of vitamin C prevent the depletion of vitamin E produced by TBHP.

**Effect of deferoxamine on the vitamin C content.** The killing of cultured hepatocytes by TBHP depends on a source of ferric iron. Chelation of the requisite pool of iron by deferoxamine prevents the cell killing by TBHP (46–48). The data in Table 2 show that deferoxamine spares the depletion of vitamin C.

In this experiment, hepatocytes were cultured overnight with or without 1 μM α-TP. The next day, the cells were pretreated with 10 mM deferoxamine for 1 hr, washed, and placed in fresh medium without either deferoxamine or α-TP. The cells were then treated with 400 μM TBHP for 1 hr, at which time the content of ascorbate and the extent of cell killing were assessed.

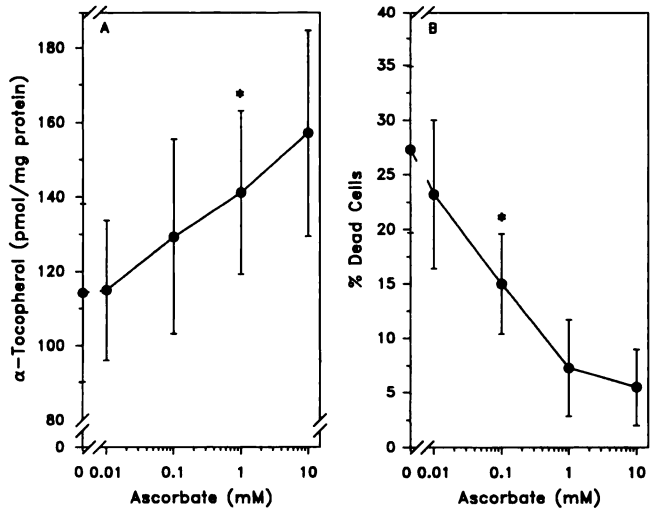


**Fig. 4.** Effect of overnight ascorbate supplementation on cellular α-T concentrations (A) and cell killing after 60 min with 500 μM TBHP (B). Hepatocytes were prepared and incubated overnight with 1 μM α-TP and 0.01–10 mM ascorbate. After 16–18 hr, cells were either washed and assayed for α-T or washed, placed in Williams' E medium without serum, additional α-TP, or additional ascorbate, and then incubated with 500 μM TBHP for 60 min. Values are the mean ± standard deviation for the results of five experiments. Cells not treated with TBHP had 158 ± 23 pmol/mg of protein α-T and 2.2 ± 0.6% cell killing. *, $p < 0.05$, compared with group not supplemented overnight with ascorbate.

TABLE 2

**Deferoxamine spares the depletion of ascorbate produced by TBHP**

Cultured hepatocytes were incubated overnight in complete Williams' E medium with or without 1 μM α-TP. After 16–18 hr, cells were pretreated with 10 mM deferoxamine. After 1 hr, the cultures were washed and serum-free Williams' E medium was added to the flasks. This medium contained neither α-TP nor deferoxamine. Cells were treated with 400 μM TBHP for 60 min. Ascorbate content and cell killing were determined as described in Materials and Methods. Values are the mean ± standard deviation for the results of five experiments.

| | Ascorbate | Cell killing |
|---|---|---|
| | *nmol/mg of protein* | *% dead cells* |
| No α-TP overnight | | |
| Control | 6.7 ± 0.7 | 2.3 ± 0.6 |
| 10 mM Deferoxamine pretreatment | 8.5 ± 1.0 | 1.8 ± 0.3 |
| 400 μM TBHP | 3.7 ± 1.2[a,b] | 40.0 ± 14.0[a] |
| Deferoxamine + TBHP | 7.8 ± 1.3 | 5.6 ± 1.3 |
| 1 μM α-TP overnight | | |
| Control | 6.7 ± 0.9 | 2.7 ± 1.2 |
| 10 mM Deferoxamine pretreatment | 8.5 ± 1.3 | 2.4 ± 0.2 |
| 400 μM TBHP | 5.9 ± 0.9[a] | 25.0 ± 10.1[a,c] |
| Deferoxamine + TBHP | 8.0 ± 1.2 | 3.7 ± 0.8 |

[a] Significantly different from other groups with similar incubations with α-TP, $p < 0.001$.
[b] Significantly lower than TBHP-alone group incubated with α-TP, $p < 0.001$.
[c] Significantly lower than TBHP-alone group incubated without α-TP, $p < 0.05$.

Pretreatment with deferoxamine prevents the loss of ascorbate produced by TBHP, with or without supplementation with α-TP, in parallel with its preservation of the viability of the cells. It should be noted that both the depletion of vitamin C and the cell killing are less in the cultures supplemented with vitamin E and treated with TBHP alone. Interestingly, there is a significantly higher ascorbate content (approximately 25%) in the control hepatocytes pretreated with deferoxamine and then placed in fresh medium for 90 min. Such a result may represent the effect of a constitutive flux of activated oxygen species on ascorbate levels.

**Effect of DPPD on the vitamin C content.** The 400 μM concentration of TBHP used in the experiment described above (Table 2) kills the cultured hepatocytes by peroxidizing membrane phospholipids (46). The data in Table 3 show that the antioxidant DPPD prevents the depletion of vitamin C.

For this experiment, hepatocytes were cultured overnight with or without 1 μM α-TP. The next day, the cells were washed and placed in fresh medium without α-TP. The cells were then treated with 400 μM TBHP, in the presence or absence of 1 μM DPPD, for 1 hr, at which time the content of ascorbate and the extent of cell killing were assessed.

With or without α-TP supplementation, DPPD spares the depletion of ascorbate produced by TBHP, in parallel with the preservation of the viability of the cells. Again, it should be noted that the extent of both depletion of vitamin C and cell killing is less in the cultures supplemented with vitamin E and treated with TBHP alone. In contrast to the effect of deferoxamine, DPPD does not produce a significantly higher ascorbate content in the control hepatocytes.

**Effect of vitamin E and vitamin C on DNA single-strand breaks.** The reaction of TBHP with ferrous iron in cells can generate a number of radical species, including the *tert*-butyl peroxyl, alkoxyl, and alkyl radicals (49). In turn, there are at least two cellular constituents that interact with

TABLE 3

**DPPD spares the depletion of ascorbate produced by TBHP**

Cultured hepatocytes were incubated overnight in complete Williams' E medium with or without 1 μM α-TP. After 16–18 hr, cells were washed and serum-free Williams' E medium without α-TP was added to the flasks. Cells were treated with 400 μM TBHP for 60 min, in the presence or absence of 1 μM DPPD. Ascorbate content and cell killing were determined as described in Materials and Methods. Values are the mean ± standard deviation for the results of three experiments.

| | Ascorbate | Cell killing |
|---|---|---|
| | *nmol/mg of protein* | *% dead cells* |
| No α-TP overnight | | |
| Control | 4.3 ± 2.8 | 3.2 ± 1.0 |
| 1 μM DPPD | 5.2 ± 2.6 | 3.6 ± 0.4 |
| 400 μM TBHP | 0.9 ± 0.3[a,b] | 72.0 ± 10.1[a] |
| DPPD + TBHP | 3.8 ± 1.7 | 4.5 ± 0.5 |
| 1 μM α-TP overnight | | |
| Control | 4.3 ± 2.2 | 2.8 ± 0.9 |
| 1 μM DPPD | 4.9 ± 2.7 | 2.7 ± 0.7 |
| 400 μM TBHP | 2.2 ± 0.9[a] | 47.0 ± 11.1[a,c] |
| DPPD + TBHP | 3.7 ± 2.0 | 5.7 ± 2.0 |

[a] Significantly different from other groups with similar incubations with α-TP, $p < 0.001$.
[b] Significantly lower than TBHP-alone group incubated with α-TP, $p < 0.001$.
[c] Significantly lower than TBHP-alone group incubated without α-TP, $p < 0.05$.

these species. The TBHP-derived radicals initiate lipid peroxidation, the event responsible for the cell killing that occurs in the present study. Alternatively, these same radicals can produce single-strand breaks in DNA. We have previously shown that such damage to DNA can occur in the absence of any loss of viability of the hepatocytes (40). The antioxidant DPPD prevents cell killing without having any effect on the rate or extent of the accumulation of DNA single-strand breaks (40). In the present study, we also use the production of DNA single-strand breaks to assess the ability of both vitamin E and vitamin C to scavenge iron-dependent TBHP-derived radicals in the intact hepatocyte.

Table 4 shows the accumulation of DNA single-strand breaks after exposure to 750 μM TBHP for 30 min. DNA single-strand breaks were quantified by their effect on the rate of alkaline unwinding of double-stranded DNA (39, 40).

TABLE 4

**α-TP supplementation does not prevent the accumulation of DNA single-strand breaks caused by TBHP**

Cultured hepatocytes were incubated overnight in complete Williams' E medium with or without 1 μM α-TP, as described in Materials and Methods. After 16–18 hr, cells either were pretreated with 10 mM deferoxamine for 1 hr and then washed or were washed after no pretreatment. Serum-free Williams' E medium without either α-TP or deferoxamine was added to the flasks and the cells were treated with 750 μM TBHP. In other flasks, serum-free Williams' E medium was added to the flasks and the cells were treated either with DPPD and TBHP or with TBHP alone. After 30 min, DNA single-strand breaks were determined by the unwinding of DNA in alkali, and they are reported as the percentage of double-stranded DNA. No cell killing was observed at 30 min. Values are the mean ± standard deviation for the results of three experiments.

| | Double-stranded DNA | |
|---|---|---|
| | −α-TP | +α-TP |
| | % | |
| Control | 62.8 ± 10.1 | 62.1 ± 5.4 |
| 750 μM TBHP | 37.3 ± 5.4[a] | 32.8 ± 1.8[b] |
| 10 mM Deferoxamine pretreatment + TBHP | 50.3 ± 13.6[c] | ND[d] |
| 1 μM DPPD + TBHP | 33.0 ± 9.0[a] | ND |

[a] Significantly different from the control group without α-TP, $p < 0.005$.
[b] Significantly different from the control group with α-TP, $p < 0.001$.
[c] Not significantly different from control groups.
[d] ND, not determined.

Control hepatocytes with or without overnight α-TP supplementation show similar rates of DNA unwinding. Treatment with TBHP results in an accumulation of DNA single-strand breaks. This DNA damage depends on a cellular source of ferric iron, because pretreatment with deferoxamine before exposure to TBHP prevents the accumulation of the single-strand breaks. As reported previously, treatment with DPPD does not prevent DNA single-strand breaks produced by TBHP at a time when there is no hepatocyte killing.

Table 5 shows the effect of an overnight incubation with 1 mM ascorbate on the accumulation of DNA single-strand breaks produced by 750 μM TBHP in hepatocytes supplemented or not supplemented with 1 μM α-TP. Control hepatocytes incubated overnight with ascorbate alone or with ascorbate and α-TP exhibit similar rates of DNA unwinding. As in Table 4, treatment with TBHP for 30 min causes an accumulation of DNA single-strand breaks. Again, supplementation with α-TP does not affect the single-strand breaks. Enrichment of vitamin E-supplemented or unsupplemented hepatocytes with ascorbate has no effect on the accumulation of DNA single-strand breaks produced by TBHP. Thus, neither vitamin C nor vitamin E effectively scavenges the TBHP-derived radicals, in intact cultured hepatocytes, that are responsible for DNA damage.

## Discussion

The present study examines the metabolism of vitamins E and C in cultured hepatocytes exposed to an oxidative stress. The underlying concern is to evaluate the nature of any interaction between these vitamins in intact cells during the course of oxidative cell injury. The data presented above document the antioxidant properties of vitamin C and support the conclusion that ascorbate acts independently of vitamin E. Stated differently, there is no evidence that ascorbate acts as a reductant of the vitamin E radical.

In intact rats, the liver synthesizes and secretes vitamin C. In cultured hepatocytes, no increase occurs either in the total intracellular content of ascorbate or in the concentration of total ascorbate in the culture medium (data not shown) over the time course of the experiments in the present study. Thus, *de novo* synthesis of vitamin C by the hepatocytes is minimal and unlikely to influence the data obtained.

The ascorbate content of intact rat liver is 10.8 nmol/mg of

TABLE 5

**Ascorbate supplementation does not prevent the accumulation of DNA single-strand breaks caused by TBHP**

Cultured hepatocytes were incubated overnight in complete Williams' E medium with or without 1 μM α-TP and/or 1 mM sodium ascorbate, as described in Materials and Methods. After 16–18 hr, cells were washed and serum-free Williams' E medium without α-TP or ascorbate was added to the flasks. Cells were treated with 750 μM TBHP for 30 min. DNA single-strand breaks were determined by the unwinding of DNA in alkali and are reported as the percentage of double-stranded DNA. No cell killing was observed at 30 min. Values are the mean ± standard deviation for the results of eight experiments.

| | Double-stranded DNA | |
|---|---|---|
| | −α-TP | +α-TP |
| | % | |
| Overnight ascorbate (1 mM) | 56.9 ± 9.5 | 56.1 ± 11.0 |
| 750 μM TBHP | 29.3 ± 7.1[a] | 27.9 ± 11.3[a] |
| Overnight ascorbate + TBHP | 29.9 ± 11.5[a] | 33.8 ± 13.5[a] |

[a] Significantly different from the respective control group, $p < 0.001$.

protein [calculated from the reported value of 1.62 nmol/mg of wet weight (32) and assuming that protein is 15% of wet weight]. The cultured hepatocytes used in the present study contain a total of 6.1 nmol of ascorbate/mg of protein. In these cells, reduced vitamin C represents 80–90% of the total ascorbate, with 10–20% present as oxidized vitamin C, DHAA (Table 1). This is similar to the percentage of DHAA reported in rat liver (32).

After a 16–18-hr incubation, the vitamin C content of cultured hepatocytes does not differ significantly from that of freshly isolated cells (Table 1). In contrast, α-T decreases by 85% during the overnight incubation of the hepatocytes (30) (Fig. 2). In addition, maintaining a physiological concentration of α-T by supplementation of the culture medium does not affect the ascorbate content of the cells (Tables 1–3). Thus, a 1-order of magnitude change in the vitamin E content of the cultured hepatocytes produces no change the cellular content of vitamin C.

Vitamin E-deficient hepatocytes are more sensitive than vitamin E-supplemented hepatocytes to the oxidative stress imposed by TBHP (Fig. 1B). However, increasing the content of vitamin C in both vitamin E-replete and vitamin E-deficient cells increases the resistance of hepatocytes to TBHP (Fig. 1B). Augmenting the vitamin C content of the medium from 0.01 to 10 mM increases the cellular content of ascorbate by 1 order of magnitude (Fig. 1A). Importantly, a higher cellular content of vitamin C protects the hepatocytes from oxidative injury by TBHP irrespective of the vitamin E content of the cells. In other words, ascorbate is an effective antioxidant in hepatocytes with either a physiological content of α-T or a virtual absence of this vitamin. Thus, the ability of vitamin C to protect liver cells from TBHP-induced cell killing does not depend on the vitamin E content of the cells.

This conclusion is not weakened by the fact that enrichment with an increasing content of ascorbate in hepatocytes with normal levels of vitamin E prevents the loss of α-T in response to TBHP (Fig. 4). This sparing of α-T by supplementation with ascorbate does not necessarily denote a direct interaction between the two vitamins. Rather, the data in Figs. 1 and 4 reflect the ability of an increased cellular content of vitamin C to independently reduce the oxidative stress that is responsible, in turn, for the depletion of vitamin E.

Additional evidence for the independence of the antioxidant actions of α-T and ascorbate is the time course of the depletion of these vitamins in hepatocytes undergoing oxidative injury (Fig. 2). If the action of ascorbate were to reduce the tocopheroxyl radical, one would expect a loss of vitamin C to precede the depletion of vitamin E. Such a scenario has been reported when cellular organelles or model membranes are oxidized *in vitro* in the presence of both vitamins (12, 19, 20). However, in intact hepatocytes, α-T depletion precedes that of ascorbate, as well as the death of the cells (Fig. 2). Furthermore, the loss of vitamin C is not observed before leakage of lactate dehydrogenase from the cells, indicating that the decline in ascorbate is likely due to the loss of integrity of the plasma membrane. This conclusion is supported by the observation that the total (reduced plus oxidized) ascorbate content declines in the same manner as the reduced ascorbate content (data not shown). This result argues against recycling as a factor in the decline of reduced ascorbate.

There is a prompt decrease in the GSH content of the cells upon exposure to TBHP. During the first 30 min, this loss is the same in vitamin E-sufficient and -deficient cells. After 30 min, the rate and extent of GSH loss are greater in the vitamin E-deficient hepatocytes. This most likely reflects the greater loss of viability evident at these later times in vitamin E-deficient cultures. The conversion of GSH to GSSG during TBHP metabolism is an iron-independent event (46). Preincubation of cells with deferoxamine does not change the extent of GSH decline or the accumulation of GSSG in the medium after exposure to TBHP. Thus, the loss of GSH in the present study reflects its conversion to GSSG during the catabolism of TBHP by GSH peroxidase. There is no evidence for loss of GSH as a result of its reaction with TBHP-derived radicals.

The loss of protein sulfhydryls produced by $H_2O_2$ occurs by an iron-independent mechanism and is completely accounted for by the accumulation of GSH-protein mixed disulfides (50). Thus, we can again attribute, in large part, the loss of protein thiols in the present study to the metabolism of TBHP, rather than to reaction with TBHP-derived radicals.

Supplemental vitamin C has a similar effect on the manifestations of the oxidative injury of suspended hepatocytes produced by the bipyridilium herbicide diquat (23). The depletion of vitamin E, the peroxidation of cellular lipids, and the extent of cell killing are reduced by 1 mM ascorbate in hepatocytes depleted of GSH by inhibition of GSH reductase with bischloroethylnitrosourea. Because diquat also reduces the vitamin C content of the cells before the other manifestations of oxidative injury, an interaction between α-T and ascorbate is postulated (23). However, treatment of the suspended hepatocytes in the absence of bischloroethylnitrosourea results in an identical depletion of vitamin C without, in this case, any change in vitamin E content. Thus, the data do not document an interaction between α-T and ascorbate and are entirely consistent with the independent antioxidant action of supplemental vitamin C observed in the present study.

Supplementation of ODS rats, which do not synthesize vitamin C, with dietary vitamin C produces higher concentrations of vitamin E in several tissues (27). On the basis of these data, an interrelationship between ascorbate and α-T is claimed to have been documented in intact animals. The data presented in that report do not address the ability of vitamin C to reduce the vitamin E radical. Rather, they document that supplemental ascorbate spares the depletion of vitamin E, which is similar to the effects of vitamin C reported in the present study. The independent antioxidant properties of vitamin C readily explain the sparing of α-T consumption.

Studies in guinea pigs, which, like humans, do not synthesize vitamin C, did not find evidence for an interaction between vitamin E and vitamin C (28). The uptake and loss of radiolabeled vitamin E were determined in nine tissues in animals receiving diets with high, normal, or low vitamin E and/or high, normal, or low vitamin C contents. The sparing of vitamin E by vitamin C was negligible, compared with the metabolic process that utilizes vitamin E. In another study, alteration of the diet similarly did not produce evidence of interaction between α-T and ascorbate (29).

Others have proposed mechanisms whereby reduction of

the α-T radical occurs without the involvement of vitamin C. The tocopheroxyl radical is reported to be reduced by an enzymatic process dependent upon NAD(P)H. Mitochondria, liver microsomes, human platelets, and human erythrocyte membranes demonstrate such recycling of α-T (2, 20, 51, 52). In addition, GSH-dependent regeneration of vitamin E has been proposed (2, 53, 54).

The killing of cultured hepatocytes by oxidative injury depends on a cellular source of ferric iron (46–48). Accordingly, the ferric iron chelator deferoxamine protects hepatocytes against TBHP. Deferoxamine prevents the decline of ascorbate due to TBHP (Table 2). One explanation is that the loss of vitamin C is a consequence of the generation of TBHP-derived radicals. However, there is no evidence that protective concentrations of ascorbate effectively scavenge the *tert*-butyl radical species that are responsible for DNA damage in cultured hepatocytes. Strand breaks occur despite other studies that show reaction rates of ascorbate (and α-T) with carbon-based and oxygen-based radicals (4, 6, 7, 55). A more likely explanation is that the loss of ascorbate is due to the damage to the plasma membrane. This is supported by the observations that no decline in the ascorbate content is seen until after the cells begin to die (Fig. 2) and the ascorbate content correlates with the degree of cell killing (Fig. 2; Tables 2 and 3).

The inability of either physiological α-T concentrations or high concentrations of ascorbate to prevent the formation of DNA single-strand breaks indicates that neither vitamin C nor vitamin E reacts effectively with TBHP-derived radicals to prevent DNA single-strand breaks. As mentioned, TBHP produces single-strand breaks in DNA by a mechanism that also depends on a cellular source of ferric iron (Table 4) (40). In the absence of either lipid peroxidation or a loss of viability, damage to DNA can be used as an indirect assay for the formation of *tert*-butyl radical species (40). Thus, in the presence of the antioxidant DPPD, which prevents both lipid peroxidation and cell killing, single-strand breaks in DNA still appear (Table 4) (40). In the present study, there was no difference in the extent of formation of DNA single-strand breaks after TBHP treatment of hepatocytes that were sufficient or deficient in vitamin E (Table 4). Thus, similarly to DPPD, physiological α-T concentrations do not adequately scavenge TBHP-derived radicals that cause DNA damage at 30 min. Likewise, a concentration of ascorbate that greatly reduces lethal cell injury even after 90 min (Fig. 1) does not prevent the formation of DNA single-strand breaks either in the presence or in the absence of vitamin E supplementation (Table 5).

The protection by DPPD, α-T, and ascorbate against the cell killing by TBHP implicates lipid peroxidation as a mechanism of lethal injury (Fig. 2; Table 3). Previous experiments have demonstrated that lipid peroxidation is prevented or greatly reduced by DPPD and α-T (30). In addition, DPPD also prevents the decline of ascorbate content in the presence or absence of vitamin E. As with deferoxamine, it is not clear whether the mechanism by which DPPD spares ascorbate is based on the direct antioxidant effect of DPPD or its subsequent effect on the prevention of cell killing. In summary, the present data support the hypothesis that vitamins E and C act as independent antioxidants and that ascorbate does not reduce the α-T radical back to α-T in cultured rat hepatocytes.

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