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Facts About the Artifacts in the Measurement of Oxidative DNA Base Damage by Gas Chromatography–Mass Spectrometry

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Recently, several papers reported an artifactual formation of a number of modified bases from intact DNA bases during derivatization of DNA hydrolysates to be analyzed by gas chromatography–mass spectrometry (GC/MS). These reports dealt with 8-hydroxyguanine (8-OH-Gua), 5-hydroxycytosine (5-OH-Cyt), 8-hydroxyadenine (8-OH-Ade), 5-hydroxymethyluracil (5-OHMeUra) and 5-formyluracil that represent only a small percentage of the 20 or so modified DNA bases that can be analyzed by GC/MS. Removal of intact DNA bases by prepurification of calf thymus DNA hydrolysates using HPLC was shown to prevent artifactual formation of these modified bases during derivatization. It needs to be emphasized that the procedures for hydrolysis of DNA and derivatization of DNA hydrolysates used in these papers substantially differed from the established procedures previously described. Furthermore, a large number of relevant papers reporting the levels of these modified bases in DNA of various sources have been ignored. Interestingly, the levels of modified bases reported in the literature were not as high as those reported prior to prepurification. Most values for the level of 5-OH-Cyt were even lower than the level measured after prepurification. Levels of 8-OH-Ade were quite close to, or even the same as, or smaller than the level reported after prepurification. The same holds true for 5-OHMeUra and 8-OH-Gua. All these facts raise the question of the validity of the claims about the measurement of these modified DNA bases by GC/MS.

A recent paper reported a complete destruction of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde) by formic acid under the conditions of DNA hydrolysis prior to GC/MS. The complete destruction of FapyGua and FapyAde by formic acid is in disagreement with the data on these compounds in the literature. These two compounds were measured by GC/MS following formic acid hydrolysis for many years in our laboratory and by other researchers with no difficulties. These facts clearly raise the question of the validity of the claims made about the previous measurements of these compounds by GC/MS.

Keywords: Derivatization, DNA repair, formamidopyrimidines, gas chromatography–mass spectrometry, 5-hydroxycytosine, 8-hydroxyguanine

INTRODUCTION

Oxidative DNA damage is implicated in mutagenesis, carcinogenesis and aging (reviewed in [1]). Among oxygen-derived species, hydroxyl radical (•OH) is highly reactive and can produce a number of modifications in DNA including base lesions by a variety of mechanisms (reviewed in

[2–5]). Hydrated electron (e^-_{aq}) and H atom produced by ionizing radiation from water in cells also react with DNA bases producing a number of modified bases.[3] The types and yields of DNA modifications profoundly depend on the free radical-generating system, experimental conditions, and the presence or absence of oxygen.[4] A number of analytical techniques have been used to identify and quantify a variety of products of DNA (reviewed in [6,7]). The detection of a product by most techniques is carried out with no specific structural evidence. Generally, one product or a limited number of products are measured at a time. The measurement of a single product such as 8-hydroxy-2′-deoxyguanosine (8-OH-dG) as done by HPLC with electrochemical detection (ECD) may be misleading, because oxidative damage generates many lesions in DNA at the same time.[2–4] Furthermore, product yields and ratios of product yields to one another differ depending on the type of DNA-damaging agent and experimental conditions.[4,8]

ANALYSIS OF DNA BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY

For over a decade, we have shown that gas chromatography–mass spectrometry (GC/MS) is a well-suited technique for the measurement of oxidative damage to DNA[9,10] (reviewed in [11,12]). GC/MS can be applied to DNA itself or directly to chromatin.[11,12] Of the analytical techniques used for the detection of DNA damage, GC/MS is the only technique capable of positive identification and quantification of a large number of base products from all four DNA bases in a single DNA sample at the same.[10–12]

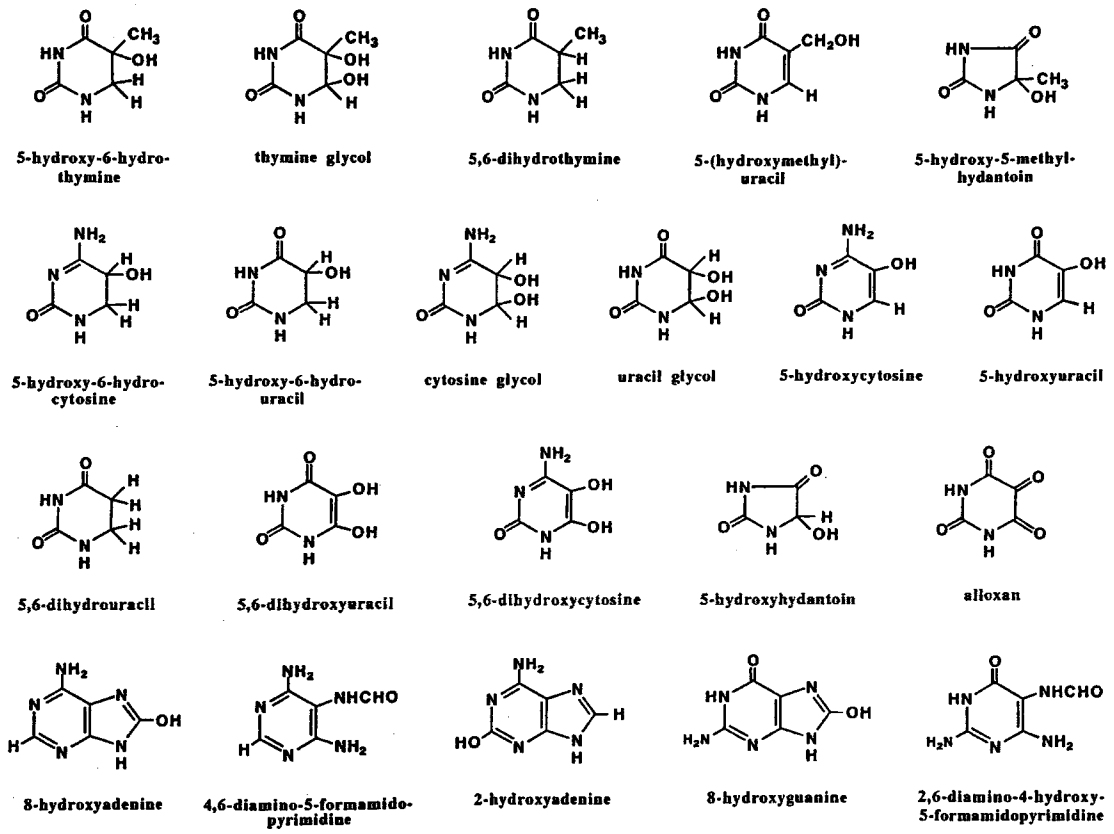

FIGURE 1 Structures of modified DNA bases that can be measured by GC/MS.

Figure 1 illustrates the structures of modified DNA bases that can be measured by GC/MS. The analysis of DNA sugar products and DNA-protein cross-links by GC/MS has also been described (reviewed in [11,12]). The yields of products in Figure 1 and their ratios to each other substantially depend on the DNA-damaging agent as well as other reaction conditions such as the presence or absence of oxygen (reviewed in [4]). For this reason, the measurement of multiple products of all four DNA bases permits the precise comparison of DNA damage caused by a variety of DNA-damaging agents.[4] This may prevent possible misleading conclusions obtained by measurement of a single product as done by other techniques such as HPLC–ECD. Excellent examples of this fact are two recent works, which clearly demonstrated the necessity of measurement of multiple products at the same time.[8,8a] In these cases, the measurement of 8-OH-dG only would have led to misleading results on the activity of vitamin C *in vivo*.

For GC/MS analysis, DNA is first hydrolyzed to release intact and damaged bases from the sugar–phosphate backbone, and formic acid is used for this purpose.[10–12] Subsequently, the hydrolysates are derivatized to obtain volatile derivatives of intact and damaged bases, which are suitable for GC/MS analysis. For this purpose, trimethylsilylation is the mode of derivatization most frequently used.[13] The measurement at low concentrations of modified DNA bases is performed using GC/MS with selected-ion monitoring (SIM).[11,14] The quantification is best achieved by isotope-dilution mass spectrometry (IDMS) using stable isotope-labeled analogues of modified bases as internal standards.[14,15]

ARTIFACTS DURING GC/MS ANALYSIS? HOW MANY PRODUCTS ARE AFFECTED?

Recently, several papers reported an artifactual formation of five modified bases from corresponding intact DNA bases during derivatization of DNA hydrolysates prior to GC/MS[16–18] (reviewed in [19]). These modified bases were 8-hydroxyguanine (8-OH-Gua), 5-hydroxycytosine (5-OH-Cyt), 8-hydroxyadenine (8-OH-Ade), 5-hydroxymethyluracil (5-OHMeUra) and 5-formyluracil. It should be pointed out that these products are only a few of the many products that can be measured by GC/MS (Figure 1).

8-Hydroxyguanine

The formation of 8-OH-Gua by oxidation of guanine in DNA hydrolysates during trimethylsilylation has been reported.[16,17] The presence of oxygen in derivatization mixtures increased the amount of 8-OH-Gua. Purging the mixtures with nitrogen prevented the formation of 8-OH-Gua, but not completely at longer derivatization times. No evidence was provided to indicate whether oxygen was completely removed from derivatization mixtures.[17] Furthermore, the procedures used for hydrolysis and derivatization substantially differed from those described previously for GC/MS analysis of modified DNA bases. The procedural differences may have led to the high values observed for 8-OH-Gua following derivatization at high temperature. Derivatization has also been performed at room temperature.[16] Under these conditions, the levels of 8-OH-Gua in calf thymus DNA have been shown to be similar to those obtained by analysis using HPLC–ECD.[16] On the other hand, it is possible that derivatization may have been incomplete since 8-OH-Gua is not soluble under the conditions used, although trifluoroacetic acid was added to derivatization mixtures to dissolve the purines. No evidence for a complete derivatization at room temperature was provided. A subsequent work reported that the addition of trifluoroacetic acid to DNA hydrolysates prevents accurate measurement of pyrimidine-derived modified bases.[20]

Removal of intact bases from DNA hydrolysates by HPLC or immunoaffinity chromatography prior to derivatization GC/MS led to lower

levels of 8-OH-Gua that were similar to those measured by HPLC–ECD.[17] However, it was assumed that HPLC–ECD provided correct levels of 8-OH-Gua in DNA, although possible drawbacks and the unacceptable variability of this assay have been known for some time (reviewed in [6,7,21]). Furthermore, the levels of 8-OH-Gua were compared to only a few previously given levels in the literature. Unfortunately, the authors neglected to refer to the vast majority of publications, which had reported much lower levels of 8-OH-Gua.[22–32] Subsequent work using GC/MS[33–35] also reported levels of 8-OH-Gua, which were close to those reported in Refs. [16,17]. Background levels of 8-OH-Gua were measured by other techniques without derivatization. Levels found were similar to those reported originally using GC/MS.[36–38] For example, guanase digestion was used to remove guanine from formic acid-hydrolysates of DNA. This was followed by measurement of 8-OH-Gua using GC/MS and HPLC–ECD.[36,37] The levels obtained for untreated calf thymus DNA or γ-irradiated DNA by both techniques were very similar, but these levels were about 4.5 times higher than the level of 8-OH-dGuo measured by HPLC–ECD following enzymatic hydrolysis. These results indicate that enzymatic hydrolysis may not completely release 8-OH-dGuo from DNA.[7,21]

5-Hydroxycytosine, 8-Hydroxyadenine and 5-Hydroxymethyluracil

Artifactual formation of 5-OH-Cyt, 8-OH-Ade, 5-OHMeUra and 5-formyluracil during derivatization has recently been reported.[18,19] Additionally, Cadet *et al.* have reported artifactual formation of 8-OH-Gua.[19] Since 5-formyluracil has not been measured in our laboratory, it will not be discussed. Removal of intact DNA bases by prepurification of calf thymus DNA hydrolysates using HPLC was shown to prevent artifactual formation of these modified bases during derivatization. However, the procedures for hydrolysis and derivatization described in the literature have not been followed. Unfortunately, the authors failed to mention the significant differences in the experimental procedures.[18,19] Moreover, levels of the modified bases measured by GC/MS prior to prepurification were compared to only two values given in the literature. A large number of relevant papers reporting levels of these modified bases in DNA were not cited. Table I shows the reported values before and after prepurification of DNA hydrolysates,[18,19] and levels of the same modified bases in the literature. This is a partial listing due to space limitation. It should be pointed out that the literature values in Table I were obtained without prepurification of DNA hydrolysates and with derivatization under nitrogen atmosphere at temperatures such as 120°C. An examination of Table I clearly shows that the levels of 5-OH-Cyt and 8-OH-Ade in the literature were not as high as those reported prior to prepurification.[18,19] Most values for the level of 5-OH-Cyt were even lower than the level measured after prepurification.[18,19] Levels of 8-OH-Ade were never as high as 157 lesions/10^6 DNA bases but were close to, the same as, or smaller than the value after prepurification. The same holds true for 5-OHMeUra and 8-OH-Gua (Table I). In fact, all the literature levels of 5-OHMeUra shown in Table I were smaller than that measured prior to prepurification.[18,19] The levels of 8-OH-Gua were not as high as 410 lesions/10^6 DNA bases. A number of these values were quite close to those obtained after prepurification.[18,19] Again, it should be pointed out that the literature values in Table I and elsewhere were not cited by Douki *et al.* and Cadet *et al.*[18,19] Furthermore, there was no emphasis on the fact that the artifactual formation observed in their laboratory was restricted to only four compounds among the many compounds listed in Figure 1. All these facts raise the question of the validity of the claims about the measurement of DNA base damage by GC/MS presented in those papers.[18,19]

TABLE I Levels of four modified bases in DNA (lesions/10^6 DNA bases) as measured by GC/MS in different laboratories

5-OH-Cyt	8-OH-Ade	8-OH-Gua	5-OHMeUra		Reference
70	158	410	31	*direct*	[18,19]
24	12	25	—	*prepurification*	[18,19]
—	23	65	—		[8]
—	6	32	—		[22]
—	10	30	—		[23]
10	27	35	3		[24]
7, 10	4,7	9,13	4		[26]
5, 5, 12	10, 6, 20	25, 26, 29	4, 5, 6		[27]
8	16	37	7		[28]
—	25	45	—		[29]
32	26	26	8		[30]
20	5	22	14		[31]
32	38	32	22		[32]
4	10	26	2		[33]
15	15	70	—		[34]
14, 16	17, 20	18, 22	6, 12		[35]
24	29	77	7		[43]
19	15	47	3		[46]
10	6	60	7		[47]
9, 10, 22	11, 16, 19	22, 31, 37	1, 1, 5		[48]
19	38	96	—		[49]
22	22	90	10		[50]
22	16	48	—		[51]

DERIVATIZATION AT ROOM TEMPERATURE VERSUS AT HIGH TEMPERATURE

As mentioned above, the derivatization of 8-OH-Gua was performed by Hamberg and Zhang at room temperature with a prior addition of trifluoroacetic acid to DNA hydrolysates to make 8-OH-Gua soluble.[16] Recently, the effect of temperature on the derivatization of multiple products from all four DNA bases was studied.[20] Three different temperatures were used including room temperature. Levels of eleven products were measured. Higher background levels of 8-OH-Cyt, 5-OHMeUra, 8-OH-Ade and 8-OH-Gua were observed at higher temperatures, whereas the levels of the remaining seven modified bases were not affected by an increase in temperature. Formyluracil was not measured. Purging with nitrogen prior to derivatization decreased the levels of the above products at high temperatures, although levels obtained at room temperature were still lower. This study confirmed that the artifactual formation of modified bases is restricted to only four modified bases among the 20 or so modified bases detectable by GC/MS, in contrast to the claims made by Cadet *et al.*[19] It was also reported that the addition of trifluoroacetic acid to derivatization mixtures was not suitable for analysis of pyrimidine-derived lesions.

Table II shows the levels of 8-OH-Cyt, 5-OHMeUra, 8-OH-Ade and 8-OH-Gua in commercial calf thymus DNA measured at three different temperatures.[20] The levels of the latter two compounds measured in human lymphocytes following room temperature derivatization are also shown.[8] For comparison, the levels of the four compounds previously published in the literature are given. It should be pointed out that the latter levels were measured following derivatization at high temperatures such as 120°C. An examination of Table II reveals that the levels of 8-OH-Cyt, 5-OHMeUra, 8-OH-Ade and

TABLE II Levels of four modified bases in DNA (lesions/10^6 DNA bases) as measured by GC/MS following various derivatization temperatures

5-OH-Cyt	8-OH-Ade	8-OH-Gua	5-OHMeUra		Reference
157	159	272	34	140°C	[20]
64	62	82	13	90°C	[20]
40	30	30	7	room temperature	[20]
—	23	65	—	room temperature	[8]
—	6	32	—		[22]
—	10	30	—		[23]
10	27	35	3		[24]
7,10	4,7	9,13	4		[26]
5,5,12	10,6,20	25,26,29	4,5,6		[27]
8	16	37	7		[28]
—	25	45	—		[29]
32	26	26	8		[30]
20	5	22	14		[31]
32	38	32	22		[32]
4	10	26	2		[33]
15	15	70	—		[34]
14,16	17,20	18,22	6,12		[35]
24	29	77	7		[43]
19	15	47	3		[46]
10	6	60	7		[47]
9,10,22	11,16,19	22,31,37	1,1,5		[48]
19	38	96	—		[49]
22	22	90	10		[50]
22	16	48	—		[51]

8-OH-Gua published in the literature are smaller than or quite similar to those obtained at room temperature. Some levels of 8-OH-Gua and 5-OHMeUra were higher. In this context, of course, one should keep in mind that different lots of calf thymus DNA and DNA samples from different sources may contain varying amounts of modified bases. The data in Table II clearly indicate that derivatization at high temperature does not always cause artifactual formation of those four products if oxygen is successfully removed from derivatization mixtures. It is also clear from Table II as well as from Table I that artifacts of derivatization may vary between laboratories and depend on the experimental conditions, especially on the exclusion of oxygen during derivatization. If one is not certain that oxygen can be excluded entirely, the derivatization at room temperature perhaps may be more suitable. However, one should keep in mind that not all products may be soluble and/or derivatized completely at room temperature, as was emphasized by England *et al.*[20] The facts discussed in this section contrast the claims by Cadet *et al.*[19]

PREPURIFICATION OR NOT?

As discussed above, Tables I and II present comparisons of the levels of four modified bases in DNA, which were obtained with or without prepurification,[18] or derivatization at room temperature,[8,20] with those previously published levels measured using high temperature derivatization. These comparisons clearly show that there is no need for prepurification of DNA hydrolysates prior to derivatization if oxygen is excluded from derivatization mixtures or derivatization is done at room temperature. High levels of the four aforementioned products measured by Douki *et al.*[18,19] without prepurification may have been caused by the presence

of oxygen in derivatization mixtures and/or experimental conditions, which were different from those previously published. Prepurification is a tedious procedure and requires the use of stable isotope-labeled analogues of modified bases. The work by England *et al.*[20] demonstrated that the use of non-labeled internal standards may provide similar values to those obtained using stable isotope-labeled analogues as internal standards. These standards are very expensive to obtain and may not be available in all laboratories. Even if they are available, the data in Tables I and II clearly demonstrate that there is no need for a prepurification of DNA hydrolysates prior to derivatization if the derivatization is performed under proper experimental conditions.

IS CALF THYMUS DNA CELLULAR DNA?

During the Copenhagen meeting, an argument was put forward that calf thymus DNA was not cellular DNA and thus the results obtained with it could not be compared to those in the literature obtained with DNA from cultured cells and tissues. Firstly, calf thymus DNA is cellular DNA, as it was isolated from calf thymus, a tissue as any other tissue. Secondly, even if this argument were true, then it would be illogical to dismiss all the literature values of the aforementioned modified bases, which were measured by GC/MS in cellular DNA without so-called prepurification. Unfortunately, that is exactly what has been done in the review article by Cadet *et al.*[19] Furthermore, the argument that calf thymus DNA may contain metal ions such as iron and copper ions, which may cause artifactual formation of modified bases, does not make any sense in this case. It is true that calf thymus DNA may contain metal ions and this has been clearly demonstrated previously.[39] However, Douki *et al.* did not attempt to remove metal ions from calf thymus DNA prior to hydrolysis and derivatization.[18] Therefore, the artifactual formation of modified bases during hydrolysis and/or derivatization may have been due to metal ions. For these reasons, the literature values cannot be dismissed on the basis of the results obtained with calf thymus DNA, as was done by Cadet *et al.*[19]

FACTS ABOUT ANALYSIS OF FORMAMIDOPYRIMIDINES BY GC/MS

A recent paper by Douki *et al.*[40] reported the complete destruction of 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua) and 4,6-diamino-5-formamidopyrimidine (FapyAde) by formic acid (88% or 60%) under the conditions of DNA hydrolysis. The results are in disagreement with previously published literature data regarding these compounds. FapyGua and FapyAde have been measured by GC/MS following formic acid hydrolysis for many years in our laboratory and by other researchers with no difficulties. Even the publication that reported first the application of GC/MS to the measurement of oxidative DNA base damage showed the separation of these compounds by GC and reported the mass spectra of their trimethylsilyl (TMS) derivatives recorded during GC/MS analysis.[10] Subsequently, the identification and quantification of these compounds by GC/MS have been reported for many types of DNA including isolated DNA, isolated chromatin, or DNA isolated from cultured cells or tissues. It is not the purpose of this article to review all the data in the literature. However, a few examples will be presented to clarify recent misleading claims about the analysis of FapyGua and FapyAde.[19,40] Figure 2 illustrates the ion-current profiles of the TMS derivatives of FapyGua and its stable isotope-labeled analogue recorded during GC/MS analysis of a trimethylsilylated formic acid-hydrolysate of γ-irradiated chromatin.[15] The dose–yield plot for the formation of FapyGua formed in γ-irradiated chromatin is shown in Figure 3. These data contrast a recent claim by

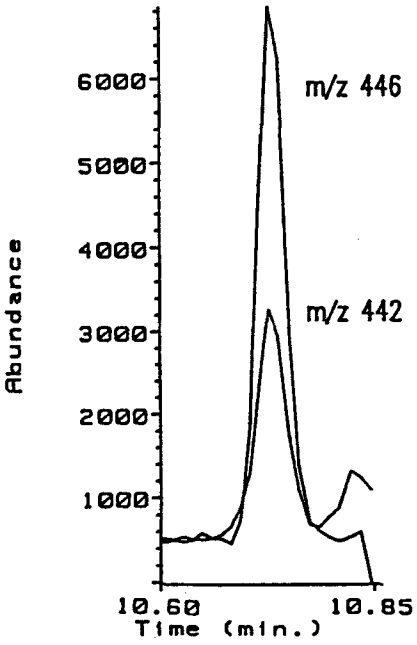

FIGURE 2 Ion-current profiles of the trimethylsilyl derivatives of FapyGua (m/z 442) and its stable isotope-labeled analogue (m/z 446) recorded during GC/MS analysis of γ-irradiated chromatin.[15]

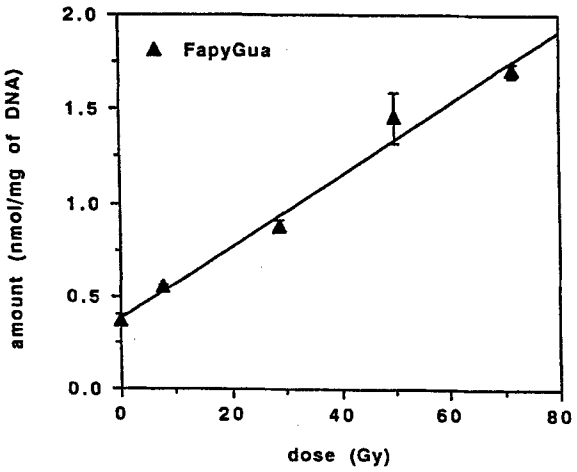

FIGURE 3 Radiation dose–yield plot of FapyGua formed in γ-irradiated chromatin.[15] One nmol of a lesion/mg of DNA corresponds to ≈32 lesions/10^5 DNA bases.

Douki *et al.*[40] that the only information on the formation of FapyGua in γ-irradiated DNA had been done without the use of the labeled internal standard by citing an earlier work from our

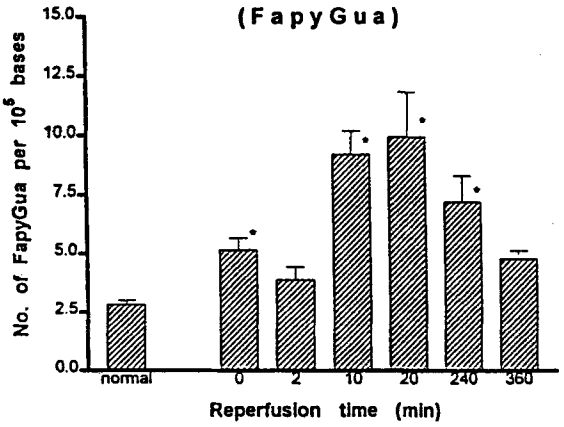

FIGURE 4 Formation and repair of FapyGua in mouse forebrain following ischemia-reperfusion.[34]

laboratory.[41] Moreover, we have also shown the formation of FapyGua and other purine and pyrimidine lesions in isolated chromatin by exposure to γ-irradiation under various gaseous conditions.[42] The formation of FapyGua and FapyAde in cultured human cells exposed to ionizing radiation has also been demonstrated.[43] Malins and Gunselman[29] showed the formation of FapyGua and FapyAde in the DNA of wild fish exposed to toxic chemicals. We demonstrated the formation and cellular repair of FapyGua and FapyAde, and other lesions in H_2O_2-treated cultured human cells using GC/MS with formic acid hydrolysis of cellular DNA.[33] As Figure 4 illustrates, the formation and repair of FapyGua has been shown in mouse forebrain following ischemia-reperfusion.[34] In similar studies, Spencer *et al.* and Abalea *et al.* demonstrated the formation and repair of these compounds among other lesions in human respiratory tract epithelial cells and in primary rat hepatocyte cultures, respectively.[44,45] There are many other examples of the application of GC/MS with formic acid hydrolysis to the measurement of these compounds in DNA. The procedures used by Douki *et al.*[40] included the hydrolysis of DNA with formic acid in sealed vials without the removal of oxygen and the removal of formic acid from DNA hydrolysates under vacuum at room

temperature. In contrast, other studies performed the formic acid hydrolysis in evacuated and sealed tubes and removed formic acid from DNA hydrolysates by lyophilization at low temperature. Unfortunately, these authors did not mention the significant differences between the procedures. Moreover, they did not cite the many studies in the literature that have successfully measured FapyGua and FapyAde by GC/MS using formic acid hydrolysis. These facts question the validity of the recent claims about the previous measurements of these compounds.[19,40] At present, it is not clear as to why Douki *et al.*[40] were not able to prevent the destruction of these compounds. Oxygen, which was not removed from hydrolysis vials may have played a role. Our experience with these compounds shows that hydrolysis of DNA with formic acid must be done in evacuated and sealed tubes, and DNA hydrolysates must be lyophilized in order to avoid any artifacts.

FACTS ABOUT THE MEASUREMENT OF DNA REPAIR BY GC/MS

More than a decade ago, we introduced the concept of measurement of DNA repair by GC/MS.[10] Since then, GC/MS has extensively been used for the study of the substrate specificities of DNA glycosylases.[52–60] These included *E. coli* Fpg and Nth proteins, T4 endonuclease V, *E. coli* and human uracil glycosylases, *S. pombe* and human Nth proteins, *S. cerevisiae* Ogg1 protein and *Drosophila* ribosomal S3 protein. The great advantage of GC/MS is its capacity to simultaneously identify and quantify a multitude of base lesions in the same DNA sample. For this reason, the technique readily determines which lesions are excised or not excised from DNA by a given DNA glycosylase under the same conditions. Simultaneous measurement of excision rates of lesions can also be achieved.[57–60] Repair experiments involving DNA with multiple lesions are more representative of the circumstances in a cell than those with an oligonucleotide containing a single lesion.

For the purpose of determining the substrate specificity of a DNA glycosylase, damaged DNA containing multiple lesions is incubated with the enzyme and then precipitated with ethanol. This is followed by separation of DNA pellets and supernatant fractions. Pellets are analyzed by GC/MS after hydrolysis and supernatant fractions without hydrolysis because DNA glycosylases release free modified bases.[61] Removal of substrate lesions from pellets and appearance of lesions in supernatant fractions prove the activity of the enzyme on corresponding lesions.[52–60] Typical Michaelis–Menten kinetics of excision are also determined.[57–60] As examples, Figure 5 illustrates excision of 8-OH-Gua, FapyGua and FapyAde by *E. coli* Fpg protein from damaged DNA.[57] The amounts removed from the pellet fractions by the enzyme were accounted for by the appearance of these compounds in the supernatant fractions of the same samples. This proved that 8-OH-Gua, FapyGua and FapyAde are substrates of this enzyme. Other base lesions were not significantly excised from damaged DNA samples by this enzyme.[57] Figure 6 illustrates Lineweaver–Burk plots for excision of 8-OH-Gua and FapyGua from γ-irradiated DNA by *S. cerevisiae* Ogg1 protein.[60] The usefulness of GC/MS has been demonstrated for the determination of DNA repair in cells.[33,34,44,45] As examples, Figure 7 illustrates formation and repair of FapyGua and 8-OH-Gua in human cells exposed to H_2O_2.

Recently, Cadet *et al.* questioned the validity of the determination of substrate specificities of DNA glycosylases by GC/MS.[19] However, no data were presented that would disagree with the application of GC/MS to the studies of DNA repair. As was explained above, the excision of a lesion by a given DNA glycosylase is determined by measurement of the amounts of the lesion in DNA pellets and in supernatant fractions following enzymatic treatment and precipitation with ethanol. As Figure 5 and the published data[52–60]

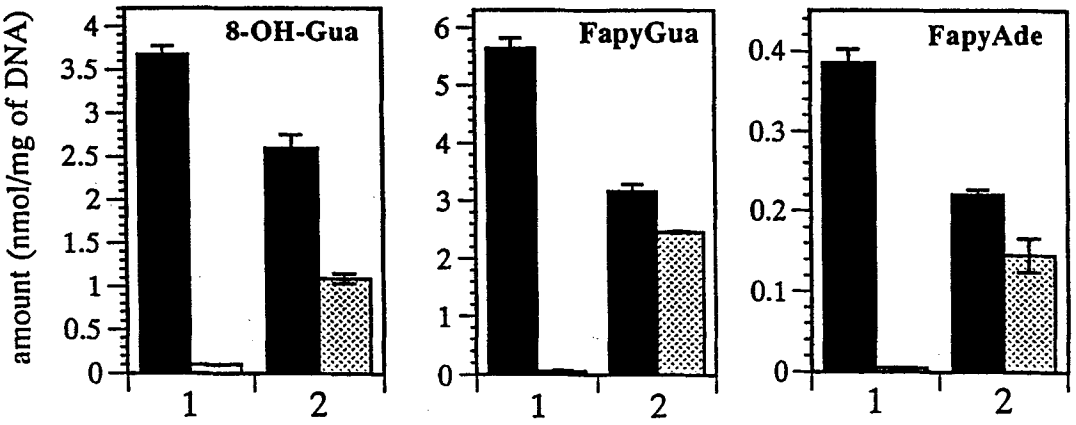

FIGURE 5 Excision of 8-OH-Gua, FapyGua and FapyAde by *E. coli* Fpg protein from DNA treated with H_2O_2/Fe(III)-EDTA.[57] Dark columns, pellets; light columns, supernatant fractions. 1, Incubation without Fpg protein; 2, incubation with Fpg protein. Each column represents the mean (± standard deviation) of the values obtained from the analysis of three independently prepared samples. One nmol of a lesion/mg of DNA corresponds to ≈32 lesions/10^5 DNA bases.

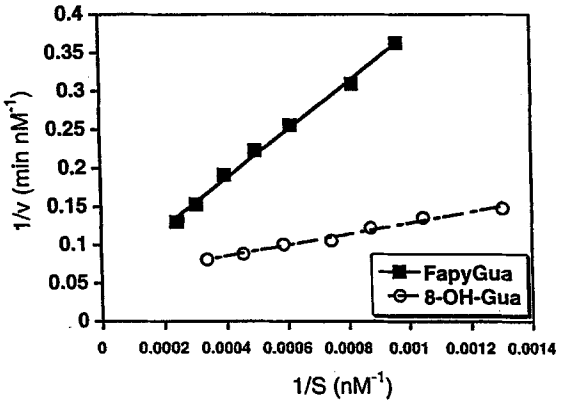

FIGURE 6 Lineweaver–Burk plots for excision of 8-OH-Gua and FapyGua by *S. cerevisiae* Ogg1 protein from γ-irradiated DNA.[60] [S], concentration of 8-OH-Gua or FapyGua; v, initial velocity. The amounts of products found in supernatant fractions were used for initial velocity.

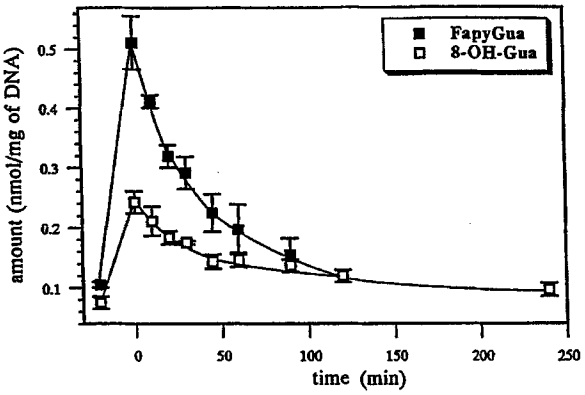

FIGURE 7 Formation and repair of FapyGua and 8-OH-Gua in human cells exposed to H_2O_2 as determined by GC/MS.[33]

clearly show, the excision of a lesion is proved by the similarity of the amount of the lesion removed from the pellet fractions to the amount found in the supernatant fractions of the same DNA sample. Subsequently, supernatant fractions are used for determination of excision kinetics.[57–60] These fractions do not contain DNA, which is precipitated by ethanol. Furthermore, supernatant fractions are not hydrolyzed. However, they are derivatized following lyophilization and then analyzed by GC/MS. Therefore, it is not possible to artifactually generate any of substrate lesions that are detected and quantified using supernatant fractions. Even if some lesions are artifactually generated in DNA pellets to some extent, it would make no difference, because the proof of the removal of a lesion is based on significant differences between control samples and those treated with the enzyme. All these facts have been explained in relevant published papers and clearly question the validity of the claims made by Cadet *et al.*[19]

CONCLUSIONS

The scientific facts presented in this article indicate the following points:

1. A thorough review of the published data clearly shows that recent claims about the artifactual formation of modified bases were made without citing the vast majority of the data.
2. In those claims, it has not been emphasized that artifactual formation during derivatization of DNA hydrolyzates is restricted to only four modified bases among the many pyrimidine- and purine-derived lesions.
3. There has been no emphasis discussing the substantial differences between experimental procedures that could account for the artifactual formation. The previously reported levels of those four modified bases were smaller than, similar or close to those after prepurification of DNA hydrolyzates prior to derivatization.
4. The facts presented in this article indicate that there is no need for prepurification of DNA hydrolyzates, if the derivatization is carried out under proper experimental conditions.
5. The same is true for analysis of formamidopyrimidines by GC/MS. In the past, these compounds have been successfully analyzed by GC/MS. This fact has been ignored in recent claims indicating that formamidopyrimidines were destroyed during formic acid hydrolysis. Moreover, the fact that there were substantial differences between hydrolysis conditions has not been mentioned. A thorough review of published data clearly show that these compounds are hydrolyzed by formic acid without destruction if the hydrolysis is performed under proper conditions.
6. It is clear that different results may be obtained in different laboratories. Furthermore, different techniques may provide different results (other papers, this volume). Data obtained in various laboratories should be compared by discussing all relevant data in the literature and all scientific facts including experimental differences between laboratories. In taking this approach, misleading claims can be eliminated.

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