Vitamin A and C compounds permitted in supplements differ in their abilities to affect cell viability, DNA and the DNA nucleoside deoxyguanosine

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In accordance with the European Parliament and Council’s directive, vitamin A and C supplements can include any of four (vitamin A) or five (vitamin C) specified compounds. This study focuses on these compounds and compares their abilities to affect the DNA and viability of cells in culture, but also their potencies to chemically oxidise the DNA nucleoside deoxyguanosine (dG). To study the vitamins’ strict chemical oxidation potencies, dG was exposed to vitamin solution and the amount of the oxidation product 8′-hydroxydeoxyguanosine (8-oxodG) formed was estimated using a high-performance liquid chromatography system with electrochemical and ultraviolet detection. The vitamin’s ability to cause DNA damage to promyelocytic leukaemia cells (HL-60), as detected by strand breaks, alkaline labile sites and formamidopyrimidine DNA glycosylase (FPG)-sensitive sites was, after vitamin exposure, measured using the comet assay and cytotoxicity was estimated using trypan blue staining. The results highlight that vitamin A and C compounds found in supplements do have different properties, chemically as well as in a cellular system. Among the vitamin C compounds, ascorbic acid, sodium ascorbate and calcium ascorbate stood out, causing both oxidation to dG and cytotoxicity to cells. The vitamin A compounds retinol, retinyl acetate and retinal (a breakdown product found in vivo) caused oxidation of dG, while retinal was the only compound causing cytotoxicity, giving rise to an almost complete cell death. β-carotene caused, as the only vitamin compound, a small increase in FPG-sensitive sites. It is concluded that even though the compounds are found in supplements do have different properties linked to oxidation, cytotoxicity and DNA damage.

Introduction

Vitamins are essential dietary constituents required for a functioning metabolism in humans. It is very common that individuals consume vitamin supplements, in addition to, or even instead of, dietary sources such as fruits and vegetables. Fruits and vegetables rich in vitamins with antioxidant properties generally protect against oxidative stress (an imbalance between oxidising agents and the protective mechanisms of the cell) and related diseases (1). Antioxidants have largely been accredited as the fruit and vegetable component responsible for this effect, but this hypothesis has in recent years become questioned since epidemiological studies have shown ambiguous results concerning the health effects from antioxidants and other vitamins. While antioxidants supplemented (14 vitamins, 12 minerals) to a malnourished population indicated a decreased risk of total cancer and mortality (2), several studies on populations with normal nutrient levels have shown a number of antioxidant supplements to have no significant effect on the health (3, 4). Supplementation studies have also revealed harmful health effects by antioxidants, even increasing cancer morbidity and mortality risks (5–8).

In the present study, focus has been given on vitamin A and vitamin C, due to their previously observed abilities to act as pro-oxidants in vitro (9, 10). In accordance with the European Parliament and Council’s directive (2002/46/EC), vitamin A or C supplements can include any of four (vitamin A) or five (vitamin C) specified compounds (Table I). The manufacturers are not required to specify which of these compounds that are present in the supplements. To compound the situation, a variety of vitamin A- and vitamin C-related metabolites and isomers are also found in vivo. Vitamin A is a generic term that includes a number of preformed retinoids (e.g. retinol and retinyl esters) and provitamin A carotenoids (e.g. β-carotene, α-carotene and β-cryptoxanthin) with biological retinol activity. During absorption, distribution and metabolism, retinol and retinyl esters are predominant while retinoic acids and retinal (carotenoids are cleaved to retinal) play essential roles in vitamin A’s biological activities in maintaining cell growth, differentiation and night vision and retinyl esters for vitamin A storage (11). The bioactive form of vitamin C is ascorbic acid (AA) (and its anion, ascorbate), needed as a scavenger against free radicals and as a cofactor in a number of enzymes required for the biosynthesis of, for example, collagen, carnitine and neurotransmitters. Its oxidised form, dehydroascorbic acid (DHAA), is also present in vivo and is reduced to AA intracellularly (11). Specific attention in the present study has been given to the different vitamin A and C compounds found in supplements and their oxidative effects related to DNA.

A key site of radical and oxidative attack of DNA is at the 8-position of guanine (G), the base most susceptible to oxidation due to its low redox potential. The most frequently studied guanine and 2′-deoxyguanosine (dG) oxidation products are 8-oxoguanine (8-oxoG) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG), respectively. 8-oxodG is a pro-mutagenic lesion inducing G:C→T:A transversions and the most common biomarker for oxidative stress (12). 8-oxodG levels have been associated with both inflammation, ageing as well as several forms of cancer (13). Antioxidants have the ability to scavenge radicals and protect biomolecules such as lipids, proteins and DNA against oxidative...
damage, avoiding oxidative stress. In addition to their scavenging properties, some antioxidants can promote antioxidant enzymes or even repair existing DNA damage by, e.g., reducing the guanyl radical, which is formed as an intermediate during guanine oxidation (14). On the contrary, their ability to react with electrons also enables antioxidants to act as pro-oxidants—causing oxidative stress opposed to preventing it (9,10).

The aim of the present study was to investigate whether vitamin A and C compounds, respectively, permitted in supplements differ in their potencies to cause chemical pro-oxidant effects on dG as well as DNA damage, formamido pyrimidine DNA glycosylase (FPG)-sensitive sites (oxidative lesions) and cytotoxicity. In addition to these compounds, retinal and DHAAs were also included as these might be formed as degradation products (and are found in vivo).

Materials and methods

Vitamins

The choice of vitamin A and C compounds investigated was based on the European Parliament and Council’s directive 2002/46/EC stating which compounds that are permitted in supplements (Table I). The following vitamins were used (+)-calcium t-ascorbate dihydrate, β-carotene, DHAA, t-ascorbic acid, t-ascorbic acid 6-palmitate, retinal, retinyl acetate, retinyl palmitate (RP) and (+)-sodium t-ascorbate (all from Sigma–Aldrich, St Louis, MO, USA), stored at −20°C. Vitamin solutions were freshly prepared for each experiment. Any vitamin compound classified as ‘vitamin A’ was first dissolved in dimethyl sulphoxide (DMSO) and then diluted in either buffer (for dG studies) or supplemented RPMI medium (for cell studies, see below), with the exception of RP, which was dissolved in ethanol. The final concentration of DMSO or ethanol was <0.5%. Ascorbic acid 6-palmitate (AA6P) was also dissolved in an equivalent amount of ethanol, while all other C vitamins were dissolved in a buffer or supplemented RPMI only. RP and AA6P were dissolved in ethanol due to lower solubility. It could be the case that the relatively low solubility could affect the outcome of the assays, but, on the other hand, visually it was observed that they were solubilised. The vitamin concentrations used were based on normal plasma concentrations of retinol [2 μM (11)] and AA [50 μM (9)], and for strict physicochemical comparison, equivalent concentrations were used for all vitamin A and C compounds, respectively. AA6P was, however, only analysed at 50 μM and below due to solubility problems at higher concentrations.

Chemicals

The following chemicals were used in addition to vitamins: agarose type VII, albumin from bovine serum (BSA), 2-deoxyguanosine monohydrate and 8-hydroxydeoxyguanosine (Sigma–Aldrich), ammonium acetate, sodium dihydro phosphate-2-hydrate (Riedel-de Haén, Seelze, Germany), di-sodium hydrogen phosphate dodecahydrate, sodium acetate and zinc chloride (Merck, Darmstadt, Germany), FPG (Prof. A.R. Collins, Department of Nutrition, School of Medicine, University of Oslo, Norway), Triton X-100 (LSS; MP Biomedical, Illkirch, France), Chelex® 100 resin (Bio-Rad Laboratories, Hercules, CA, USA) and photosensitiser Ro 19-8022 (F. Hoffmann-La Roche, Basel, Switzerland).

Incubation of dG
dG (400 μM) in acetate buffer (0.3 M ammonium acetate, 0.03 M Zn2+ , chlex treated, pH 5.3), phosphate buffer (0.1 M, chlex treated, pH 5.3) or acetate buffer without Zn2+ (otherwise as above) was exposed to vitamin dissolved in the same buffer. Incubation was performed for 1 h at 37°C (total incubation volume 400 μl) where after the solutions were immediately frozen at −80°C until analysis by high-performance liquid chromatography with electrochemical and ultraviolet detection (HPLC-EC/CV) was carried out. In order to minimise oxidation of dG, the buffer and dG solution were kept on ice during the mixing and until exposure. The zinic content of the acetate buffer was, after acidification, verified using a flame atomic absorption spectrometer with a Perkin Elmer AAnalyst 800 instrument; the detection limit was 2 μM.

HPLC-EC/CV analysis of 8-oxodG

The HPLC-EC/CV system was washed (0.08 ml/min) with methanol and milli-Q-water (4:1) overnight prior to analysis, followed by re-equilibration using HPLC eluent (10% v/v methanol, 20 mM sodium acetate, pH 5.3) at 0.30 ml/min for a minimum of 30 min. The EC-cell was connected to the HPLC system and the flow was adjusted to 0.75 ml/min prior to analysis. New calibration curves for 8-oxodG and dG using standards were generated on each day of analysis. Concentrations of dG (110, 490 and 1370 μM) and 8-oxodG (1, 2, 4 and 32 mM) standards or stock solutions were determined through calculations based on the absorbance, extinction coefficients (dG: 13 000/M/cm at 254 nm, 8-oxodG: 9700/M/cm at 294 nm (15) and Lambert Beer’s Law. Vitamin solutions and standards were eluted for 20 min using the HPLC eluent (0.75 ml/min), with an injection volume of 100 μl. The EC-chromatograms were smoothed using the Stavinsky-Golalz method. The HPLC-EC/CV system is further described by Nagy et.al. (16), where the pump has been replaced by a Scantec pump 100 (Knauer, Berlin, Germany).

Cell culture and exposure

The human promyelocytic leukaemia cell line HL-60 was cultured in RPMI 1640 medium supplemented with (+) t-glutamine, 25 mM HEPES and 10% heat-inactivated foetal bovine serum, in a humidified atmosphere at 37°C and 5% CO2. Cells were split twice a week, and the viability confirmed to be at least 94% using trypan blue staining. At the time of splitting, 0.08 million cells were seeded per well on a 24-well plate and were exposed to vitamin, dissolved and vortexed in supplemented RPMI (total incubation volume 1 ml), for 24 h, at 37°C, 5% CO2 and a humidified atmosphere. The HL-60 cell line was originally obtained from DSMZ, Braunschweig, Germany, and passages 18–42 were used.

Cytotoxicity analysis (trypan blue staining)

The trypan blue stain penetrates damaged cell membranes allowing discrimination between damaged (unviable) and intact (viable) cells. In order to terminate exposure and to achieve a denser cell suspension, cells were centrifuged at 200 × g and 4°C for 4 min, resuspended in 200 μl supplemented RPMI medium and placed on ice. Incubation was then performed with trypan blue stain (1:1 v/v) for 3 min at room temperature. A total of at least 100 cells were counted using a Bürker chamber, and a percentage of unviable (stained) cells was calculated as a measure of cytotoxicity.

The comet assay (single cell gel electrophoresis)

DNA damage in terms of strand breaks (SBs) and alkaline labile sites (ALSs) were detected using the alkaline comet assay. Cells were also treated with the enzyme FPG, enabling FPG-sensitive sites to be identified (including oxidised purines such as 8-oxoguanine). After exposure, cells were harvested by centrifugation at 210 × g and 4°C for 4 min, washed with phosphate-buffered saline (PBS), centrifuged again and resuspended in 100 μl PBS. Cells were mixed with 0.75% low-melting-point agarose and embedded on a microscope slide precoated with 0.3% agarose. After solidification on a cold plate, cells were lysed in lysis buffer (1% Triton X-100, 2.5 M NaCl, 10 mM Tris, 0.1 M EDTA, pH 10) for 1 h. Slides were washed and equilibrated in enzyme buffer (0.1 M KCl, 0.5 mM EDTA, 40 mM HEPES and 0.2 mg/ml BSA, pH 7.9), 3 × 5 min and dried before FPG treatment was initiated. Thirty microlitres of FPG (for FPG treatment) or enzyme buffer (for DNA damage: SBs and ALSs) added to each gel and incubation was performed at 37°C for 30 min in a humidified atmosphere. DNA was then unwinded in alkaline solution (0.3 M NaOH, 1 mM EDTA) for 40 min, where after electrophoresis was performed for 30 min in the same, but fresh, solution (600 ml), causing cell fragments to migrate towards the anode. The slides were neutralised in EDTA twice, and milli-Q-water once, 5 min in each, dried overnight and fixedate in methanol for 5 min, all at room temperature. The FPG was diluted 1:3000 in enzyme buffer before use. Electrophoresis was performed at 1.15 V/cm in dark using a Comet electrophoresis tank (Comet-20; SCIE-PLAS, Warwickshire, UK) that was kept cold by the provided cooling system. All incubations performed in solutions were carried out in dark and on ice, unless otherwise stated.

Positive controls.

For regular comets, SBs and ALSs were induced by H2O2. For FPG treatment) or enzyme buffer (for DNA damage: SBs and ALSs). Incubation was performed for 1 h at 37°C (for FPG treatment) or 30 min in the same, but fresh, solution (600 ml), causing cell fragments to migrate towards the anode. The slides were neutralised in EDTA twice, and milli-Q-water once, 5 min in each, dried overnight and fixedate in methanol for 5 min, all at room temperature. The FPG was diluted 1:3000 in enzyme buffer before use. Electrophoresis was performed at 1.15 V/cm in dark using a Comet electrophoresis tank (Comet-20; SCIE-PLAS, Warwickshire, UK) that was kept cold by the provided cooling system. All incubations performed in solutions were carried out in dark and on ice, unless otherwise stated.

Table I. Compounds permitted in supplements by the European Parliament’s directives (2002)

<table>
<thead>
<tr>
<th>Vitamin A</th>
<th>Vitamin C</th>
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<tbody>
<tr>
<td>Retinol</td>
<td>t-α-ΑΑ</td>
</tr>
<tr>
<td>Retinyl acetate</td>
<td>Sodium t-ascorbate</td>
</tr>
<tr>
<td>RP</td>
<td>Calcium t-ascorbate</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>Potassium t-ascorbate</td>
</tr>
<tr>
<td>L-Ascorbyl-6-palmitate</td>
<td>—</td>
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</tbody>
</table>

*Not included in the present study due to it being less accessible for purchase.

| Compound | Solubility problems at higher concentrations. AA6P was, however, only analysed at 50 μM and below due to solubility problems at higher concentrations. |
200 \times g$ and 4°C, supernatant was removed and the cells were exposed to 200 μM H$_2$O$_2$ in PBS for 5 min on ice (to avoid repair of the initiated damage), in darkness. For FPG-sensitive sites, the photosensitizer Ro 19-8022 (Ro) and visible light, giving rise predominately to 8-oxoguanine (17), was used as positive control. Ro (0.4 μM) in PBS was added to the cells (harvested as above), and the cells were irradiated with a 500-W tungsten halogen lamp, for 5 min on ice. Exposures were terminated by centrifugation as above, and the cells were resuspended in PBS. The same steps as for vitamin-exposed cells were then followed (see above).

Scoring of cells: The cells were examined using a fluorescence microscope (Olympus BH-2 with a ×20 apochromatic objective) and the Komet 4.0 software (Kinetic Imaging Ltd). Fifty cells were assessed in duplicates for each run (i.e. 100 cells per sample) and % DNA in tail was used as a measure of DNA damage. FPG-sensitive sites were calculated by subtracting the value of % DNA in tail of comet not treated with enzyme from the value obtained when enzyme treatment was performed. Prior to scoring, the slides were stained with ethidium bromide (EtBr) solution (1 μg/ml in 1× TAE buffer) for 5 min at room temperature, excess EtBr was washed away in pure TAE buffer and coverslips were placed over the slides.

The presence of 0.5% ethanol or DMSO in controls did not affect the % DNA in tail as determined by statistical analysis after three repeated experiments. Therefore, controls with supplemented medium only were used throughout the comet assay experiments.

Statistical analysis
Unpaired two-tailed Student’s $t$-test with unequal variance was used for statistical analysis. For each vitamin, four to six independent experiments were conducted for dG studies, four for cytotoxicity analysis (five to seven for AA6P) and three to four for comet assay runs. Outliers were identified using the Q-test at 95% confidence interval, and the entire run was consequently removed and rerun.

### Results

#### Oxidation to dG

**Vitamin A.** The normal plasma level of retinol is in the range of 2–2.2 μM (11). In acetate buffer (with 0.03 M Zn$^{2+}$), retinol and retinal were the only vitamin A compounds to significantly increase oxidation of dG at this concentration (2 μM), as well as at 6 and 20 μM, when compared to control (Figure 1A). At the two highest concentrations, retinol acetate (RA) also induced a significant increase in 8-oxodG formation. Neither RP nor β-carotene caused any significant changes in oxidation of dG at the concentrations analysed.

When instead dissolved in phosphate buffer, in order to evaluate if buffer type affected the oxidation potency of the vitamin compounds, the effects of 2 μM retinol and retinal were significantly reduced ($P < 0.05$). Retinol still caused an increase in 8-oxodG formation when compared to control, while retinal no longer did. The control value for retinol also changed significantly between the different buffers (but not for any of the other controls). RA caused a significant increase in 8-oxodG formation even at 2 μM, although the level of 8-oxodG formed, did not statistically differ from that achieved in acetate buffer.

**Vitamin C.** With the exception of AA6P, all other vitamin C-related compounds [AA, DHAA, sodium ascorbate (SA) and calcium ascorbate (CA)] caused a significant increase in the oxidation of dG, in acetate buffer (with 0.03 M Zn$^{2+}$), when compared to controls. This was the case at vitamin concentrations equivalent to normal plasma levels of AA (50 μM), as well as at 150 and 500 μM (Figure 1B).

The pro-oxidant effects of AA and SA were further enhanced when phosphate buffer was used instead of the acetate buffer ($P < 0.05$). The mean value of dG oxidation caused by CA also increased several fold, the amount did, however, vary between runs and the increase was not statistically significant. Although AA6P did not cause a significant change in oxidation in either acetate or phosphate buffer compared to control, the level of oxidation of dG was significantly higher when the phosphate buffer was used ($P < 0.05$). None of the control values differed between the two buffers.

In order to evaluate whether the different effects by AA, SA and to some extent CA were due to the change of buffer type (acetate versus phosphate) or due to the difference in Zn$^{2+}$ content, an additional acetate buffer without Zn$^{2+}$ was used for comparison. In comparison to the acetate buffer containing 0.03 M Zn$^{2+}$, AA ($P < 0.05$), SA ($P < 0.01$) and CA ($P < 0.01$) caused significantly higher oxidation to dG in the modified acetate buffer, while the effect by DHAA was decreased ($P < 0.01$) and the effect by AA6P remained unchanged (Figure 1B). The levels were, however, not enhanced as much as in the phosphate buffer, and in comparison with the acetate buffer without Zn$^{2+}$, the use of phosphate buffer caused a statistically higher oxidation in the case of AA ($P < 0.01$), DHAA ($P < 0.05$) and SA ($P < 0.05$). A lowering of oxidation was seen by AA6P and no statistical difference for CA.

#### Cytotoxicity

Among the vitamin A compounds, only retinal affected the cell viability of HL-60 cells, and 20 μM gave rise to >90% non-viable cells (Figure 2A). AA, SA and CA were the C vitamins potent to cause cell death to cells at 500 μM and under this experimental set-up (Figure 2B). Due to methodological problems, AA6P was not analysed at 500 μM, and 50 μM did not cause a statistically significant increase in non-viable cells, although the mean value was increased from 6% non-viable cells in controls to 15%, with large variations between runs. For comet assay runs, a cytotoxicity <12% non-viable cells was accepted in an attempt to avoid difficulty when interpreting the results (e.g. are the effects dependent on each other?). Dose–response studies were therefore carried out for the vitamin compounds exceeding this limit. The following concentrations were determined to be used: 2 μM retinal (giving 7% non-viable cells), 250 μM of AA (8% non-viable cells) and SA (7% non-viable cells), 150 μM CA (8% non-viable cells) and 5 μM AA6P (7% non-viable cells).

#### DNA damage (comet assay)

None of the vitamin A or C compounds caused any DNA damage in terms of SBs and ALSs as measured by the alkaline comet assay, at the concentrations tested (Figure 3). Neither did any of the vitamin compounds cause FPG-sensitive sites, with one exception, β-carotene induced a statistically significant increase in FPG-sensitive sites (Figure 4). When the limit of 12% cytotoxicity was exceeded, SA and CA still did not cause SBs/ALSs or FPG-sensitive sites at 500 μM, while AA caused an increase of FPG-sensitive sites (from 7.7% DNA in tail in controls to 10.5%, $P < 0.05$) (data not shown). Twenty micromolars of retinal caused such a high cytotoxicity that analysis via the comet assay was not possible.

#### Discussion

Regulations from the European Parliament and Council state that a supplement containing vitamin A can include any of four, specified, different compounds (Table I). In the case of vitamin C, five different compounds are permitted. The manufacturers are not required to specify which of these compounds are present in the supplements. As a result,
consumers are generally not aware of which specific vitamin compound they are consuming. This study showed that, even though the compounds are given the same name (vitamin A or C, for chemical structures, see Figures 5 and 6), they do have different properties linked to oxidation, cytotoxicity and DNA damage. Hence, it is possible that they affect preparation, storage and metabolism of the supplements differently.

Physiological concentrations of retinol and AA were used based on normal plasma levels (9,11). We also included concentrations up to 10 times normal plasma levels since both plasma and specific tissues/cells can have much higher concentrations (11,18). Intravenous administration of AA, for therapeutic purposes, can, for example, produce plasma levels as high as 20 mM (400 times normal levels) (18).

Vitamin A’s
Two (retinol and RA) of the four vitamin A compounds permitted in supplements showed chemical oxidation potency

**Fig. 1.** Oxidation of dG by (A) vitamin A and (B) vitamin C compounds. The amount of 8-oxodG formed per $10^6$ dG by the different compounds after dG was exposed to them at 37°C for 1 h, as determined by HPLC-EC/UV. Either acetate buffer containing 0.03 M Zn$^{2+}$, phosphate buffer or acetate without Zn$^{2+}$ was used as solvent (chelex treated, pH 5.3). Each bar represents the average of a minimum of four independent experiments ± standard deviation. Statistical significance was made in relation to the respective control (0 μM vitamin); *$P<0.05$, **$P<0.01$, ***$P<0.001$. The normal plasma concentration of retinol and AA is 2 μM (11) and 50 μM (9), respectively.
while their effects on the cellular level was less dramatic, as summarised in Table II. β-Carotene induced a small but significant increase in FPG-sensitive sites, while no other vitamin A compound affected DNA damage or FPG-sensitive sites. Retinal stood out, with the property to cause strict chemical oxidation to dG as well as cytotoxicity to cells, causing almost complete cell death.

Retinal is not a constituent of vitamin A supplements, but it is a direct breakdown product and metabolite from all of the vitamin A compounds that are found in supplements (Figure 7). It is essential in vivo where it has vitamin A activity and is formed as an intermediate when retinol is oxidised to the highly bioactive retinoic acid (11). Retinal exists both as 11-cis and all-trans, the latter being the more stable isomeric form and therefore the preferred isomer formed from retinol, β-carotene, RA and RP, as well as the form used in this study. No effect on DNA damage (i.e. SBs and ALSs) or FPG-sensitive sites was seen by retinal in the present study. Retinal clearly has the ability to induce oxidation of biomolecules, such as oxidising dG to 8-oxodG, which suggests an underlying oxidative mechanism not detectable in the cell-based experiments to be behind the cytotoxicity by retinal. Furthermore, other studies have reported retinal to increase the level of 8-oxodG in cultured cells, as compared to retinol, at similar and lower concentrations to what was used in this paper (2 and 5 μM) (10).

Vitamin A toxicity (hypervitaminosis A) is well documented, during which retinoid plasma transporters become saturated, plasma levels of retinoids such as retinyl esters and retinoic acid (normally not present in plasma) are elevated and the integrity of biological membranes affected (11,19). Trypan blue staining, the cytotoxicity assay used in the present study, identifies cells whose membranes are no longer intact. Since membrane integrity is affected during vitamin A toxicity, it is possible that cytotoxicity by retinal contributes to the toxicity or that some other shared mechanisms underlie both scenarios.
As a possible secondary product from retinol, retinal was hypothesised to contribute to the chemical oxidation observed from retinol in the present study. However, retinal showed a lower potency than retinol to induce oxidation on dG, and it can be concluded that retinol itself has oxidation potency.

β-Carotene exposure gave rise to an increase in FPG-sensitive sites (including 8-oxoguanine) in cells in the present study, although the increase was small. Surprisingly, β-carotene did not oxidise dG (giving rise to 8-oxodG), indicating that the effect of β-carotene observed in cells cannot be attributed to a strict chemical oxidation. It is possible that breakdown product(s) of β-carotene caused the effect or that differences in environmental factors affected the action of β-carotene. In fact, a breakdown product from β-carotene (β-apo-8-carotenal) has been reported to cause DNA damage in retinal pigment epithelial cells (20). Other studies have shown β-carotene to have pro-oxidant properties in vitro, and circumstantial factors such as unphysiologically high oxygen tension and high β-carotene concentration are believed to shift the antioxidant pro-oxidant equilibrium towards the latter (21), suggesting that in vivo conditions might not promote pro-oxidant effects. β-Carotene has also been pinpointed as responsible for the increase in lung cancer among smokers and asbestos workers in the α-Tocopherol β-Carotene Cancer Prevention trial and the β-Carotene and Retinol Efficacy trial (6,7,21). The mechanisms behind these adverse effects are, however, still not clear but it has been indicated that breakdown products play an important role (21).

**Vitamin C’s**

All but one of the vitamin C compounds permitted in supplements were clearly potent to oxidise dG under the experimental conditions used in this study, regardless of buffer type. The same compounds (AA, SA and CA) also induced cytotoxicity of cells, while DNA damage and FPG-sensitive sites were unaffected, as summarised in Table II. DHAA, the oxidised form of AA, also oxidised dG in most buffers but did not affect the cells.

It is clear that several vitamin C compounds have the ability to oxidise molecules and hence act as pro-oxidants through chemical reactions alone. The surrounding milieu did, as observed by others (22), also highly influence this capability. When present in phosphate buffer, AA and SA produced a several-fold higher increase in the oxidation of dG when compared to acetate buffer. The oxidation potency of CA also increased, although not statistically significant, since the amount of 8-oxodG formed varied between runs. The two buffers differed in salt content and concentration, but the acetate buffer also contained 0.03 M Zn$^{2+}$, whereas the phosphate buffer did not. By including an acetate buffer without Zn$^{2+}$, it could be concluded that the presence of Zn$^{2+}$ was an important contributor to the oxidative effects by all vitamin C compounds. Some evidence has suggested that zinc ions may be able to decrease DNA damage in cell culture and in vivo, possibly by acting as an antioxidant (23), as observed with AA, SA and CA in the present study. However, Zn$^{2+}$ enhanced the DHAA-induced oxidation of dG. Since the levels of dG oxidised by AA, SA and CA in phosphate buffer were much higher than in any of the acetate buffers, other factors such as salt content [the stability of AA can be affected by the type of salt (24)] and concentration must also have influenced the differences in oxidation potency, in addition to the Zn$^{2+}$ content. AA and its analogues can take part in Fenton reactions, react with reactive oxygen species and oxidise other molecules readily. AA6P on the other hand is more stable (25) and the lack of reactivity by AA6P in the assays used in this study was therefore not unexpected.

AA, SA and CA also caused cytotoxicity of cells, CA with the strongest effect, confirming what others have previously reported concerning AA and SA (26,27). DNA damage (SBs and ALSs) and oxidative lesions (FPG-sensitive sites) were not affected by either SA or CA, indicating that the cytotoxicity by these vitamin compounds was initiated by other factor(s)/mechanisms. Due to the vitamin C compounds’ distinct chemical oxidative effect on dG, it remains possible, even for SA and CA, that oxidative mechanisms, possibly involving the oxidation of dG, not detectable under the cellular experimental conditions play a role in the cytotoxicity. In the case of AA, an induction of FPG-sensitive sites was seen at the highest concentration. Since the cytotoxicity limit set for the comet

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**Fig. 4.** Fpg-sensitive sites. The effect of (A) vitamin A and (B) vitamin C compounds on FPG-sensitive sites of HL-60 cells after 24-h exposure, as determined by an enzymatic version of the comet assay. Each bar represents the average of a minimum of three independent experiments ± standard deviation. Statistical significance was made in relation to the respective control (0 μM vitamin); *P < 0.05, **P < 0.001. The normal plasma concentration of retinol and AA is 2 μM (11) and 50 μM (9), respectively.
Vitamin A compounds

Fig. 5. Chemical structures of the vitamin A compounds analysed.

assay was exceeded at this concentration, it cannot be concluded if these oxidative lesions underlie the cytotoxicity or not, but vitamin C has been seen to cause DNA damage in other studies (22). The formation of \( \text{H}_2\text{O}_2 \) accumulation intracellularly has previously been concluded as one responsible mechanism.

Vitamin C is taken up by cells through the transport of DHAA via facilitated glucose transporters and through the transport of the reduced form of vitamin C (ascorbate) via a sodium dependent transport system (11). Since one of the major routes is through the uptake of DHAA, it was somewhat surprising that DHAA did not affect the cells in the present study while the other vitamin C compounds did. HL-60 cells have the ability to stabilise extracellular ascorbate (28) and it is possible that the sodium-dependent receptors worked with preference under the conditions in this experimental set-up and that DHAA was taken up to a lesser extent. In addition, DHAA forms a bicyclic structure in aqueous solution and predominately exists as the bicyclic hemiketal hydrate, while AA remains in the monocyclic form, contributing further to differences in their physiochemical properties (29). This might have contributed to the less pronounced effects, in comparison to AA, SA as well as CA, on both dG oxidation and cytotoxicity by DHAA.

To what extent vitamin C causes pro-oxidation \textit{in vivo} is still uncertain. Carr and Frei (30) reviewed the literature in 1999 and found evidence for \textit{in vivo} antioxidant action on lipids by vitamin C, while its effect on proteins and, to an even greater extent, DNA remained uncertain. Since then, additional studies and reviews have been conducted, but the uncertainty remains (9,18,31,32). Many authors consider that the presence of transition metals is required for vitamin C to cause oxidation, whether extra- or intracellularly (33). On the other hand, lipid
oxidation has been shown to decrease in guinea pigs following ascorbate supplementation, even in the presence of an iron overload (31). In the present study, we observed strict chemical pro-oxidative properties by several vitamin C compounds (including AA) in a chemical system where no transition metals had been added. All buffers were chelex treated to remove metal ions such as Cu$^{2+}$ and Fe$^{2+}$. Even though we made precautions to exclude transition metals, it remains possible that trace amounts were present as contaminants in chemicals and equipment used, which is also a possibility in the cellular system. Therefore, it is not possible to conclude for certain if the pro-oxidation and cytotoxicity by vitamin C compounds were aided by transition metals or not. In the cellular system, no DNA damage was, however, observed even in the presence of possible metal contaminants in both equipment and cell medium.

Strict chemical potencies of the vitamin compounds were studied by evaluating their ability to oxidise dG, thereby forming 8-oxodG, which is a pro-mutagenic lesion. If left unrepaired, the change is potentially fatal to cells. The same lesion was investigated in a cellular system, as part of FPG-sensitive sites, in an attempt to resemble a more physiological situation. In the DNA of cells, dG is clearly less accessible to attack than in the chemical experimental layout using dG, and the results obtained cannot be extrapolated to an in vivo situation. However, in case of access, there are also specific DNA-binding proteins and histones in close proximity to nucleosomal DNA, which can donate an electron to guanyl radicals (14,34). Although the in vitro cellular system is closer to an in vivo situation, it is important to note that it, too, is a model system. In addition, the HL-60 cells can be induced to differentiate by different agents, including DMSO, retinoids and AA. The concentration used for DMSO was higher (1.5–3 times) when compared to this study (35), and the exposure time of AA was shorter (six times) (36), leading to a lower differential effect by both agents in the present study. For retinoids, 1 μM of retinoic acid has been shown to
Table II. Summary of the results on vitamin A’s and C’s

<table>
<thead>
<tr>
<th>Vitamin A’s</th>
<th>Oxidation of dG&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Cytotoxicity</th>
<th>DNA damage&lt;sup&gt;b&lt;/sup&gt;</th>
<th>FPG-sensitive sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol</td>
<td>↑</td>
<td>↑</td>
<td>—</td>
<td>—</td>
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<tr>
<td>Retinyl acetate</td>
<td>↑</td>
<td>↑</td>
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<tr>
<td>RP</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>↑</td>
</tr>
<tr>
<td>Vitamin C’s</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>DHAA</td>
<td>↑</td>
<td>↑</td>
<td>—</td>
<td>—</td>
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<tr>
<td>AA</td>
<td>↑</td>
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<tr>
<td>SA</td>
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<td>CA</td>
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<td>↑</td>
<td>↓&lt;sup&gt;c&lt;/sup&gt;</td>
<td>—</td>
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<tr>
<td>AA6P</td>
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<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

The arrows (↑) indicate an increase in damage caused by the vitamin, compared to control, the Em dashes (—) corresponds to no effect. For more details, see Figures 3–6.

<sup>a</sup>Generalised, not always the case at all concentrations and in all buffers; for more details, see Figure 3.
<sup>b</sup>SBs and ALSs.
<sup>c</sup>At 500 μM, AA caused an increase in FPG-sensitive sites, possibly due to high cytotoxicity.
<sup>d</sup>Tested at a lower concentration than the other vitamin C’s (50 μM, instead of 500 μM), due to solubility problems.

to induce cytotoxicity in cultured human cells, and to some extent oxidative lesions. To investigate the mechanisms of vitamin supplements are highly relevant since there are many publications showing an increased morbidity, mortality as well as cancer risk in human populations (5–8).

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**References**


