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Can Oxidative DNA Damage be Used as a Biomarker of Cancer Risk in Humans? Problems, Resolutions and Preliminary Results from Nutritional Supplementation Studies

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Damage to DNA by oxygen radicals and other reactive oxygen/nitrogen/chlorine species occurs *in vivo* despite the presence of multiple antioxidant defence and repair systems. Such damage is thought to make a significant contribution to the age-related development of cancer. Modulation of oxidative DNA damage by diet thus constitutes a "biomarker" putatively predictive of the effect of diet on cancer incidence, provided that DNA damage can be accurately quantitated by validated methods. Current issues addressed in this article include the problems of artifactual DNA oxidation during isolation and analysis, the relative merits of different analytical methods, the advantages and disadvantages of relying on measurement of 8-hydroxydeoxyguanosine (8OHdG, 8-oxodG) as an index of oxidative DNA damage, and the limited data that are so far available on how diet can affect "steady-state" levels of oxidative DNA damage in humans. It appears that such damage can be modulated by vegetable intake, although the effects of vegetables may be mediated by components different from the "classical" antioxidants vitamin C, α-tocopherol and β-carotene.

Keywords: Oxidative DNA damage, hydroxyl radical, singlet oxygen, reactive oxygen/chlorine/nitrogen species, nitric oxide, peroxynitrite, antioxidants

WHY DO WE MEASURE OXIDATIVE DNA DAMAGE?

There is considerable evidence that damage to DNA by oxygen radicals and other reactive species occurs *in vivo*. First, low levels of base damage products have been detected in DNA isolated from all aerobic cells examined to date.[1–12] Second, multiple enzyme systems capable of repairing oxidative DNA damage exist *in vivo*, and knockout of these systems increases mutation frequency.[13–19] Third, several oxidized DNA bases can be detected in human urine.[20–22]

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Although some of them may arise from the diet, it is widely believed that urinary levels of 8-hydroxydeoxyguanosine (8OHdG) are a marker of "whole body" oxidative DNA damage.[22]

The "steady-state" levels of DNA base damage products reported in DNA isolated from mammalian cells vary over a wide range,[8,10,11] but the total is often in the range of 1 oxidation product per 10^5 DNA bases, and many published values are higher than this. One oxidized base per 10^5–10^6 "normal bases" is a significant number by comparison with the levels of other DNA lesions that are related to cancer. For example, levels of benzpyrene diol epoxide-DNA adducts in lung carcinomas of smokers ranged from 0.65 to 5.33 per 10^6 DNA bases.[23,24] If, as some studies suggest, the true steady-state levels of DNA base oxidation products are higher (Table I) then the risk of mutation would be expected to be correspondingly greater, although this is still an area of debate. For example, much oxidative damage could conceivably reside in non-expressed DNA, both "junk DNA" and in silent genes. The existence of transcription-related repair mechanisms (reviewed in Ref. [19]) suggests that a particular effort may be made to remove errors when DNA is being unwound for transcription and perhaps for replication.

Hence it is widely believed that ongoing damage to DNA by "reactive species" is a significant contributor to the age-related development of cancer.[2,5,7,8,28–34] It follows that diets or dietary supplements that decreased such damage should have an anticancer effect.[35] In addition, direct chemical modification of DNA is only one of the mechanisms by which reactive species can lead to mutations or promote carcinogenesis. Some reactive species (e.g. H_2O_2, $O_2^{\bullet -}$) at low levels can promote cell proliferation.[36] Oxidation of lipids induced by reactive species can generate end-products, such as malondialdehyde and unsaturated aldehydes, that can bind to DNA to generate mutagenic adducts.[37–39] Damage to DNA polymerases and DNA repair enzymes by reactive species might raise error rates during DNA replication, and slow down repair processes.[33,34,40,41]

SOURCES OF OXIDATIVE DNA DAMAGE

The diatomic oxygen molecule, O_2, is a free radical.[1] Oxygen is insufficiently reactive to oxidize DNA directly at a measurable rate at body temperature, in part because its two unpaired electrons have parallel spin and this "spin restriction" precludes direct acceptance of two electrons by O_2.[1] However, oxidation will occur given long enough, and this is an enormous problem in attempts to use PCR to amplify "ancient

TABLE I GC–MS and HPLC measurement of 8-OH-guanine in commercial calf thymus DNA after acid hydrolysis (values were converted when needed on the basis of 1 nmol/mg of DNA = 318/10^6 bases, 1 ng of 8-OH guanine = 5.98 pmol and each base is 25% of the total in DNA. HPLC determination of 8-hydroxy-2-deoxyguanosine after enzymic hydrolysis of commercial calf thymus DNA has resulted in values of 23.4–1006 pmol/mg of DNA in the literature)

Analytical technique	8-OH-guanine (pmol/mg of DNA)	Derivatization conditions
HPLC	53	No derivatization, electrochemical detection
GC–MS	43	30 min, derivatization under argon at 23°C
	336	30 min, derivatization under argon at 140°C
	500–1000	30 min, derivatization under N_2, at 130°C
	45–53	30 min, 130°C, pre-purification
	2426	30 min, 130°C, no removal of air
	210	60 min, derivatization under N_2, at 90°C
	56	2 h, derivatization under N_2 at 23°C in presence of ethanethiol

Note: Levels using FPG protein-induced nicks in cellular DNA give 0.007–0.38 lesions/10^6 base pairs.[10,26] Values using the comet assay in human lymphocytes were 0.03/10^5 dG compared with 0.43/10^5 dG for the same samples analyzed by HPLC.[27] Table modified from Ref. [25].

DNA",[42] where considerable oxidation will have occurred over centuries. Some products of oxygen reduction, such as superoxide radical ($O_2^{\bullet -}$) and the non-radical hydrogen peroxide (H_2O_2) also seem incapable of reacting with DNA directly, except perhaps at levels way above their physiological range (reviewed in Refs. [2, 11]). This is not surprising given the generally-poor reactivity of $O_2^{\bullet -}$ in aqueous solution,[3] and is perhaps fortunate, since it is often suggested that 1–3% of the O_2 we breathe in forms $O_2^{\bullet -}$ and H_2O_2. One per cent of basal human O_2 consumption is ~150 mmol/day, a substantial amount.[43]

By contrast, the singlet states of O_2 readily oxidize guanine, the most oxidizable base in DNA.[6,44,45] Hydroxyl radical, $OH^{\bullet}$, generates a multiplicity of products from all four DNA bases.[2,3,9,46–49] For example, $OH^{\bullet}$ can attack guanine at several positions.[3,9] Figure 1 shows how attack of $OH^{\cdot}$ at position 8 generates an 8-hydroxyguanine radical (C8–OH adduct radical) that can have various fates depending on the redox state of the environment. Anoxic conditions and the presence of reducing agents favour reduction and ring opening, whereas the amount of 8-hydroxyguanine produced by a given amount of free radical attack on C8 of guanine in DNA rises as

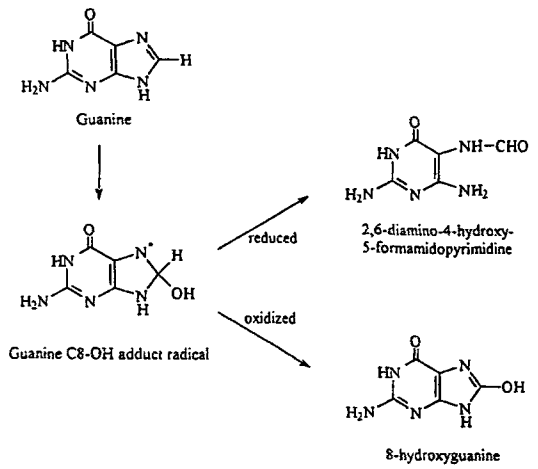

FIGURE 1 Formation and fate of the radical generated by attack of hydroxyl radical at position 8 of the purine base guanine. Oxidation generates 8-hydroxyguanine, whereas reduction steps lead to a ring-opened product.

O_2 levels increase.[49] Figure 2 shows the structures of some of the many other oxidation products generated when DNA is exposed to $OH^{\bullet}$.

Addition of H_2O_2 to mammalian cells often produces DNA damage, usually assessed by strand breakage (reviewed in Ref. [2]). However, H_2O_2 is not thought to react directly with DNA. Analysis of the pattern of damage to the purines and pyrimidines of DNA isolated from H_2O_2-treated cells confirms that some or all of this damage is caused by conversion of H_2O_2 to $OH^{\bullet}$ within the cell nucleus.[2,4,142] By contrast, the DNA strand breakage observed in a leukaemic cell line treated with therapeutic levels of the anthracycline antibiotic doxorubicin did not involve $OH^{\bullet}$, since levels of the DNA base oxidation productions typical of $OH^{\bullet}$ did not increase.[50]

Other reactive species cause different patterns of damage to DNA. This has been established for singlet O_2,[6,26,44] $ONOO^-$,[51,52] peroxyl radicals,[53] hypochlorous acid, HOCl[54] and nitryl chloride, NO_2Cl.[55] Hence it is possible to use the pattern of chemical damage observed in the bases of DNA isolated from cells and tissues in order to gain information about which reactive species could have caused DNA damage *in vivo*.[2] This can be illustrated by studies on neurodegenerative disease. For example, DNA isolated from the brains of patients with senile dementia of the Alzheimer type shows a pattern of purine and pyrimidine damage suggestive of attack by $OH^{\bullet}$,[56] whereas in senile dementia of the Lewy body type elevations in deamination products, suggestive of attack by reactive nitrogen species, are observed in addition.[57] By contrast, in Parkinson's disease there is a selective oxidation of guanine, ruling out $OH^{\bullet}$ as a damaging species.[58]

Nitric Oxide and its Derivatives

Although nitric oxide is a free radical, $NO^{\bullet}$, it may be insufficiently reactive to attack DNA directly.[51,52] By contrast, oxidation products of $NO^{\bullet}$, such as N_2O_3, HNO_2 and $ONOO^-$,

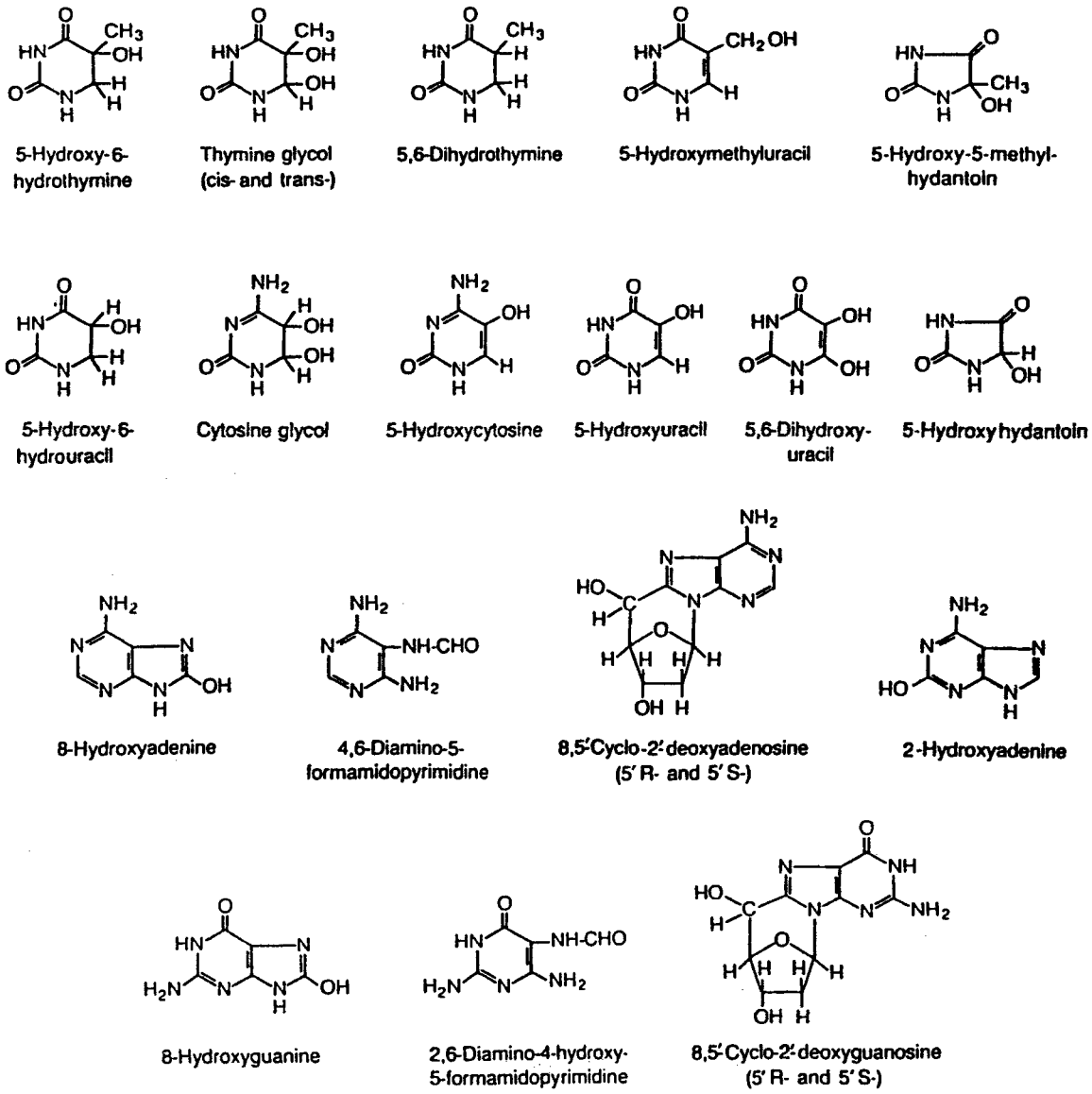

FIGURE 2 Structure of some of the oxidation products of DNA. These modified bases (except for 5,6-dihydrothymine, a product of attack on thymine by hydrogen radicals or hydrated electrons) are formed in DNA subjected to attack by hydroxyl radicals.

can nitrate and/or deaminate DNA and cause strand breakage and mutations. For example, $ONOO^-$ can induce G → T transversions whereas deamination of adenine to hypoxanthine can result in transitions (AT → GC) since hypoxanthine can pair with cytosine.[59–68] Spontaneous deamination reactions also occur in DNA over the long human lifespan.[42] Living organisms have therefore evolved enzymes that can remove deamination products of cytosine (uracil), adenine (hypoxanthine) and guanine (xanthine) from DNA to decrease the risk of mutagenicity.[13–15]

Estimates of total daily production of oxides of nitrogen in the healthy human body are about 1 mmol/day (compare this with ~150 mmol/day of reactive oxygen species: see above), based on steady-state levels of plasma NO_3^- and NO_2^- in subjects placed on diets free of these substances.[69] Also, generation of oxides of nitrogen in the stomach by reaction of salivary (and dietary) NO_2^- with gastric acid, initially to form HNO_2, may be an important antibacterial mechanism.[70] However, excess production of "reactive nitrogen species", e.g. as a result of *H. pylori* infection, chronic inflammation or excessive consumption of NO_2^--rich foods, may enhance the risk of gastrointestinal cancer.[64,68]

STEADY-STATE LEVELS OF DNA DAMAGE IN HUMAN TISSUES: REAL OR ARTIFACT?

If so many different DNA repair systems exist *in vivo*, why should there be any damage found in DNA isolated from human tissues? The obvious possibility, that the damage is not real but is created during the extraction and/or analysis of the DNA, must be examined seriously. Damage could occur if

(1) DNA is oxidized during isolation,
(2) DNA is oxidized during preparation for analysis,
(3) DNA is oxidized during the analysis itself.

Procedures such as phenol extraction, dialysis of isolated DNA, storage of tissues prior to DNA extraction, and nuclease digestion of DNA to liberate oxidized bases for HPLC analysis could all conceivably result in artifactual oxidation, to an extent that may vary widely between laboratories. For example, data from my own laboratory show that phenol does not increase oxidative modification during DNA isolation from human tissues, whereas data from some other laboratories suggest the opposite.[71] Such variation may arise from background levels of contaminating transition metal ions in water supplies and reagents, use of partially-oxidized phenol etc.[1,71–74] There is a wide variation in levels of reported base modifications in DNA between different laboratories[11] and sometimes from the same laboratory at different times.[8] Most laboratories studying DNA oxidation use HPLC analysis of enzymically-hydrolyzed DNA to examine levels of 8OHdG, but steady-state levels reported from different laboratories, ostensibly using the same method, vary over almost an order of magnitude (see papers in this special issue). Some laboratories use gas chromatography-mass spectrometry (GC/MS) to measure 8-hydroxyguanine and other base modification products in DNA after acid hydrolysis.[9–12,46,47,75,76] The acid hydrolysis removes the deoxyribose from the base, so that 8-hydroxyguanine (8OHG) is measured instead of 8OHdG (Figure 3 explains the nomenclature in detail). Levels of 8OHG in mammalian DNA as measured by GC/MS have usually been greater, and sometimes much greater, than those measured as 8OHdG by HPLC.[11] Table I gives some illustrative data. Levels of oxidized DNA bases measured in cells by enzyme digestion assays, including the comet assay[10,27] tend to be lower than those revealed by HPLC, whereas ^{32}P-postlabelling methods give higher values than HPLC,[77,78] although damage to DNA by the radioactive isotope must be considered.[79] Another factor that must be taken into account in using GC/MS is that any RNA contaminating the DNA will be hydrolysed by acid, so that some RNA oxidation products, including 8OHG, could be counted as DNA oxidation products. Hence DNA for GC/MS analysis must first be freed of RNA, by ribonuclease treatment. Another problem is that results of different methods are often expressed in different terms. Table II gives some conversion factors that may prove helpful.

Nevertheless, even with the most elaborate precautions taken to date, and assuming that the lowest values of steady-state levels of oxidative damage reported in DNA isolated from human cells and tissues are correct (an issue that can be

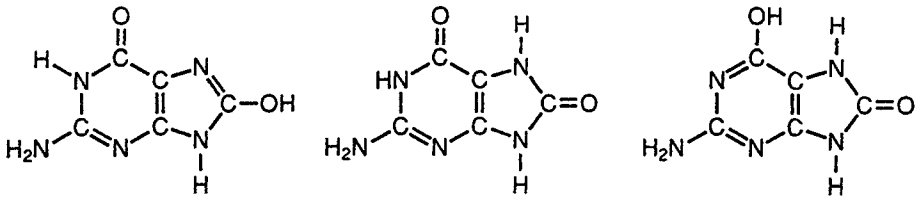

FIGURE 3 Chemical structure of the base 8-hydroxyguanine. 8-Hydroxydeoxyguanosine is the nucleoside, i.e. 8-hydroxyguanine with 2'-deoxyribose still attached to it. The base undergoes keto-enol tautomerism, which favours the 6,8-diketo form. Hence 8-hydroxyguanine is often called 8-oxo-7-hydroguanine, or 8-oxo-guanine for short. The nucleoside would be 8-oxo-7-hydrodeoxyguanosine, abbreviated to 8-oxodeoxyguanosine.

TABLE II Some conversion factors for assessing DNA damage

1 nmol of 8OHdG per mg DNA is
– 10^3 pmol 8OHdG/mg
– 318 8OHdG/10^6 DNA bases
1 8OHdG per 10^5 guanines in DNA is
– 2.2 8OHdG/10^6 DNA bases
1 8OHdG/10^6 bases is about 6000 8OHdG per cell assuming 3×10^9 base pairs per cell

Note: The advice of Dr. Miral Dizdaroglu in establishing these conversion factors is gratefully acknowledged.

debated, since there is no a priori reason to rule out the possibility that the assays that give the lowest numbers are simply failing to detect some of the lesions present), the numbers of base oxidation products measured (Table I) are still consistent with the concept that oxidation is a major cause of "spontaneous" mutation and a major contributor to the age-related development of cancer.

IS HYDROXYLATED GUANINE IN CELLULAR DNA OR IN URINE A VALID BIOMARKER OF OXIDATIVE DNA DAMAGE?

Since its introduction by the pioneering work of Kasai *et al.*,[7,80] aided by the development of a simple electrochemical detection method linked to HPLC,[81] the measurement of 8OHdG (or 8-oxodG: the nomenclature is reviewed in Figure 3) has become the most widely-used technique for assessing oxidative DNA damage. Factors supporting its measurement include

(i) the availability of a sensitive assay,[81]
(ii) its formation in DNA by a range of reactive species, e.g. $OH^{\bullet}$ and $^1O_2$,[6,46]
(iii) the likely importance of this lesion *in vivo*, as reflected by its known mutagenicity in inducing transversions, as well as the multiple mechanisms that appear to have evolved to remove it from DNA, or prevent its incorporation into DNA.[5,7,17,18]

Nevertheless, some points must be borne in mind when interpreting the results of 8OHdG measurements, as summarized in Figure 4.

(1) *Levels of 8OHdG in DNA are a balance between formation and repair*, e.g. 8OHdG levels could rise not only because of a rise in oxidative DNA damage, but also because of a decline in repair rate. The measurement of urinary 8OHdG excretion, as an assessment of "whole body" DNA damage[82] could be combined with 8OHdG measurements in cellular DNA to approach this question. For example, administration of 2-nitropropane to rats led to elevated tissue levels of 8OHdG, which then decreased, accompanied by a rise in urinary 8OHdG excretion.[82] Smoking raises 8OHdG levels in both cellular DNA[83] and in urine.[22] Hence both nitropropane and cigarette smoke produce rises in steady-state

PROBLEMS WITH 8-HYDROXYDEOXYGUANOSINE (8-OXODEOXYGUANOSINE)

- A balance between repair and synthesis
- Not generated in significant amounts by several reactive species (e.g. HOCl, NO_2Cl, $ONOO^-$)
- Destroyed by several reactive species (e.g. HOCl, $ONOO^-$)
- Formation from [8OHdG]* affected by redox state of cell
- Oxidative DNA damage not always reflected in rises in 8OHdG in nutritional supplementation studies.

FIGURE 4 Some of the points to consider when interpreting levels of 8-hydroxydeoxyguanosine in DNA.

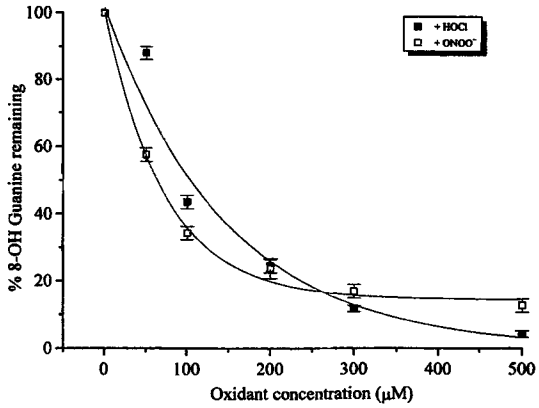

FIGURE 5 Effect of hypochlorous acid or peroxynitrite on levels of 8-hydroxyguanine in DNA. DNA (1.0 mg/ml) in 50 mM K_2HPO_4-KH_2PO_4 buffer pH 7.4 was exposed to an $OH^\bullet$ generating system (H_2O_2 [2.8 mM]/$CuCl_2$ [100 μM]/ascorbate [100 μM]) for 1 h at 37°C. After dialysis with water for 24 h, the oxidised DNA solution was made to 0.5 mg/ml in 100 mM K_2HPO_4–KH_2PO_4 buffer pH 7.4 and incubated at 37°C for 15 min. After this time either HOCl or $ONOO^-$ was added and the mixture incubated for a further hour before dialysis against water for 24 h. DNA exposed to the $OH^\bullet$ generating system typically contained between 40–50 nmol 8-OH guanine/mg DNA. The addition of "decomposed" $ONOO^-$ did not affect the levels of 8-OH guanine. Data courtesy of Dr. Matthew Whiteman.

levels of oxidative DNA damage *in vivo*, which are not due to decreased repair. Indeed, the rate of repair (at least as indicated by 8OHdG excretion) has accelerated, although not to the extent that could prevent a rise in steady-state 8OHdG levels. Administration of a homogenate of cooked Brussels sprouts to rats decreased both urinary 8OHdG excretion and the levels of 8OHdG in kidney DNA,[84] suggestive of a real fall in net oxidative DNA damage induced by this vegetable. Endurance exercise decreased 8OHdG levels in lymphocytes and colon in dogs,[85] but has been reported to increase urinary 8OHdG excretion in humans and other animals,[86] suggestive of increased repair activity. Indeed, regular physical exercise may stimulate DNA repair.[87]

In summary, *observations of steady-state levels, or of urinary excretion rates of 8OHdG, alone should be interpreted with caution*. For example, an agent that increased 8OHdG excretion rates might be interpreted as "bad" (being thought to increase DNA damage), but it might in fact be "good" (if it stimulated repair and so decreased steady-state 8OHdG levels in DNA).

(2) *8OHdG levels are not always a true marker of oxidative attack on guanine residues in DNA. They can be affected by the redox state of the cell and the mixture of reactive species present*. Attack on DNA by species such as $OH^\bullet$, $^1O_2$, $RO_2^\bullet$, $ONOO^-$, NO_2Cl and HOCl produces a multiplicity of products. For the last four species, 8OHdG is not one of the major products formed.[51–55,65] Indeed, $ONOO^-$ and HOCl can destroy pre-formed 8OHdG in DNA, since 8OHdG is easily oxidizable (Figure 5).

Hydroxyl radical can attack guanine at several positions. Attack at C8 generates an intermediate radical that can be oxidized or reduced (Figure 1). Only if oxidation takes place will 8OHdG be formed. Hence the amount of 8OHdG resulting from a fixed amount of free radical attack on C-8 of guanine in DNA will vary depending on the redox state of the cell, e.g. it will be lower under hypoxic conditions.[49] The ratio is also affected by the presence of transition metal ions under oxic conditions, e.g. copper ions cause more oxidation than iron ions.[141] An example of the former may occur in Parkinson's disease, where an apparent rise in 8OHG is matched by a fall in FApy-guanine levels, so that the sum of oxidative damage products of guanine is approximately normal in most parkinsonian brain regions.[58] These changes could result from a redox shift in the brain towards more oxidizing conditions. The existence of such a shift is supported by significant falls in levels of GSH and rises in other oxidative damage products in Parkinson's disease.[88,89]

Another important factor is that 8OHG/8OHdG are themselves subject to oxidation. Figure 5 shows what happens to levels of 8OHG in DNA treated with HOCl or $ONOO^-$: there is a concentration-dependent loss of measurable 8OHG. This is perhaps not unexpected. Guanine is the most oxidizable base in DNA, especially if adjacent to another guanine.[90] Both 8OHdG and 8OHG are more oxidizable than guanine itself; indeed, this allows their electrochemical detection, without interference by guanine or guanosine, at low voltages.[81] Given that at sites of, for example, chronic inflammation, multiple reactive species are generated, it is quite feasible for some 8OHG to be lost. Preliminary evidence has been obtained that this can occur in white blood cell DNA in patients suffering from rheumatoid arthritis.[55]

(3) *Oxidative DNA damage can occur without changes in 8OHdG, and can increase even when 8OHdG levels fall.* Podmore *et al.*[91] showed that administration of vitamin C (500 mg daily) to healthy human volunteers raised levels of 8-hydroxy-adenine, but *decreased* levels of 8OHG, in lymphocyte DNA, i.e. the effect of ascorbate on oxidative DNA damage could have been misinterpreted if only 8OHG had been measured. A more striking example is provided by a study[92] of the effects of supplementation of healthy well-nourished humans with iron (as ferrous sulphate, $FeSO_4$) plus ascorbate (Table III). The supplementation had no significant effect on levels of 8OHG in white blood cells at 6 weeks, and tended to decrease it at 12 weeks (arrows in Table III). Even at 6 weeks, the supplementation decreased levels of some bases, e.g. 5-chlorocytosine (measured as 5-chlorouracil in these experiments). 5-Chlorocytosine is a product of attack on DNA by reactive chlorine species and its fall is presumably because ascorbate is a good scavenger of such species.[93] However, levels of the hydantoins and FAPy-guanine rose markedly at 6 weeks, as did the total base damage to DNA.

Table IV shows data from another study, the effect on oxidative DNA damage in human white cells of a triple supplementation of healthy well-nourished subjects, with ascorbate, α-tocopherol and β-carotene.[94] Again, levels of hydantoins rose after 4 weeks but normalized on continued supplementation.

TABLE III The effects of supplementation of healthy, well-nourished volunteers with 14 mg iron (as $FeSO_4$) and either 60 mg vitamin C (Group A) or 260 mg vitamin C (Group B) for 12 weeks on DNA base damage in white cells

Base product	Group A (nmol/mg DNA)			Group B (nmol/mg DNA)		
	0 wk	6 wk	12 wk	0 wk	6 wk	12 wk
5-Cl Uracil	0.06 ± 0.020	0.01 ± 0.010*	0.005 ± 0.0033*	0.06 ± 0.027	0.02 ± 0.011*	0.01 ± 0.011*
5-OH Me Hydantoin	0.22 ± 0.087	1.0 ± 0.43*	0.13 ± 0.054*	0.23 ± 0.084	1.1 ± 0.31*	0.16 ± 0.13
5-OH Hydantoin	0.12 ± 0.044	0.51 ± 0.21*	0.09 ± 0.044	0.12 ± 0.044	0.59 ± 0.24*	0.08 ± 0.025
5-OH Uracil	0.11 ± 0.039	0.05 ± 0.016*	0.04 ± 0.011*	0.11 ± 0.024	0.05 ± 0.0077*	0.04 ± 0.013
5-OH Me Uracil	0.03 ± 0.014	0.02 ± 0.0077	0.05 ± 0.012*	0.04 ± 0.012	0.02 ± 0.0085*	0.05 ± 0.011
5-OH Cytosine	0.10 ± 0.019	0.05 ± 0.014*	0.15 ± 0.054*	0.09 ± 0.013	0.05 ± 0.026*	0.12 ± 0.034*
Thymine glycol (cis)	0.35 ± 0.22	0.25 ± 0.10	0.87 ± 0.45*	0.30 ± 0.15	0.30 ± 0.087	0.73 ± 0.20*
Thymine glycol (trans)†	0.006 ± 0.0048	0.08 ± 0.028*	0.15 ± 0.099*	0.008 ± 0.0055	0.06 ± 0.041*	0.10 ± 0.073*
FAPy Adenine	0.54 ± 0.68	0.68 ± 0.35	1.1 ± 0.29	0.69 ± 0.72	0.95 ± 0.49	0.97 ± 0.2
8-OH Adenine	0.33 ± 0.17	0.17 ± 0.079	0.08 ± 0.049*	0.40 ± 0.27	0.18 ± 0.072*	0.07 ± 0.031*
2-OH Adenine	0.15 ± 0.077	0.20 ± 0.066	0.14 ± 0.11	0.10 ± 0.060	0.21 ± 0.064*	0.11 ± 0.024
FAPy Guanine	0.33 ± 0.22	1.8 ± 0.98*	0.24 ± 0.13	0.32 ± 0.23	1.6 ± 0.50*	0.21 ± 0.048
8-OH Guanine	0.24 ± 0.087	0.31 ± 0.081	0.12 ± 0.034*]←	0.23 ± 0.11	0.34 ± 0.12	0.15 ± 0.067]←
Total Base Damage	2.6 ± 0.77	5.2 ± 1.9*	3.1 ± 0.87	2.7 ± 0.98	5.6 ± 1.2*	2.8 ± 0.39

Note: Mean values ±SD, $n = 9$ or 10 in both groups.
* Statistically significant difference from 0 wk, $p < 0.05$ paired t-test. Data selected from Ref. [92].
† Acidic hydrolysis causes formation of *trans* from *cis*-thymine glycol, so these should probably be considered as a total.

TABLE IV The effects of supplementation of healthy, well-nourished volunteers with a mixture of ascorbate, α-tocopherol and β-carotene on levels of DNA base damage in white cells

Base product nmol/mg DNA	Day 0	Day 28	Day 56
5-OH Hydantoin	0.136 ± 0.115	0.247 ± 0.178*	0.114 ± 0.088
5-OH Me, Hydantoin	0.182 ± 0.137	0.363 ± 0.303*	0.198 ± 0.172
5-OH Uracil	0.032 ± 0.039	0.056 ± 0.036	0.039 ± 0.032*
5-OH, Me Uracil	0.019 ± 0.018	0.020 ± 0.014	0.017 ± 0.019
5-OH Cytosine	0.123 ± 0.101	0.201 ± 0.175	0.126 ± 0.127
Thymine Glycol	0.312 ± 0.268	0.508 ± 0.405	0.388 ± 0.254
FAPy Adenine	0.091 ± 0.090	0.260 ± 0.265	0.090 ± 0.090
8-OH Adenine	0.112 ± 0.090	0.157 ± 0.152	0.074 ± 0.069
2-OH Adenine	0.167 ± 0.097	0.102 ± 0.1	0.086 ± 0.054*
FAPy Guanine	0.340 ± 0.277	0.297 ± 0.288	0.224 ± 0.224
8-OH Guanine	0.146 ± 0.122	0.122 ± 0.084	0.103 ± 0.101
Total measured base products	1.76 ± 0.61	2.63 ± 1.28*	1.67 ± 0.85

Note: Data selected from Ref. [94]. * Significant changes.

The biological significance of these various rises in DNA base damage products is uncertain, but they show that relying on 8OHG alone as a marker can lead to erroneous conclusions about changes in oxidative DNA damage. The same may be true for urinary measurements: treatment of patients with adriamycin elevated excretion of 5-(hydroxymethyl) uracil but not that of 8OHdG.[96]

Can Studies of Urinary Base Excretion Help?

The steady-state level of oxidized bases in cellular DNA is a balance of damage and repair rates. The total extent of damage can, in principle, be assessed by measuring the amounts of lesions that are excised from DNA by repair processes. This has most often been attempted by urinary measurements of 8OHdG, but the validity of such studies must be considered in the light of Ref. [96]. Also, accurate analysis is not easy (see papers by Loft and Poulsen, this volume). Several other DNA base damage products are excreted in human urine, including 8-hydroxyadenine, 8OHG and 7-methyl-8-hydroxyguanine.[20] Some of these (especially 8OHG), may arise in whole or in part from diet, especially as cooking of food is likely to oxidize DNA, and oxidized bases may be absorbed after hydrolysis of DNA in the gastrointestinal tract. The level of 8OHdG in urine is presumably unaffected by the diet since nucleosides would not be expected to be absorbed from the gut, although no detailed study on this point in humans has been published. Similarly, the question of whether any 8OHdG is metabolized to other products in humans has not been rigorously addressed in the literature. Additionally, it is possible that some or all of the 8OHdG excreted in urine may arise not from DNA, but from deoxyGTP (dGTP) in the DNA precursor pool of nucleotides.[17] Because of these uncertainties, several research groups are attempting to develop alternative urinary markers of total body oxidative DNA damage. Cells supplied with oxidized bases other than 8OHdG can also mistakenly incorporate them into DNA.[95]

GAS CHROMATOGRAPHY/ MASS SPECTROMETRY: THE ARTIFACT PROBLEM

All methods of assessing oxidative DNA damage could suffer from artifacts. Artifactual oxidative damage to DNA could occur during DNA isolation (see above) and during the enzymic

digestions used to liberate 8OHdG for HPLC analysis. The question as to whether such digestions routinely liberate all the 8OHdG from DNA is also not completely resolved. Indeed, the discussions of artifactual oxidation of DNA during sample preparation that have taken place have largely focussed on GC/MS[97–100]. The unspoken assumption seems to be that HPLC analysis of 8OHdG in enzymically-hydrolyzed DNA produces the "correct" answer and that GC/MS must be "wrong" because its values for 8OHG are sometimes higher. The wide variation in values obtained by HPLC between different laboratories (e.g. as revealed by the ESCODD study; see the paper by Lunec in this issue) show that currently used HPLC-techniques are far from being a "gold standard" for validation of other techniques (also see legend to Table I). Other methods of analysis, such as ^{32}P post-labelling, and the comet assay combined with the use of repair enzymes, give answers that are different yet again. For example, the latter produce estimates of base oxidation products lower than those achieved by HPLC analysis in the same laboratory (Table I).

Some base oxidation products can be chemically-modified during the acid hydrolysis procedures used to prepare DNA for GC/MS,[9,75] e.g. a recent example is the conversion of 5-chlorocytosine into 5-chlorouracil.[54] Calibration techniques, e.g. using stable isotopes, can be used to allow for this.[101–104] The derivatization procedure can also be a source of artifacts. It is often carried out at high temperature, and failure to exclude air under these conditions will predictably result in absurdly high levels of base oxidation products,[98–100] since heating DNA bases at high temperatures is bound to cause oxidation. GC/MS as usually carried out does attempt to exclude O_2 during derivatization.[9,75] Nevertheless, a detailed study suggests that routine N_2-flushing procedures can leave sufficient residual O_2 for artifactual formation of some oxidized bases to be a problem,[25,105] at least in our laboratory. Of the many base oxidation products that can be measured by GC/MS (Figure 2), problems of artifactual oxidation of the parent base have only been identified to date with 8OHG, 8-hydroxyadenine, 5-(hydroxymethyl)uracil and 5-hydroxycytosine. Their artifactual formation can be minimized, but is not always prevented, by carrying out derivatization at room temperature.[25,105] It could presumably be prevented by more-rigorous exclusion of air, and the extent of the "artifact" may well vary between laboratories, depending on how rigorously air is excluded from the derivatization reaction mixture.

An alternative procedure is to remove undamaged bases before derivatization, since if unoxidized DNA bases are first removed, they cannot be oxidized during derivatization.[99] Their removal can be achieved by HPLC prepurification or, in the case of guanine, by guanase digestion. We have found that[25] derivatization at room temperature in the presence of ethanethiol seems to eliminate artifacts, measuring levels of 8OHG comparable to or less than levels of 8OHdG measured by HPLC or by GC/MS with prepurification to remove undamaged bases (Table I). Hong *et al.*[106] used N-phenyl-1-naphthylamine as an antioxidant to suppress derivatization artifacts. In the recent ESCODD inter-laboratory study (Lunec *et al.*, this volume) our values of 8OHG in animal DNA were lower than those recorded by many laboratories using HPLC to analyze the same blinded DNA samples. The ultimate solution will probably be the elimination of derivatization procedures by moving to LC-MS technology, which is under development in several laboratories (e.g. Ref. [107]) or to combine acidic hydrolysis of DNA with HPLC analysis.[108]

USE OF OXIDATIVE DNA DAMAGE AS A "BIOMARKER" TO IDENTIFY DIETARY "ANTI-CANCER" AGENTS

There is considerable evidence that the endogenous antioxidants synthesized by aerobes (e.g. SOD, catalase, GSH) do not completely prevent

damage by reactive species *in vivo*.[1,35,43] Hence efficient repair systems are needed. Repair of the damage done to DNA by reactive species is particularly important, as the constant assault by these species on DNA throughout the long human lifespan may contribute to the age-related development of cancers.[5,7,8,21,28–34]

Humans also obtain certain antioxidants from the diet. The physiological role of some of these is well-established (e.g. vitamin E), whereas the antioxidant role of others (e.g. the carotenoids) is uncertain as yet.[109] Some of these antioxidants can be made to exert pro-oxidant effects *in vitro*, often by interaction with transition metal ions. This is well-known for ascorbate: mixtures of ascorbate with iron salts have been used for decades to induce lipid peroxidation and other free radical reactions *in vitro* (reviewed in Ref. [110]). Several plant phenols can also interact with transition metals to produce pro-oxidant effects *in vitro*.[111–113] The physiological role (if any) of these effects is uncertain, it would presumably depend on the availability of "catalytic" metal ions.[114] This relates to another important nutritional question; what is the optimal intake of iron? Iron is essential for human health, especially in children and pregnant women, but could too much iron intake cause harm? In the healthy human body, metal ions appear to be largely sequestered into forms unable to catalyse free radical reactions.[114] Hence the antioxidant properties of ascorbate (and any plant phenolics that are absorbed) are usually thought to predominate over pro-oxidant effects.[110,114,115] However this has not been experimentally proven and the data in Table III are suggestive of pro-oxidant effects of Fe/ascorbate *in vivo* under certain circumstances. Similarly, although β-carotene can exert a range of antioxidant effects *in vitro*,[109] its actions in epidemiological intervention studies have been disappointing.[116,117]

If we accept that oxidative DNA damage is a significant contributor to the age-related development of cancer, then levels of oxidative DNA damage could be used as a "biomarker" of cancer risk. In principle, one should be able to measure levels of oxidative DNA damage in humans and examine how they are affected by changes in diet such as alterations in fruit and vegetable intake changes in consumption of saturated/polyunsaturated fats or supplementation with antioxidants (pure compounds, whole foods or even "antioxidant" herbal extracts). The optimal intake could then be determined, as could the biological relevance of putative pro-oxidant effects.[35] Such work is just beginning and obviously relies on the further development of methodology. The availability of human tissues from which DNA can be isolated is restricted: placenta, foetal material, white blood cells, skin, buccal cells, nasal epithelial cells, cells from lung lavage fluids, spermatozoa, and various tissue biopsy samples. Nevertheless, some tentative conclusions are already emerging.

(1) *There is no convincing evidence that β-carotene decreases oxidative DNA damage in humans*[118] (Table IV). Any beneficial effects of carotenoids may be exerted by alternative mechanisms,[119,120] or perhaps β-carotene is not an important anticancer agent in humans, other than as a precursor of vitamin A.[121]

(2) *Vegetables may decrease oxidative DNA damage by mechanisms unrelated to their content of β-carotene or vitamins E and C*.[123–128] The Brussel sprout is the only example to be published in detail to date.[122,123]

(3) *Dietary antioxidants may be beneficial only up to a point*. For example, the studies in Table III suggest that giving ascorbate/iron supplements to healthy subjects with high plasma levels of vitamin C increased oxidative DNA damage. However, the fact that levels normalized on continued supplementation is intriguing and perhaps suggestive of upregulation of protective mechanisms, e.g. improved sequestration of iron and/or more effective DNA repair. Is this perhaps analogous to the ability of some vegetable constituents to upregulate xenobiotic-metabolizing enzymes and thus decrease the impact of chemical carcinogens?[126–128] However, subjects

with lower plasma levels of vitamin C at the beginning of the study showed *decreases* in oxidative DNA damage with $FeSO_4$/ascorbate supplementation.[92] Supplementation of Scottish smokers who had low plasma ascorbate levels (< 30 μM) with a mixture of vitamins C, E and β-carotene decreased levels of oxidized bases in lymphocyte DNA,[129] but much less effect was observed in non-smokers (mean ascorbate > 35 μM). Of course, one cannot identify the protective constituent(s) in this combined supplementation.

Hence there may be an optimal body content of ascorbate that minimizes DNA damage and higher intakes (alone or in combination with iron) may give no additional benefit and may even be deleterious. One value of the "biomarker" approach is that it can identify what may be the optimal doses of antioxidants, and the best populations, on which to perform full-scale intervention trials.

ANTIOXIDANT ACTIONS IN THE GASTROINTESTINAL TRACT

Antioxidants may have protective actions not only after their absorption into the body, but also before that, in the gastrointestinal tract itself. For example salivary and diet-derived NO_2^- presumably react with gastric acid to produce HNO_2, which decomposes to oxides of nitrogen. Figure 6 shows the ability of HNO_2 to deaminate DNA bases: guanine is most sensitive, followed by adenine. Several phenolic compounds are powerful inhibitors of deamination, much more effective than is ascorbate.[130] Hence phenols in fruits, vegetables, wines, tea and other beverages could conceivably exert a gastro-protective effect in situations of excess production of reactive nitrogen species.[130,131] This assumes of course, that any products (e.g. nitrated phenols) resulting from their interactions with such species are not themselves toxic.

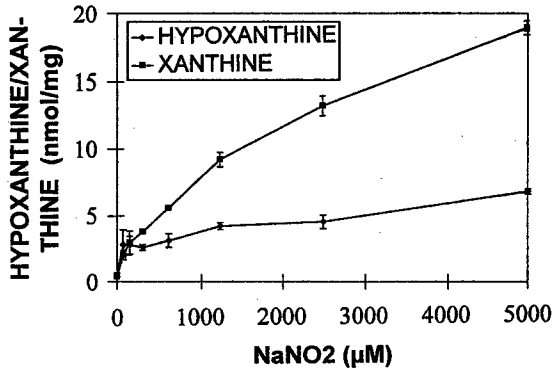

FIGURE 6 Deamination of bases in calf thymus DNA by nitrous acid, generated from $NaNO_2$ at pH 3. Data courtesy of Dr. K. Zhao. Hypoxanthine is a deamination product of adenine, xanthine a deamination product of guanine.

Unabsorbed iron and phenolic compounds (also inefficiently absorbed) end up in the faeces. It is perhaps fortunate that the colon is hypoxic, since faeces incubated under aerobic conditions generate reactive oxygen species at a high rate.[132] The ability of dietary phytates and phenolics to pass largely unabsorbed into the colon, where they may chelate iron and (in the latter case) scavenge reactive oxygen, nitrogen and chlorine species[133–138] may thus be beneficial in cases where transient rises in intracolonic oxygen tension occur and allow production of reactive species.

WHAT DOES THE FUTURE HOLD?

Studies of healthy human subjects with broadly comparable plasma levels of ascorbate, α-tocopherol and β-carotene showed that "steady state" levels of oxidative DNA damage in white cells vary over a wide range, i.e. some people have more "oxidized" DNA than others (Table V). This might be related to exposure to environmental toxins, and/or variable intake of antioxidants as yet unidentified. There may also be genetic predispositions;[138–140] it seems reasonable to suppose that variations in such parameters as

(i) endogenous rates of free radical production,
(ii) levels of antioxidant defences,

TABLE V The range for DNA base damage products measured in white blood cells from nineteen healthy human non-smokers

Base damage product	nmoles/mg DNA				
	Mean	SD	Median	Minimum	Maximum
5-Cl Uracil	0.06	0.02	0.06	0.03	0.10
5-OH Me Hydantoin	0.23	0.08	0.18	0.12	0.36
5-OH Hydantoin	0.12	0.04	0.12	0.06	0.20
5-OH Uracil	0.11	0.03	0.11	0.07	0.19
5-OH Me Uracil	0.03	0.01	0.03	0.01	0.05
5-OH Cytosine	0.10	0.02	0.09	0.08	0.14
Thymine glycol (cis)	0.32	0.18	0.26	0.11	0.75
FAPy Adenine	0.62	0.68	0.30	0.01	1.9
8-OH Adenine	0.37	0.22	0.28	0.14	0.93
2-OH Adenine	0.12	0.07	0.11	0.04	0.27
FAPy Guanine	0.32	0.22	0.42	0.12	0.64
8-OH Guanine	0.24	0.10	0.23	0.06	0.39
Total base damage	2.6	0.87	2.4	1.3	4.5

Note: No differences between male and female subjects were observed. Data compiled by Ms. Almas Rehman.

POSSIBLE GENETIC ASPECTS AFFECTING OXIDATIVE DNA DAMAGE

- Rate of ROS/RCS/RNS production
- Levels of antioxidant defence, both basal and extent of upregulation in response to oxidative stress
- Susceptibility of DNA to damage (e.g. conformation, type and level of DNA binding proteins, chromatin structure)
- Uptake/processing/delivery to site of action of dietary antioxidants
- Rates of repair, and extent of upregulation of repair systems in response to oxidative stress
- Efficiency of intracellular sequestration of transition metal ions

FIGURE 7 Some of the genetic factors that could affect steady-state levels of damage to DNA *in vivo*. ROS – reactive oxygen species; RCS – reactive chlorine species; RNS–reactive nitrogen species.

(iii) the activity of repair enzymes,
(iv) the ability to increase levels of defence and/or repair systems in response to oxidative stress, and
(v) the ability to take up, process and deliver dietary antioxidants to the correct site of action

are some of the many factors (Figure 7) that influence levels of oxidative DNA damage and hence, in the long term, susceptibility to cancer. Only time will reveal the truth.

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