Mutagenicity of Tocopheryl Quinones: Evolutionary Advantage of Selective Accumulation of Dietary \( \alpha \)-Tocopherol

David G. Cornwell, Marshall V. Williams, Altaf A. Wani, Gulzar Wani, Elaine Shen, and Kenneth H. Jones

Abstract: We have shown that phenolic antioxidant tocopherols are oxidized to nonarylating \( \alpha \)-tocopheryl quinone (\( \alpha \)-TQ) and arylating \( \gamma \) and \( \delta \)-TQ electrophiles. The arylating quinones stimulate apoptosis and are highly cytotoxic in mammalian cells. Some xenobiotic phenolic antioxidants, and mutagens, and it has been suggested that their arylating quinone metabolites are the active agents in mutagenesis related to carcinogenesis. We found that neither \( \alpha \)-nor \( \gamma \)-TQ was directly genotoxic in supercoiled-to-nicked circular DNA conversions, but these agents interacted with the cytomegalovirus reporter-driven plasmid and enhanced luciferase transfection, with \( \gamma \)-TQ > \( \alpha \)-TQ. The Ames test, using \( \gamma \)-TQ and a number of Salmonella strains, showed no evidence of bacterial mutagenesis. \( \gamma \)-TQ was highly cytotoxic and \( \alpha \)-TQ slightly cytotoxic in eukaryocyte AS52 cells. A guanosine phosphoribosyltransferase gene assay showed that \( \gamma \)-TQ was highly mutagenic and \( \alpha \)-TQ slightly mutagenic in AS52 cells. A review of the literature identified associations where a decrease in dietary \( \gamma \)-tocopherol \(( \gamma \)-T) diminishes and an increase in dietary \( \gamma \)-T and its quinone enhances carcinogenicity. Humans and other omnivores selectively accumulate \( \alpha \)-tocopherol, even though \( \gamma \)-T is their principal dietary tocopherol. We suggest that this selectivity confers an evolutionary advantage by limiting tissue \( \gamma \)-T, a putative precursor of the mutagen \( \gamma \)-TQ.

Introduction

Tocopherols are a family of four phenolic antioxidants \(( \alpha, \beta, \gamma, \text{and} \ \delta \) that are synthesized in plants and required by animals (1). These compounds differ in the number and position of the methyl groups on the aromatic (chromane) ring, with three methyl groups for fully substituted \( \alpha \)-tocopherol \(( \alpha \)-T), two methyl groups for partially substituted \( \beta \)- and \( \gamma \)-T, and one methyl group for partially substituted \( \delta \)-T (Fig. 1). Tocopherols are oxidized in vitro (1), and small amounts of tocopherol are oxidized in vivo (for review see Refs. 2–4) to their paraquinoes [tocopheryl quinones (TQ)]. We have found that partially substituted \( \gamma \) and \( \delta \)-TQ, unlike \( \alpha \)-TQ, function as arylating electrophiles and form Michael adducts with nucleophilic thiol groups (2–4). This reaction, which is common to all arylating quinone electrophiles (5), may be a basis for genotoxicity and mutagenicity. Butylated hydroxyanisole (BHA), a model xenobiotic phenolic antioxidant, is widely used in foods and is, like all phenolic antioxidants, oxidized to its paraquinone. In turn, this paraquinone functions as an arylating electrophile and forms Michael adducts with thiol groups (6).

In reviewing the literature, we noted a number of similarities between tocopherols and their quinones and BHA and its paraquinone. Work reported as early as 1933 (7), which has been confirmed repeatedly in our laboratory and elsewhere (2–4,8–10), showed that tocopherols enhanced cell proliferation. A number of studies summarized in the recent literature also show that BHA enhanced cell proliferation (11,12). Arylating tocopheryl quinones are highly cytotoxic agents (2–4,13) that stimulate apoptosis associated with cytochrome \( c \) release and caspase activation (4). Similarly, BHA is involved in apoptosis associated with cytochrome \( c \) release and caspase activation (14). BHA is readily metabolized to its quinone (15), and there is increasing evidence that this arylating quinone is the active metabolite (6,16).

A number of reports in the literature find associations between specific phenolic antioxidant tocopherols and their quinones and cancer. As a general rule, the plasma \( \alpha \)-T level varies inversely with cancer (for review see Ref. 17). Plasma \( \gamma \)-T levels vary directly or inversely with cancer, depending on the specific cancer (17–20). When tocopherols are destroyed by oxidation, presumably to tocopheryl quinones, oils containing \( \gamma \)-T, but not \( \alpha \)-T alone, show cytotoxicity and carcinogenicity in feeding experiments (21,22). When tocopherols are extracted, under specific conditions that promote oxidation, from oils containing \( \gamma \)-T, cancers are in

---

D. G. Cornwell is affiliated with the Department of Molecular and Cellular Biochemistry, M. V. Williams with the Department of Molecular Virology, Immunology, and Medical Genetics, A. A. Wani and G. Wani with the Department of Radiology, and K. H. Jones with the Department of Anatomy and Medical Education, College of Medicine, The Ohio State University, Columbus, OH 43210. E. Shen is affiliated with Xenometrix, Discovery Partners International, Inc., San Diego, CA 92121.
duced in feeding experiments (23). Similarly, many recent studies show a relationship between BHA and its quinone and the induction of cancer, particularly in the rat forestomach (6, 12, 24–26).

In the present investigation, we have examined arylating and nonarylating tocopheryl quinones for genotoxicity and mutagenicity in bacterial and mammalian systems. We hypothesized that a difference in mutagenicity between nonarylating and arylating tocopheryl quinone metabolites might explain the evolutionary benefit of the selective retention of \( \alpha \)-T in the body (27), even though \( \gamma \)-T is the major tocopherol found in the diet of Americans (28).

Methods

Tocopheryl Quinones

d-\( \alpha \)-Tocopherol (99.6%) was purchased from Mann Research (New York, NY), and d-\( \gamma \)-tocopherol (92.6%) was kindly supplied by Tama (Tokyo, Japan). \( \alpha \)- and \( \gamma \)-TQ were synthesized by oxidation with \( \text{FeCl}_3 \) from parent tocopherols and purified on a silica gel 60 column as previously described (2, 3).

DNA Modification and Nicking Assay

Supercoiled pCMV-Taq DNA (10 \( \mu \)g) was modified in a 100-\( \mu \)l reaction for 3 h at room temperature with increasing concentrations of \( \alpha \)- or \( \gamma \)-TQ or anti-BPDE (racemic 7,8-dihydroxy-anti-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrane; Midwest Research Institute, NCI Kansas City Repository, Kansas City, MO), a highly reactive arylating electrophile metabolite of benzo[a]pyrene that exhibits genotoxicity (29) and induces mutagenesis in the Ames test (5). DNA samples were precipitated with ethanol, washed with 70% ethanol to remove traces of adhering reagents, and dissolved in Tris-EDTA buffer. After quantitation of the DNA, 100-ng samples were analyzed on 1% agarose gels as previously described (2, 3).

Cell Transfection and Reporter Assay

Human colon adenocarcinoma HCT116 cells (kindly provided by Dr. Bert Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD) were grown in a humidified atmosphere of 5% \( \text{CO}_2 \) in Dulbecco’s modified Eagle’s medium (GIBCO-BRL, Bethesda, MD) supplemented with 10% fetal calf serum (Atlanta Biological, Atlanta, GA) and penicillin-streptomycin (GIBCO-BRL). Cells were transfected as described earlier (30).

Exponentially growing cells (3 \( \times \) 10^5) were plated in triplicate in 35-mm dishes 18–20 h before plasmid transfection and then transfected with 1 \( \mu \)g/dish modified or unmodified pCMV-Taq DNA using FUGENE 6 transfection reagent (Promega, Madison, WI) according to the manufacturer’s instructions. After 5 h, the transfection mix was removed and cultures were supplied with fresh medium for another 8 or 24 h. Cells were harvested and lysed in 100 \( \mu \)l of luciferase cell culture lysis reagent (Promega). Luciferase activity from 20 \( \mu \)l was assayed in a standard assay system (Promega). Reference standards and negative controls were run in each experiment, and the luminescence was recorded with a luminometer (model TD-20/20, Turner Designs, Sunnyvale, CA).

Bacterial Cell Mutagenesis

Mutagenic potential in bacterial cells was assessed using the Ames II mix (Xenometrix, Boulder, CO), an equimolar mix of six \textit{Salmonella typhimurium} strains constructed to detect six possible base-pair transitions or transversions (31). A separate \textit{S. typhimurium} strain (TA98) was used to detect frame-shift mutations (31). This test as used in our study does not identify deletions. All strains were defective in \textit{rfA} (cell wall lipopolysaccharide, to increase strain permeability to bulky molecules) and \textit{uvrB} (DNA excision repair) and contained the pKM101 plasmid, which increases the ability to perform mutagenic lesion bypass repair during
DNA replication. Mutagenic events revert engineered histidine auxotrophy to histidine prototrophy, allowing cell survival in histidine-deficient medium.

A microplate liquid culture was used (32). Overnight cultures were exposed to γ-TQ in ethanol for 90 min in the presence or absence of 4.5% 9α rat liver biotransformation enzymes (Aroclor-1254 induced; Moltox, Boone, NC) and tested in triplicate. After exposure, a pH-based medium lacking histidine was added, each treatment was reaquiquoted to 48 wells and incubated at 37°C for 48 h, and wells containing live Salmonella colonies were counted. Negative controls were exposed to ethanol alone and tested ($n = 8$). Values are means ± SD, and a one-tailed Student’s $t$-test was used to estimate a significant difference ($P < 0.05$) between treatment and control. Positive controls were 4-nitroquinolino-$N$-oxide, 2-nitrofluorene, and 2-aminoanthracene. These controls were shown in our laboratory to be the most consistent in producing dose-dependent reversions at the concentrations tested for the Ames II mix and TA98 (31,32).

Mammalian Cell Mutagenesis

Mutagenesis, which we define as a change in the original DNA sequence, was measured with AS52 cells (kindly provided by Dr. K. Tindall, North Carolina Biotechnology Center, Research Triangle Park, NC), which lack the normal X-linked mammalian hypoxanthine-guanine phosphoribosyltransferase ($hprt$) gene but contain a single functional copy of the Escherichia coli xanthine-guanine phosphoribosyltransferase ($gpt$) gene stably integrated into the genome (33). Cells were maintained as described previously (33,34), and in all experiments, cell viability was $>95\%$ by relative cloning efficiency. Cell treatment (35,36) was modified, because γ-TQ was cytotoxic and cytostatic with AS52 cells. Briefly, cells were washed and plated for 24 h before treatment (Day −1). Cells were treated with the agent or ethanol alone for 5 h on Day 0 and then washed and incubated overnight. Cytotoxicity was determined on Day 1. Cells were subcultured on Days 3 and 6 with ethanol and γ- and α-TQ and on Days 4 and 8 with γ-TQ. Selection was performed on Day 10 by treating 10<sup>6</sup> cells with 6-thioguanine, incubating the cells for 14 days, and examining the cells for development of 6-thioguanine-resistant clones, which could have resulted from interchromosomal deletions, mitotic recombinations, gene-chromosomal conversions, and multilocus deletions (33–36). Cytotoxicity and relative cloning efficiencies were performed as previously described (33,34), and a specific concentration/incubation time was selected where viability after a 5-h treatment exceeded 35%, the optimal condition for a mutation frequency experiment (35,36).

Results

Genotoxicity

Treatment of plasmid DNA with increasing concentrations of α- or γ-TQ did not cause any detectable conversion of supercoiled DNA to nicked circular DNA (Fig. 2). The supercoiled bands of the treated samples were indistinguishable from the control untreated plasmid. For comparison, arylating anti-BPDE exhibited a dose-dependent conversion of the supercoiled DNA to the nicked form. About 50% of the supercoiled form showed nicking at 50 µM anti-BPDE, and quantitative conversion occurred at $>200$ µM anti-BPDE.

To investigate whether tocopheryl quinones modified the plasmid without appreciably nicking the duplex, host cell reactivation experiments were carried out in HCT116 cells. The host cell reactivation assay used in this study consisted of modified or unmodified reporter plasmid encoding the cytomegalovirus (CMV)-driven luciferase gene. The assay is based on the principle that any covalent modifications of plasmid DNA will strongly block transcription, and these lesions must be repaired in order for the reporter gene to be expressed (37,38). Thus the luciferase activity can be apparent only to the extent of damage being repaired on transfection and recovery of functional plasmid in a given cell. We treated CMV-driven luciferase reporter plasmid with 100 µM α-TQ and 100 µM γ-TQ or 50 µM anti-BPDE and then transiently transfected undamaged and damaged plasmid into HCT116 cells. Data clearly show suppression of transcription of anti-BPDE-treated plasmid owing to heavy modification and consequent inactivation of the plasmid. Even after 24 h within host cells, the anti-BPDE plasmid was unable to recover from the transcription-blocking damage (Fig. 3). On the other hand, there was no such inactivation of the plasmid modified with the α- and γ-TQ. In fact, there was an increase in the activity of the plasmid treated with either agent at 8 and 24 h (Fig. 3). This increase was more pronounced with γ-TQ (60%) than with α-TQ (20%). It appears that the quinones interacted with the plasmid and somehow altered the conformation of the plasmid in a manner that facilitated transcription with the host cell. The nature of this alteration, which led to a reproducible increase in the transcriptions of quinone-treated plasmids, is not clear and requires an additional in-depth analysis.

Bacterial Cell Mutagenesis

In the Ames II test, as used in this study (Table 1), positive controls showed concentration-dependent increases in revertants with the Ames II mix and TA98. γ-TQ over a wide concentration range (0.9–440 µM, where 220 and 440 µM exceeded the solubility limit) did not show a significant increase in the number of revertants. These data for the cytoxic arylation quinone derivative of the phenolic antioxidant γ-T confirm and extend earlier studies where an Ames test failed to show mutagenesis with the phenolic antioxidant BHA (6,25).

Mammalian Cell Mutagenesis

Cytotoxicity was estimated by the cloning efficiencies of AS52 cells treated with ethanol alone and different concen-
Figure 2. Unlike a genotoxic electrophile, \emph{anti}-benzo[\textalpha{}\textalpha{}]pyrene diolepoxide (BPDE), \textgreek{\textgamma{}}-T, \textalpha{}-TQ, and \textgreek{\textgamma{}}-TQ have no effect on supercoiled plasmid DNA. Bands represent supercoiled (SC) and nicked circular (NC) DNA. Lane M contains size markers; Lane C contains medium alone.
trations of γ-T and α- and γ-TQ (Fig. 4). Ethanol had no effect on cloning efficiency compared with cells grown in medium alone (97.4 ± 3.8%). γ-TQ was highly cytotoxic, showed a significant (P < 0.01, Scheffé’s test) dose-dependent decrease from medium at all concentrations, and was significantly different (P < 0.01, Scheffé’s test) from γ-T and α-TQ at all concentrations. Visual examination showed that colonies in culture treated with γ-TQ were smaller than colonies in other treatment groups, a clear indication that γ-TQ was a cytostatic as well as a cytotoxic agent. At 34 µM, γ-T and α-TQ differed from ethanol alone and from each other (P < 0.05, Scheffé’s test). Concentrations used for the mutagenesis assay were selected from cytotoxicity data where there was >35% viability after a 5-h treatment (35,36).

Spontaneous mutation frequencies for the gpt gene were 12.7 ± 3.3, 15.8 ± 5.4, and 17.2 ± 4.5 (mean ± SD) for treatments with 5, 10, and 50 µl of ethanol alone, respectively, and did not differ from nontreated controls. A parent phenolic antioxidant, γ-T, had no effect on cloning efficiency compared with cells grown in medium alone (97.4 ± 3.8%). γ-TQ was highly cytotoxic, showed a significant (P < 0.01, Scheffé’s test) dose-dependent decrease from medium at all concentrations, and was significantly different (P < 0.01, Scheffé’s test) from γ-T and α-TQ at all concentrations. Visual examination showed that colonies in culture treated with γ-TQ were smaller than colonies in other treatment groups, a clear indication that γ-TQ was a cytostatic as well as a cytotoxic agent. At 34 µM, γ-T and α-TQ differed from ethanol alone and from each other (P < 0.05, Scheffé’s test). Concentrations used for the mutagenesis assay were selected from cytotoxicity data where there was >35% viability after a 5-h treatment (35,36).

Spontaneous mutation frequencies for the gpt gene were 12.7 ± 3.3, 15.8 ± 5.4, and 17.2 ± 4.5 (mean ± SD) for treatments with 5, 10, and 50 µl of ethanol alone, respectively, and did not differ from nontreated controls. A parent phenolic antioxidant, γ-T, had no effect on cloning efficiency compared with cells grown in medium alone (97.4 ± 3.8%). γ-TQ was highly cytotoxic, showed a significant (P < 0.01, Scheffé’s test) dose-dependent decrease from medium at all concentrations, and was significantly different (P < 0.01, Scheffé’s test) from γ-T and α-TQ at all concentrations. Visual examination showed that colonies in culture treated with γ-TQ were smaller than colonies in other treatment groups, a clear indication that γ-TQ was a cytostatic as well as a cytotoxic agent. At 34 µM, γ-T and α-TQ differed from ethanol alone and from each other (P < 0.05, Scheffé’s test). Concentrations used for the mutagenesis assay were selected from cytotoxicity data where there was >35% viability after a 5-h treatment (35,36).

Spontaneous mutation frequencies for the gpt gene were 12.7 ± 3.3, 15.8 ± 5.4, and 17.2 ± 4.5 (mean ± SD) for treatments with 5, 10, and 50 µl of ethanol alone, respectively, and did not differ from nontreated controls. A parent phenolic antioxidant, γ-T, had no effect on cloning efficiency compared with cells grown in medium alone (97.4 ± 3.8%). γ-TQ was highly cytotoxic, showed a significant (P < 0.01, Scheffé’s test) dose-dependent decrease from medium at all concentrations, and was significantly different (P < 0.01, Scheffé’s test) from γ-T and α-TQ at all concentrations. Visual examination showed that colonies in culture treated with γ-TQ were smaller than colonies in other treatment groups, a clear indication that γ-TQ was a cytostatic as well as a cytotoxic agent. At 34 µM, γ-T and α-TQ differed from ethanol alone and from each other (P < 0.05, Scheffé’s test). Concentrations used for the mutagenesis assay were selected from cytotoxicity data where there was >35% viability after a 5-h treatment (35,36).

Discussion

There is little evidence that genotoxic phenolic antioxidants such as benzoquinone and benzhydroquinone (39) and

---

Table 1. Revertant Wells Obtained With Ames II Test Using Ames II Mix and TA98 With Ethanol Alone, γ-TQ, and Positive Controls

<table>
<thead>
<tr>
<th></th>
<th>Ames II mix</th>
<th>TA98</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-TQ&lt;sup&gt;α&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethanol, µM</td>
<td>0.6 ± 0.7</td>
<td>1.1 ± 1</td>
</tr>
<tr>
<td>0.9</td>
<td>0.3 ± 0.6</td>
<td>1.8 ± 2.0</td>
</tr>
<tr>
<td>1.8</td>
<td>1.3 ± 1.5</td>
<td>2.3 ± 1.5</td>
</tr>
<tr>
<td>4.4</td>
<td>0.7 ± 0.6</td>
<td>3.0 ± 2.0</td>
</tr>
<tr>
<td>17.6</td>
<td>0.3 ± 0.6</td>
<td>4.0 ± 1.0</td>
</tr>
<tr>
<td>44</td>
<td>0.7 ± 0.6</td>
<td>4.0 ± 1.5</td>
</tr>
<tr>
<td>88</td>
<td>0</td>
<td>4.0 ± 2.7</td>
</tr>
<tr>
<td>220</td>
<td>0.3 ± 0.6</td>
<td>3.7 ± 2.1</td>
</tr>
<tr>
<td>440</td>
<td>0.7 ± 1.2</td>
<td>3.7 ± 2.1</td>
</tr>
<tr>
<td>4-NQO, µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>15</td>
<td>2.6</td>
</tr>
<tr>
<td>0.7</td>
<td>21</td>
<td>2.6</td>
</tr>
<tr>
<td>1.3</td>
<td>35</td>
<td>2.6</td>
</tr>
<tr>
<td>2.6</td>
<td>48</td>
<td>2.6</td>
</tr>
<tr>
<td>2-AA, µM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>9</td>
<td>3.2</td>
</tr>
<tr>
<td>6.5</td>
<td>20</td>
<td>3.2</td>
</tr>
<tr>
<td>12.9</td>
<td>32</td>
<td>6.5</td>
</tr>
<tr>
<td>25.9</td>
<td>40</td>
<td>12.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Values are means ± SD, expressed as number of revertant wells per 48 wells. γ-TQ, γ-tocopheryl quinone; 4-NQO, 4-nitroquinoline-N-oxide; 2-AA, 2-aminoanthracene-N-oxide; 2-NF, 2-nitrofluorene.

<sup>b</sup>: γ-TQ did not have a significant effect (P > 0.05 Student’s t-test) on number of revertant wells.
philic amine groups in DNA. However, we find facilitated transcriptions, with $\gamma$-TQ > $\alpha$-TQ, showing that these agents interact in some way with DNA. This observation should be further explored.

Early mutagenesis studies with the phenolic antioxidant BHA in bacterial systems yielded negative results (6,25), except for one study in a nonstandard system using *Staphylococcus aureus* (43). These studies used less-sensitive bacteria, i.e., strains with fewer base-pair and frame-shift substitutions than the *Salmonella* strains used in the present study (31,32), and they did not always use metabolic activators. Furthermore, these earlier studies focused on the phenol, rather than its quinone metabolite. Our studies provide no evidence for mutagenicity with $\gamma$-TQ in a newer and more-sensitive version of the Ames test, leading to the conclusion that arylating tocopheryl quinone electrophiles, unlike arylating agents such as *anti*-BPDE (5), are not mutagens in prokaryocytes or are agents that cause deletions that would not be identified by the Ames test as used by us.

Studies with AS52 cells provided new information concerning the mutagenicity of tocopherols and their quinones. The arylating electrophile $\gamma$-TQ was highly mutagenic at a low concentration, whereas the nonarylating electrophile $\alpha$-TQ was less mutagenic at a 10-fold increase in concentration. Similar to BHA and its quinone, tocopheryl quinones express clastogenic activity, and their mutagenicity pattern in eukaryocytes ($\gamma$-TQ > $\alpha$-TQ) follows the same pattern as their interaction with plasmid DNA.

The role of $\gamma$-T as a putative mutagen may explain the evolutionary benefit of the selective accumulation of $\alpha$-T in animals. We have calculated from food consumption surveys (28) an $\alpha$-T-to-$\gamma$-T ratio of 0.2 for tocopherols in foods as they are supplied in the American diet. Yet $\alpha$-T-to-$\gamma$-T ratios vary from 4.7 to 6.4 in serum (17,20,44), and it is possible to double this ratio in diets supplemented with $\alpha$-T (45). The selective retention of $\alpha$-T as opposed to $\gamma$-T will limit the availability of $\gamma$-T for the synthesis of its mutagenic arylating quinone electrophile.

A review of the literature identifies many studies where an increase or a decrease in $\gamma$-T and/or its quinone and, conversely, a decrease or an increase in $\alpha$-T are found in a number of disparate observations associated with cytotoxicity and cancer. In one very early study (21), wheat germ (rich in $\gamma$-T), treated with FeCl$_3$, which presumably oxidized the tocopherol to its quinone (2,3), was highly cytotoxic in animals, whereas cod liver oil (rich in $\alpha$-T), treated in the same way, showed little cytotoxicity. Another study found that chicks fed wheat germ treated with FeCl$_3$, developed lymphoblastomas (22). Similarly, rats fed crude vitamin E obtained by wet ether extraction of wheat germ developed malignant sarcomas (23). This experiment could not be repeated with crude vitamin E obtained from wheat germ under conditions where tocopherol oxidation would be minimal, namely, hydrocarbon extraction or cold press extraction (46–49). Therefore, cytotoxicity and the generation of

![Figure 4](image-url). Relative cloning efficiency of AS52 cells treated with $\gamma$-T, $\alpha$-TQ, or $\gamma$-TQ. $\gamma$-TQ was significantly different ($P < 0.001$, Scheffé’s test) from control, $\gamma$-T, and $\alpha$-TQ at all concentrations. $\gamma$-T and $\alpha$-TQ at 34 µM were significantly different ($P < 0.01$, Scheffé’s test) from control and from each other. Each data point represents 4 replicates.

<table>
<thead>
<tr>
<th>Concentration, µM</th>
<th>$\gamma$-T</th>
<th>$\alpha$-TQ</th>
<th>$\gamma$-TQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.4</td>
<td>12.7 ± 10.6</td>
<td>19.0 ± 15.1</td>
<td></td>
</tr>
<tr>
<td>6.8</td>
<td>81.1 ± 41.9</td>
<td>147 ± 46.9*</td>
<td></td>
</tr>
<tr>
<td>34</td>
<td>303 ± 147†</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Mutation Frequencies for *gpt* Gene in AS52 Cells Treated With $\gamma$-T, $\alpha$-TQ, or $\gamma$-TQ

*a*: Values (means ± SD for ≥4 replicates) are expressed as TG$^r$ clones per $10^6$ cells. $\gamma$-T, $\gamma$-tocopherol.

*b*: 10.3 ± 2.9 TG$^r$ clones/10$^6$ cells with ethanol alone.

*c*: Statistical significance is as follows: *, $P < 0.05$; †, $P < 0.01$ (Scheffé’s test).

Figure 4. Relative cloning efficiency of AS52 cells treated with $\gamma$-T, $\alpha$-TQ, or $\gamma$-TQ. $\gamma$-TQ was significantly different ($P < 0.001$, Scheffé’s test) from control, $\gamma$-T, and $\alpha$-TQ at all concentrations. $\gamma$-T and $\alpha$-TQ at 34 µM were significantly different ($P < 0.01$, Scheffé’s test) from control and from each other. Each data point represents 4 replicates.
tumors may be explained by the oxidation of \( \gamma \)-T with FeCl\(_3\) or metal ions in wet ether to the arylating electrophilic quinone \( \gamma \)-TQ.

Human colonic epithelial cells are unusually rich in \( \gamma \)-T (\( \gamma \)-T-to-\( \alpha \)-T ratio < 1) (50), and this suggested to us that colorectal cancer might be associated with dietary \( \gamma \)-T. Indeed, it has been reported that colorectal cancer varies directly with the plasma \( \gamma \)-T level (20), which is one indication of dietary \( \gamma \)-T.

The neonate may be especially vulnerable to the effects of \( \gamma \)-T, since the liver synthesizes a transport protein with a high affinity for \( \alpha \)-T that is responsible for the selective accumulation of \( \alpha \)-T, and this transport protein is very low in neonatal liver (51,52). Studies with infants show that the diet is an important determinant of plasma \( \alpha \)- and \( \gamma \)-T levels (52). Breast milk is rich in \( \alpha \)-T (\( \alpha \)-T-to-\( \gamma \)-T ratio = 8.6), and the plasma of breast-fed infants is rich in \( \alpha \)-T (\( \alpha \)-T-to-\( \gamma \)-T ratio = 9.0). Infant formulas are rich in \( \gamma \)-T (\( \alpha \)-T-to-\( \gamma \)-T ratio = 0.5), and the plasma of bottle-fed infants contains significantly more \( \gamma \)-T (\( \alpha \)-T-to-\( \gamma \)-T ratio = 2.6) than the plasma of breast-fed infants. A number of studies show that breast-fed infants have significantly fewer childhood cancers than bottle-fed infants (53–55). This correlation is usually discussed as an example of the interaction between antibodies in mother’s milk and the infant immune system. Is it possible that the tocopherol phenolic antioxidant \( \gamma \)-T supplied in infant formula results in the synthesis of the quinone mutagen \( \gamma \)-TQ and that this leads to carcinogenesis?

The selectivity conferred by the \( \alpha \)-T transport protein is enhanced by the exclusion of \( \gamma \)-T from the diet, and indeed the absence of this tocopherol from olive oil (28) may contribute to the beneficial effects that have been reported for the Mediterranean diet (56). A recent study dissociated the “olive oil” effect from oleic acid and related it to other unrecognized factors (57), which we suggest may include diminished \( \gamma \)-T in the Mediterranean diet.

The effects of \( \gamma \)-T may be tissue specific. Thus an increase in plasma \( \gamma \)-T is associated with a lower incidence of prostate cancer, which suggests a beneficial effect for this tissue from increases in dietary \( \gamma \)-T (18,19). Animals have evolved to exclude tocopherol precursors of cytotoxic and mutagenic arylating quinones, and there may be optimal levels for \( \alpha \)- and \( \gamma \)-T. We suggest that significant modifications in the dietary levels of specific tocopherols may be premature, especially in infants, until the mutagenic and cytotoxic effects of arylating tocopheryl quinones, including the cytotoxic quinone methide of the metabolite tocored (3) and possibly a quinone oxidation product of the metabolite \( \gamma \)-carboxyethyl hydroxyxchroman (58,59), are more fully explored in specific tissues and in different metabolic states, which include glutathione levels, glutathione S-transferase levels, and levels of specific cytochrome P-450 enzymes, which may be involved in \( \gamma \)-carboxyethyl hydroxyxchroman formation (60).

Acknowledgments and Notes

The authors thank Zongcheng Jiang for tocopherol quinone synthesis, Warren Erdahl for preparing the graphics, and Judith Schwartzbaum for making helpful suggestions. Address correspondence to D. G. Cornwell, Dept. of Molecular and Cellular Biochemistry, The Ohio State University, 333 Hamilton Hall, 1645 Neil Ave., Columbus, OH 43210.

Submitted 11 December 2001; accepted in final form 7 March 2002.

References
