

Minireview

## Are we sure we know how to measure 8-oxo-7,8-dihydroguanine in DNA from human cells?

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### Abstract

The most commonly measured marker of oxidative DNA damage is 8-oxo-7,8-dihydroguanine (8-oxoGua) or its deoxyribonucleoside (8-oxodGuo). Published estimates of the concentration of 8-oxoGua/8-oxodGuo in DNA of normal human cells vary over a range of three orders of magnitude. Analysis by chromatographic methods (GC-MS, HPLC with electrochemical detection (ECD) or HPLC-MS/MS) is beset by the problem of adventitious oxidation of guanine during sample preparation. An alternative approach, based on the use of the DNA repair enzyme formamidopyrimidine DNA N-glycosylase (FPG) to make breaks in the DNA at sites of the oxidised base, gives much lower values. ESCODD, the European Standards Committee on Oxidative DNA Damage, has been testing the ability of different laboratories using a variety of methods to measure 8-oxoGua in standard samples of 8-oxodGuo, calf thymus DNA, pig liver, oligonucleotides, and HeLa cells, and in lymphocytes isolated from blood of volunteers.

- HPLC-ECD is capable of measuring 8-oxodGuo induced experimentally in calf thymus DNA or HeLa cells with high accuracy. However, there is no sign of consensus over the *background* level of this damage, suggesting that, even though standard extraction procedures were used, variable oxidation of Gua is still occurring.
- GC-MS failed to detect a dose response of induced 8-oxoGua and cannot be regarded as a reliable method for measuring low levels of damage.
- HPLC-MS/MS as yet has not proved capable of measuring low levels of oxidative DNA damage.
- FPG-based methods seem to be less prone to the artefact of additional oxidation. Although they can be used quantitatively, they require careful calibration and standardisation if they are to be used in human biomonitoring.
- The background level of DNA oxidation in normal human cells is likely to be around 0.3–4.2 8-oxoGua per 10<sup>6</sup> Gua.
- An effort should be made to develop alternative, validated methods for estimating oxidative DNA damage.

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### Background: oxidative DNA damage and cancer

It is partly due to Bruce Ames that we began to take fruit and vegetables seriously. In a series of papers

published in the late 1980s and early 1990s, he and colleagues highlighted some contradictions in genotoxicity and cancer aetiology that have still not been fully accounted for. Many constituents of plant foods have given positive results in the Ames mutagenicity test, and some have even been shown to induce cancer in experimental animals. Yet, obviously, plant foods are not seriously hazardous for human health. With a few exceptions, such as aflatoxin, mutagenic compounds in

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food have not been demonstrated to cause human cancer. The objection can be raised that the concentration of a mutagenic food component that has a deleterious effect is in excess of the concentrations that are likely to be present in the body after ingestion in food. This may, in general, be true. But the total amount of proven mutagenic/carcinogenic compounds taken in each day by the average human, according to Ames [1], amounts to grams.

At about the same time, a meta-analysis of epidemiological studies of cancer by Doll and Peto [13] estimated the contributions to cancer incidence from various causes, and concluded that diet was about as important as smoking, being at least partly responsible for a third of all cancers. As well as the constituents of food that might cause cancer, we have to consider the absence from food of factors that might prevent cancer. This brings us back to the contribution of Bruce Ames, who emphasised the importance of endogenous mutagens, and in particular reactive oxygen species, as a cause of cancer that could be counteracted by food components, and an obvious candidate for the protective agent is the class of compounds known as antioxidants.

Meanwhile, the epidemiologists had gone further and identified carotenoids as prime candidates for cancer prevention, on the basis of population studies that included an analysis of micronutrient antioxidants in the diet or measurements of plasma concentrations, as well as some experimental studies on animals and cultured cells that backed up the observational evidence [25]. It was suggested that supplementation studies might usefully be conducted, with  $\beta$ -carotene, to see whether cancer incidence could actually be decreased.

### Detecting oxidative DNA damage

Is there really enough oxidative damage to the DNA in our bodies to cause cancer? Many reports have suggested that there is. 8-oxo-7,8-dihydroguanine (8-oxo-Gua)<sup>2</sup> or its deoxynucleoside (8-oxodGuo) is the most commonly measured oxidation product, and probably hundreds of reports of its concentration in human DNA exist. The literature has been reviewed [2,7] and only a few examples need be given here. Malins and Haimanot [23], using GC-MS with selected ion monitoring, reported levels of 8-oxoGua per 10<sup>6</sup> guanines in breast cancer tissue averaging around 1650, a 9 $\times$  increase over the level in non-cancerous tissue. Podmore et al. [27] found 300 per 10<sup>6</sup> guanines in human lymphocytes, also

using GC-MS. HPLC with electrochemical detection is a popular method introduced more than 17 years ago [18]. Dandona et al. [9] found 33 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) per 10<sup>6</sup> dGuo in human mononuclear leukocytes, but a much lower level—2.4 per 10<sup>6</sup>—was reported by Nakajima et al. [24]. (HPLC measures the concentration of 8-oxodGuo in an enzymic hydrolysate, while for GC-MS the DNA is generally acid-hydrolysed to bases, which are derivatised before being submitted to analysis.)

In the mid-1990s, a different, non-chromatographic approach was developed. This depended on the use of enzymes—bacterial endonucleases involved in DNA repair—that have specificity for certain kinds of oxidative base damage, converting the oxidised base to a DNA break. Formamidopyrimidine DNA glycosylase (FPG) is one such enzyme, which recognises 8-oxoGua, as well as formamidopyrimidines (ring-opened purines resulting from oxidation) [4]. Endonuclease III, on the other hand, excises oxidised pyrimidines [3]. The damaged base is removed, leaving a baseless sugar (apurinic/apyrimidinic or AP site) which is converted to a break by an associated AP lyase. If the digestion is carried out on intact cellular DNA (i.e., on carefully lysed cells), the breaks produced can be measured using one of these three unrelated techniques:

*The comet assay (single cell alkaline gel electrophoresis)* [8]. Cells are embedded in agarose on a microscope slide and lysed in buffer with 2.5 M NaCl and 1% Triton X-100, which remove membranes, cytoplasm, and most nuclear proteins (including histones), leaving the DNA in supercoiled form as nucleoids. The gel-embedded nucleoids are digested with FPG or endonuclease III (alongside nucleoids treated with enzyme buffer alone) and the gels are then placed in an alkaline solution prior to electrophoresis at high pH. DNA loops in which supercoiling has been relaxed by strand breakage extend out from the nucleoid to form a comet-like image, viewed by fluorescence microscopy with a DNA-staining dye such as ethidium bromide or DAPI. The relative amount of DNA in the tail of the comet reflects the number of DNA breaks in a quantitative way.

*Alkaline elution* [14]. Cells are gently lysed above a microporous filter and eluted by passing an alkaline buffer through. Rather like spaghetti in a sieve, the (denatured) DNA molecules pass through the pores, and the smaller the fragments are, the more quickly they manage to fall through the sieve. Thus, the rate of elution of DNA reflects the frequency of strand breaks, and—as with the comet assay—calibration depends on comparison with the behaviour of DNA containing known frequencies of breaks introduced by ionising radiation.

*Alkaline unwinding* [21]. Cells are subjected to a defined period of lysis at high pH, which denatures the

<sup>2</sup> Abbreviations used: 8-oxodGuo, deoxynucleoside; 8-oxoGua, 8-oxo-7,8-dihydroguanine; ECD, electrochemical detection; FPG, formamidopyrimidine DNA glycosylase.

DNA and allows the strands to separate, to an extent that (for a given time of alkaline treatment) depends on the frequency of breaks. The solution is neutralised, which causes DNA that has not separated to renature, but separated strands remain single-stranded. The DNA is fragmented by sonication, and the resulting mixture of single- and double-stranded fragments is separated by hydroxyapatite chromatography. The more breaks were present, the more DNA appears as single-stranded fragments. Again, external calibration is needed to make the assay truly quantitative.

All three of these methods of measuring breaks have been used with FPG to measure oxidised purines in normal human cells and have given remarkably similar results, around 0.5 8-oxoGua residues per  $10^6$  guanines [7].

That there was a discrepancy between the levels of damage reported using different techniques was not immediately obvious, since different methods (and different laboratories) favoured different ways of expressing their results—as femtomoles of 8-oxoGua per microgram of DNA, nanomoles per mg, 8-oxodGuo per  $10^5$  or  $10^6$  dGuo, 8-oxodGuo per  $10^6$  base pairs, or as the number of lesions per  $10^9$  Da of DNA, or per cell. However, when these were converted to common units, a range of three orders of magnitude in estimates of the background level of 8-oxoGua in the DNA of normal human cells was readily apparent [2,7]. In general, GC-MS tended to report the highest values, HPLC rather lower, and the enzymic methods lowest of all. At that time, it was becoming clear that very careful precautions must be taken to prevent oxidation of guanine during the preparation of DNA for analysis by chromatography. The derivatisation step required for GC-MS, during which the DNA is incubated for a prolonged period at high temperature, was thought to be particularly vulnerable.

#### ESCODD: a concerted attempt to address the problems associated with measuring 8-oxodGuo

A meeting of many of the European researchers engaged in measuring oxidative DNA damage was held at the Rowett Research Institute in Aberdeen, Scotland, in early 1997, and as a result the European Standards Committee on Oxidative DNA Damage (ESCODD) was set up, to try to identify the sources of error and variability in assaying 8-oxoGua, to produce standard and validated protocols, and to reach a consensus on the likely level of oxidative damage in normal cellular DNA. ESCODD received funding from the European Commission as a Concerted Action in 1999, and for 3 years operated a coordinated programme, distributing samples to the 28 participating laboratories for analysis, holding regular meetings, preparing standard operating

procedures, and facilitating exchanges of scientists between laboratories.

We began by sending out standard solutions of 8-oxodGuo for analysis by HPLC-ECD (both coulometric and amperometric), HPLC-MS/MS, and GC-MS. Fig. 1 shows that about half the laboratories/methods achieved results that were close to the actual concentrations in the standards [15].

Next, calf thymus DNA (obtained from Sigma) was distributed to members, to examine the variability resulting from different methods of preparation for chromatography. The median value for all of our determinations was 61.6 8-oxoGua/ $10^6$  Gua, and almost 1/2 of reported results were within 20% of the median. Two methods (one GC-MS, one HPLC) gave consistently very high figures for 8-oxoGua/ $10^6$  Gua—even though standard 8-oxodGuo solution was supplied to everyone [15].

Calf thymus DNA was treated with three different concentrations of the photosensitiser Ro 19-8022 and light to induce additional 8-oxoGua by specific singlet oxygen oxidation of guanine bases [31], and identical samples were distributed to ESCODD laboratories. The aim was to test the ability of different methods to detect the dose–response. It should be noted that the doses of damage were relatively high, though they were superimposed on an already high background level of oxidation of the DNA. Applying the criterion that increases in 8-oxodGuo should be detected across the whole range of concentrations of Ro 19-8022, six of 19 laboratories using HPLC-ECD detected the dose–response, compared with one of the two using HPLC-MS/MS, and none of three using GC-MS [15].

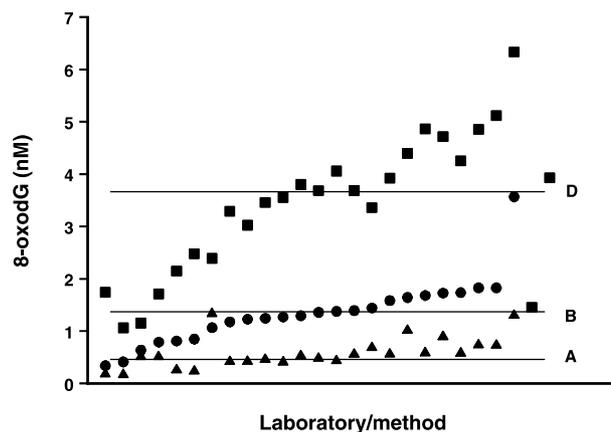


Fig. 1. Inter-laboratory comparison of chromatographic analysis of 8-oxodGuo solutions A (0.46 nM) (triangles), B (1.37 nM) (circles), and D (3.66 nM) (squares). The sets of values for the three solutions, aligned vertically, each representing one laboratory; the results are arranged in increasing order of the values reported for sample B. From [15] with permission from Taylor and Francis (<http://www.tandf.co.uk/journals/titles/10715762.html>).

Throughout the ESCODD trials, methods for decreasing spurious oxidation of guanine during sample preparation were developed, discussed, and adopted, to varying degrees, by the participating laboratories. To evaluate these methods, we thought initially to produce an oligonucleotide containing guanine, none of which was oxidised, so that the extent of the oxidation artefact could be estimated, but practically this could not be achieved. Instead, we produced 22-oligomers that contained no guanine, but 1 or 5 8-oxodGuo. Since no guanine was present, it could not be oxidised; the concentration of 8-oxodGuo was expressed relative to dAdo rather than to dGuo. We synthesised three different 22-oligomers as depicted in Table 1, one containing one 8-oxodGuo, one containing five 8-oxodGuo, and one without 8-oxodGuo; none contained any unoxidised guanine. By mixing these oligonucleotides, it is possible to produce oligonucleotides with precise concentrations of 8-oxodGuo.

The experience with such samples in 20 different laboratories (most using HPLC with coulometric electrochemical detection, one using amperometric detection, one using liquid chromatography coupled to tandem mass spectrometry, and two using GC-MS) showed that the majority of laboratories had reasonable results, i.e., they could measure a range of concentrations of 8-oxodGuo/dAdo accurately, but also that some laboratories reported levels that were too high or too low [29]. Since not only the exact 8-oxodGuo level but also the dAdo concentration in the samples was known, it was possible to identify analytical problems relating to analysis of both 8-oxodGuo and also dAdo, not related to spurious oxidation of guanine (as there was none present). Furthermore, the exercise showed that the variability of the analysis of the ratio 8-oxodGuo/dAdo, measured as the coefficient of variation, ranged from below 1 to 67% for 8-oxodGuo and from 1 to 386% for dAdo.

In future, synthetic oligonucleotides will be invaluable for assessing the efficiency of DNA digestion, extraction, and analysis procedures. These defined oligonucleotides will act as an internal control. Our results in ESCODD point to the importance of measuring with accuracy and precision the *non-oxidised* as well as the oxidised bases.

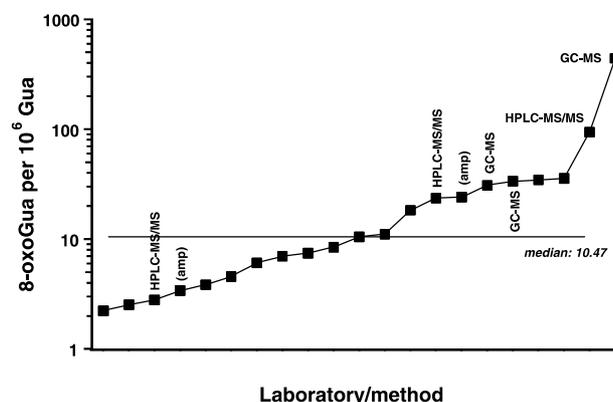


Fig. 2. Chromatographic analysis of 8-oxoGua content of pig liver DNA, arranged in order of increasing values found. HPLC with coulometric electrochemical detection (ECD) was employed except where otherwise stated; two laboratories used HPLC with amperometric ECD (amp), three used GC-MS, and three HPLC-MS/MS. A log scale was employed to accommodate the range of values. From [16] with permission from Oxford University Press.

Seeking a more biologically relevant material, we next distributed portions of pig liver, for each laboratory to extract, hydrolyse, and analyse DNA. The results were, to say the least, disappointing (Fig. 2). The median value of all determinations was 10.5 8-oxodGuo per 10<sup>6</sup>, but few laboratories were close to the median, and the range was from 2.23 to 441 [16]. This was in spite of efforts made during the course of ESCODD to improve techniques for isolating and hydrolysing DNA by including antioxidants and chelators to remove metal ions, and modifying the derivatisation procedure for GC-MS so that it was less likely to induce spurious oxidation of guanines [22,28,32].

### Comparing the enzymic and chromatographic approaches to 8-oxodGuo measurement

Up to this point, only the chromatographic methods had been included in the ring study, since the enzymic approach demands intact cellular DNA for measurement of low frequency breaks. In the next phase, HeLa cells were cultured in bulk and sent to the laboratories using the comet assay, alkaline elution, and alkaline unwinding as well as to those employing HPLC-ECD,

Table 1  
Oligonucleotide sequences

Designation	Residues			Sequence 5' → 3'
	Total	8-oxodGuo	dAdo	
Oligo ox1	22	1	6	CAT TTA CAT ATX CTT ATC ATT C
Oligo ox5	22	5	6	CAT XTA CAX ATX CTX ATC AXT C
Oligo noG	22	0	6	CAT TTA CAT ATT CTT ATC ATT C

X = 8-oxodGuo.

HPLC-MS/MS, or GC-MS [16]. Fig. 3 shows the results. The median value by chromatographic methods was 5.23 8-oxoGua/ $10^6$  dGua, with a range of 120 $\times$  between highest and lowest estimates; the median by enzymic methods was 0.79 per  $10^6$ , with an 83 $\times$  range. Although the enzymic methods depend on external calibration, it is reassuring that the three different ways of measuring the DNA breaks, calibrated independently, show agreement; the alkaline elution and alkaline unwinding results are close to the median for results with the comet assay.

The median value probably represents the best estimate according to the FPG-based methods. However, in the case of the chromatographic methods, since spurious oxidation is the likely cause of variation, the lowest values, rather than the median, are likely to be the most trustworthy. If that argument is accepted, the difference between the 'best' results with the two approaches is a factor of only 2.5-fold.

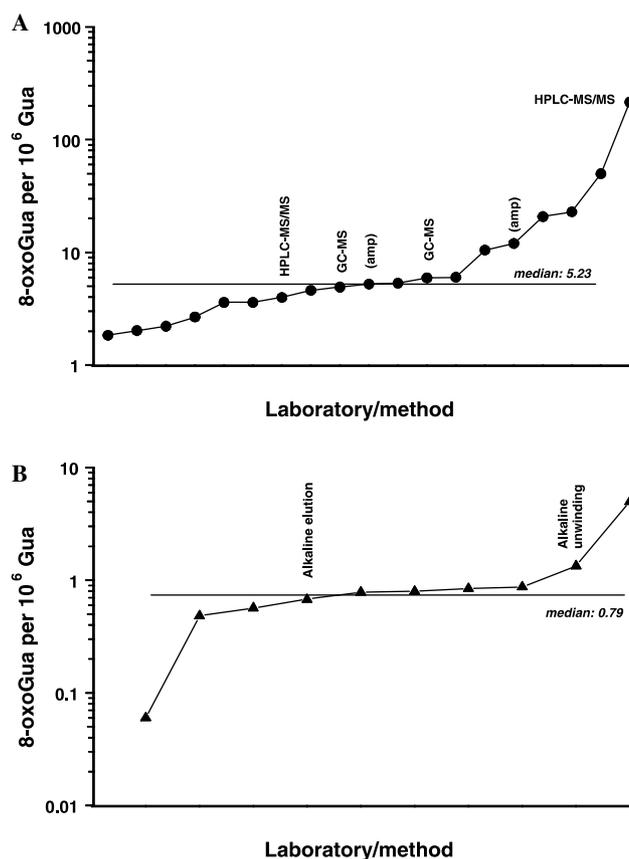


Fig. 3. Results of chromatographic (A) and enzymic (B) analysis of 8-oxoGua content of HeLa cell DNA, arranged in order of increasing values found. (A) HPLC with coulometric ECD was used except where otherwise stated. (B) The comet assay was used except where otherwise stated. Note use of log scale. From [16] with permission from Oxford University Press.

In a logical progression towards increasingly demanding tests, we next treated HeLa cells with photosensitiser plus light to induce additional guanine oxidation [17]. The concentrations of Ro 19-8022 used for chromatographic samples were higher than those used for material for enzymic analysis, since it was already known that there is a difference in sensitivity between the two approaches. The results are presented in Fig. 4. Eight laboratories used HPLC; seven of them detected the dose–response. When regression lines are calculated for the HPLC data, seven laboratories show virtually identical slopes for the dose–response curve, indicating a commendable accuracy in measuring induced DNA oxidation. However, there is a 75 $\times$  range in the  $y$ -axis intercepts, equivalent to estimates of the background level of damage, and the simplest explanation is that the problem of adventitious oxidation during sample processing has still not been overcome. Five of the 10 laboratories using the enzymic approach detected the dose response, and their slopes were not as consistent as those with HPLC, but it should be remembered that the levels of damage were many times less than those that could be detected chromatographically.

#### A critical assessment of mass spectrometry detection applied to the measurement of low levels of 8-oxoGua

The ability of GC-MS and LC-MS/MS to detect increasing amounts of 8-oxoGua in the dose–response experiments, with either calf thymus DNA or HeLa cells, was very poor compared with HPLC, even when precautions were taken to limit oxidation during sample preparation. This failing is serious, but should be seen in perspective.

The great advantage of GC-MS in the analysis of oxidative DNA damage is that it allows the identification of individual products of base oxidation. It has been used to great effect to characterise the kinds of damage caused by ionising radiation and other sources of reactive oxygen [10,11,19,30]. It has also given valuable information on the substrate specificities of DNA glycosylases involved in base excision repair of oxidative damage [12], using highly damaged DNA as substrate. The amounts of the various lesions in these studies were far in excess of what is found naturally in the DNA of living cells, and it is by no means a foregone conclusion that a method that is excellent at discriminating and measuring oxidation products in severely damaged DNA will be equally good at measuring low background levels of damage. Through ESCODD it has transpired that GC-MS is in fact the least reliable method for measuring low levels of 8-oxoGua, and it is no longer used for that purpose by ESCODD members. HPLC-MS/MS has not yet lived up to its promise. It is a relatively new technique and practical problems clearly

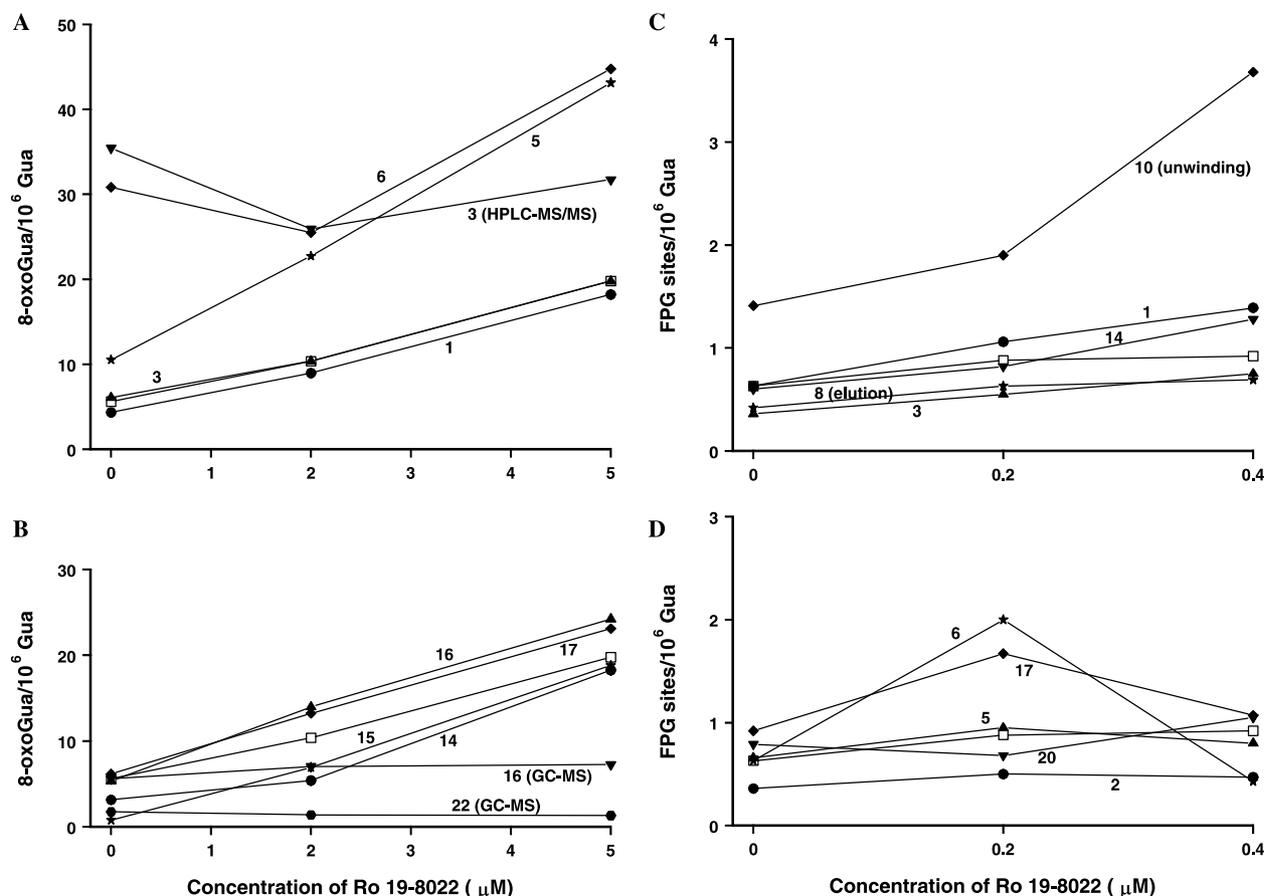


Fig. 4. Detection of 8-oxodGua dose response by chromatographic methods (A, B) and enzymically (C, D). HeLa cells were treated with different concentrations of the photosensitiser Ro 19-8022 and irradiated with visible light to induce oxidation of Gua. The chromatographic method was HPLC-ECD except where stated otherwise. The comet assay was used to measure FPG-sensitive sites, except for two laboratories which used alkaline unwinding or alkaline elution, as shown. Solid symbols represent individual laboratories, identified by numbers; median values are also shown (open squares). From [17] with permission from Elsevier Science.

still have to be resolved. Like HPLC-ECD, HPLC-MS/MS is intrinsically an accurate method. However, the still pending problem, common to all chromatographic assays, is the possibility of spurious oxidation of even a minute fraction of the relatively enormous number of guanine residues during handling of the DNA samples—despite recent major improvements in methods [28].

### The level of 8-oxodGua in human lymphocytes

The final objective of ESCODD was to measure 8-oxoGua in human lymphocytes. As part of this last exercise, we distributed identical samples of untreated HeLa cells for each laboratory to use as a reference standard for comparison with lymphocytes (ESCODD, in preparation). We compared members' own methods for extracting and hydrolysing DNA with an ESCODD-recommended protocol; there was little to choose between them. The values obtained by laboratories using the standard protocol ranged over 13-fold—about the same as the range of values found with the same samples

using the enzymic methods, and a considerable improvement on previous rounds of ESCODD. However, the fact that variation is still seen means that we have not yet solved the problem of adventitious oxidation. The enzymic methods gave similarly variable results (over a  $10\times$  range). The median concentrations of 8-oxoGua in HeLa cell DNA, using the alternative approaches, differed by a factor of 6.

Participating laboratories recruited healthy young male volunteers to give blood, from which lymphocytes were isolated. Mean values of 8-oxoGua in the lymphocytes from the volunteers in each country were used to calculate the median values for the two approaches used, i.e., HPLC and the comet assay. The median for HPLC is 4.24, with a range from 0.20 to 6.65 8-oxoGua per  $10^6$  Gua (mean value: 3.75, SD 2.37). The median value for comet assay determinations is 12-fold lower, at 0.34 (range 0.15–0.55) 8-oxoGua per  $10^6$  Gua (mean value: 0.34, SD 0.15). (Because the HeLa results were apparently so erratic, we decided that to attempt to normalise the lymphocyte results by expressing them relative to the HeLa results in the same laboratory [the

original intention] would in fact probably render the lymphocyte results less rather than more accurate.) Six laboratories measured 8-oxoGua using both HPLC and the comet assay on the same samples. Here, surely, we might hope for a correlation, indicating that we are measuring the same thing with the two approaches, and yet only one laboratory found any significant association between individual values of 8-oxoGua by HPLC and by the comet assay. A puzzle is presented here, since, although the results correlated, there was a constant factor of 12 between them. One explanation would be that FPG consistently underestimates damage, by that factor; but experiments in different laboratories in which experimentally induced oxidation was measured by both techniques strongly suggest that they are both equally effective, i.e., the enzymic approach does not underestimate damage [20,26].

Another possibility is that whatever is modulating the true background level of cellular DNA damage (presumably, the antioxidants present within the cell) is also able to affect the oxidation during sample preparation that results in the artefact. Thus, lymphocytes from an individual with high antioxidant status and a low basal level of DNA oxidation would be expected to be relatively resistant to oxidation occurring *ex vivo*. It would follow from this that it is the very earliest stage of DNA extraction that is most sensitive to oxidation.

### Future developments

We can consider ESCODD a success, since we have reduced a discrepancy of 1000× to one of only 6×–12× (depending on whether we are looking at HeLa cells or lymphocytes). But this is a qualified success; there still is a discrepancy and it apparently results in large part from still uncontrolled oxidation. It should also be kept in mind that a relatively large amount of DNA (>30 µg) is required to minimise adventitious oxidation of the nucleobases [5]. It is not at all clear what more can be done to eliminate the effect. Meanwhile, the enzymic approach, while it may be free of this artefact, has its own problems of variability between laboratories, and suffers from a dependence on an indirect calibration, using an agent such as X-rays to introduce defined numbers of breaks in the DNA. The efficiency of the FPG-mediated cleavage of oxidised DNA has also to be assessed. At least the ESCODD member laboratories are in agreement that the likely average background level of 8-oxoGua in human cells is between 0.3 and 4 per 10<sup>6</sup> guanines. This should provide a criterion by which to assess the credibility of published studies; some clearly have been measuring artefact.

In principle, it is possible to compare different procedures for isolating DNA in terms of their avoidance of spurious oxidation, by using <sup>18</sup>O-labelled 8-oxodGuo as

an internal standard. [<sup>18</sup>O]8-oxodGuo is generated from singlet oxygen released intracellularly by a water-soluble naphthalene derivative, DHPN<sup>18</sup>O<sub>2</sub>. Using HPLC-MS/MS, unlabelled and labelled nucleoside can be measured individually; spurious oxidation during sample processing will increase the ratio of unlabelled/labelled 8-oxodGuo. Several alternative DNA isolation procedures were evaluated using <sup>18</sup>O-labelled 8-oxodGuo as an internal standard [28], and the best (i.e., the method with least oxidation occurring during extraction) was adopted for the final round of ESCODD. However, this approach does not distinguish between actual background 8-oxodGuo and that which is produced by oxidation during processing, since both will be unlabelled; it simply tells us which method controls the artefact best. The approach could also be applied to calibrating the enzymic methods, by carrying out dose–response experiments similar to those described above with the photosensitiser plus light. The FPG-based methods cannot distinguish labelled and unlabelled 8-oxodGuo, but a comparison could be made between the damage induced above background at different doses of DHPN<sup>18</sup>O<sub>2</sub>, detected with FPG, and the absolute amounts of <sup>18</sup>O-labelled 8-oxodGuo measured by HPLC-MS/MS.

For many years, the idea has been current that mitochondrial DNA is more prone to oxidation than is nuclear DNA, and high levels of oxidised bases in mitochondrial DNA have been reported. Beckman and Ames [2] have thoroughly reviewed the literature and find that estimates range from 0.35 to 2 × 10<sup>4</sup> 8-oxodGuo per 10<sup>6</sup> dGuo. Clearly there are severe problems with measuring damage in mitochondrial DNA, at least equal to the problems experienced with nuclear DNA, and Beckman and Ames conclude that it is impossible to say whether mitochondrial DNA has a greater extent of oxidation than does nuclear DNA, though they suggest that there are good theoretical grounds for believing that this may be the case. Effort obviously needs to be put into eliminating spurious oxidation during isolation of mitochondrial DNA. The fact that a relatively small amount of mitochondrial DNA is normally available unfortunately increases the likelihood that any one base will be oxidised in the presence of a limited number of reactive oxygens.

### Conclusions

Bruce Ames's contribution to the debate on the significance of oxidative DNA damage is undisputed.

- The eponymous mutagenicity test was responsible for providing the evidence that, paradoxically, plant foods are loaded with potential carcinogens—and yet on the whole they are beneficial to health.
- Ames forcefully promoted the idea that endogenous DNA damage (particularly oxidation) is probably

far more important than environmental agents as a cause of mutations and is therefore a major cause of human cancer.

- He advocated the possibility that phytochemicals, with their generally antioxidant properties, might explain the cancer-protective effects of fruits and vegetables.
- On the technical side, he and his colleagues developed methods for measuring DNA oxidation and attempted to diminish the problem of adventitious oxidation by developing the chaotropic method of DNA extraction (adopted by ESCODD).

And yet, we have gone down several blind alleys in the search for the elusive answer to the conundrum Ames presented. It could be said that the emphasis on oxidation and antioxidant effects has caused us to neglect other endogenous forms of damage, such as alkylation, and to underplay the other ways in which fruits and vegetables might protect, in addition to their antioxidant effects. They are, after all, full of phytochemicals with potential roles in cell signalling and control of proliferation, modulation of gene expression, and activation and inhibition of enzymes involved in xenobiotic metabolism and DNA repair, and these possibilities are now beginning to receive the attention they deserve.

#### Advice and recommendations

1. Editors, and referees of manuscripts, should be aware of the problems associated with measuring oxidative DNA damage. They should encourage the use of standard units (8-oxoGua per  $10^6$  guanines) so that the credibility of data can be readily assessed. They should be sceptical of reports of background levels outside the range established by ESCODD (0.3–4.2 8-oxoGua per  $10^6$  Gua).
2. Researchers beginning to measure 8-oxodGuo should not expect immediate success; it has taken the best laboratories years to develop reliable techniques and still we have a problem with variable results. It is a commonplace that precision in a technique improves markedly as the operator gains experience, and it seems likely that, in the case of the 8-oxodGuo assay where spurious oxidation must be controlled by very careful working, accuracy too will improve with time, though of course attention must be paid to the choice of a tried and tested method.
3. Further work is needed to develop procedures that prevent oxidation from occurring during sample preparation for chromatographic analysis. It is still not clear what are the sensitive stages, although it seems that the earliest steps (cell isolation and lysis) may be critical.
4. Other endpoints for measuring oxidative DNA damage should be explored. Other oxidised bases (e.g., oxidised pyrimidines detected with endonuclease III) are one possibility. If the aim is simply to measure 'oxidative stress,' then perhaps a lipid oxidation product such as malondialdehyde should be considered, or the adduct formed between malondialdehyde and guanine [6].
5. Similarly, the enzymic approach, which is relatively free of the oxidation artefact, requires further investigation into the efficiency of enzymic detection of lesions, and a concerted inter-laboratory effort to find a reliable way to calibrate the assays.
6. A quality control scheme should be set up if at all possible. This would continue the work done by ESCODD, providing participating laboratories with standard samples for analysis. The samples would include oligonucleotides with defined content of 8-oxodGuo and cultured cells with different amounts of 8-oxoGua induced above background. Proficiency in measuring 8-oxoGua would be gauged by
  - Accuracy of measurement of 8-oxodGuo in oligonucleotide.
  - Accuracy of dose response slope with treated cultured cells.
  - Closeness to actual level of background oxidation in cultured cells.

Such a scheme depends on knowing what the real values are; and to be sure of that will require parallel measurements by HPLC-MS/MS and an enzymic method (probably the comet assay). In our view, journal editors and referees should demand evidence of performance in a quality assurance scheme before accepting a paper for publication.

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