Formation of cytosine glycol and 5,6-dihydroxycytosine in deoxyribonucleic acid on treatment with osmium tetroxide

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OsO₄ selectively forms thymine glycol lesions in DNA. In the past, OsO₄-treated DNA has been used as a substrate in studies of DNA repair utilizing base-excision repair enzymes such as DNA glycosylases. There is, however, no information available on the chemical identity of other OsO₄-induced base lesions in DNA. A complete knowledge of such DNA lesions may be of importance for repair studies. Using a methodology developed recently for characterization of oxidative base damage in DNA, we provide evidence for the formation of cytosine glycol and 5,6-dihydroxycytosine moieties, in addition to thymine glycol, in DNA on treatment with OsO₄. For this purpose, samples of OsO₄-treated DNA were hydrolysed with formic acid, then trimethylsilylated and analysed by capillary gas chromatography–mass spectrometry. In addition to thymine glycol, 5-hydroxycytosine (isobarbituric acid), 5-hydroxycytosine and 5,6-dihydroxyuracil (isodialuric acid or dialuric acid) were identified in OsO₄-treated DNA. It is suggested that 5-hydroxyuracil was formed by formic acid-induced deamination and dehydration of cytosine glycol, which was the actual oxidation product of the cytosine moiety in DNA. 5-Hydroxycytosine obviously resulted from dehydration of cytosine glycol, and 5,6-dihydroxyuracil from deamination of 5,6-dihydroxycytosine. This scheme was supported by the presence of 5-hydroxycytosine, uracil glycol and 5,6-dihydroxyuracil in OsO₄-treated cytosine. Treatment of OsO₄-treated cytosine with formic acid caused the complete conversion of uracil glycol into 5-hydroxyuracil. The implications of these findings relative to studies of DNA repair are discussed.

INTRODUCTION

Oxidative damage to DNA caused by reactive oxygen species, e.g. hydroxyl radicals, unless repaired by cellular repair processes, may have detrimental effects in living cells, such as mutagenesis, carcinogenesis and cell death (Painter, 1980; Ames, 1983; Friedberg, 1984; Cerutti, 1985). Oxidative damage can also be introduced into DNA by reagents such as OsO₄ and KMnO₄, which react readily with unsaturated organic compounds to give cis-glycols among other products (Hofmann, 1912; Criegee, 1936; Schröder, 1980). Investigations have shown that OsO₄ or KMnO₄ react with thymine in oligonucleotides and DNA to form cis-thymine glycol (Bayley & Jones, 1959; Burton & Riley, 1966; Beer et al., 1966; Darby et al., 1967; Iida & Hayatsu, 1971; Frenkel et al., 1981). The reactivity of OsO₄ with DNA bases other than thymine has been reported to be minimal or absent (Beer et al., 1966). However, under vigorous conditions, permanganate oxidation of DNA has been reported to lead to some ureido residues of thymine, cytosine and guanine (Bayley & Jones, 1959; Darby et al., 1967). Formation of 5-hydroxy-5-methylbarbituric acid on oxidation of thymine with KMnO₄ has also been observed (Iida & Hayatsu, 1970, 1971). The reactions of Os(VIII) reagents with cytosine in the presence of some ligands have been reported to form o xo-Os(VI) esters of uracil (Chang et al., 1981). However, no products from the reaction of OsO₄ with cytosine in DNA have yet been reported.

In the past, the production of altered bases in DNA was initiated in order to provide a suitable substrate for studies of DNA repair. OsO₄ has been used commonly as the chemical reagent of choice in studies of thymine glycol release from DNA by DNA glycosylases (Hariharan & Cerutti, 1974; Gates & Linn, 1977; Nes, 1980; Demple & Linn, 1980; Brent, 1983), on the basis of its selective conversion of thymine in DNA into thymine glycol. Typically, reaction mixtures included OsO₄-treated DNA and either cell extracts or partially purified enzymes. Those studies, however, have been conducted without any information on the possible OsO₄-induced products of DNA bases other than thymine. A complete knowledge of base alterations in OsO₄-treated DNA, in addition to thymine glycol, may be of great importance for the studies of DNA repair that use this reagent as well as other similarly acting oxidative agents in the synthesis of DNA substrates.

The present study was undertaken to elucidate other possible OsO₄-induced products in DNA. For this purpose we employed an assay that has been developed for characterization of oxidative DNA base damage (Dizdaroglu, 1984, 1985). Here we present evidence that cytosine glycol and 5,6-dihydroxycytosine moieties are formed in DNA on treatment with OsO₄.
MATERIALS AND METHODS

Materials

Calf thymus DNA, 5-hydroxyuracil (isobarbituric acid), cytosine, 5-methylcytosine, thymine, uracil, bis-(trimethylsilyl)trifluoroacetamide and 5,6-dihydroxythymine were purchased from Sigma Chemical Co. Acetonitrile was isolated from Spectrapor; membranes (trimethylsilyl)trifluoroacetamide/acetonitrile coated with black, coloured Linn, Oxidation of isobarbituric acid (2%)

was carried out with a fused-silica capillary (2.5 m × 0.2 mm internal diam.) coated with cross-linked SE-54 (5% phenylmethylsilicone; film thickness 0.11 μm) (Hewlett-Packard). Helium was used as the carrier gas with a linear velocity of 23.4 cm/s through the column. The split ratio was 10:1. Mass spectra were taken at 70 eV. The temperature of the ion source was approx. 200 °C.

Separations were carried out with a Hewlett-Packard model 1090 microprocessor-controlled liquid chromatograph equipped with a model HP 1040A high-speed spectrophotometric detector and a 10 cm × 0.21 cm Hypersil ODS microbore reversed-phase column (particle size 5 μm; Hewlett-Packard). For semi-preparative separations, a Supelcosil LC-8-DB column (25 cm × 1 cm; particle size 5 μm; Supelco) was used.

RESULTS AND DISCUSSION

DNA samples were hydrolysed with formic acid and subsequently analysed by capillary g.c.-m.s. by using a method described previously (Dizdaroglu, 1985). Fig. 1 shows a total-ion chromatogram obtained from a trimethylsilylated formic acid hydrolysate of OsO₄-treated DNA. Peaks 1–4, 13 and 14 represent the trimethylsilyl derivatives of phosphoric acid, 2-deoxyribose, thymine, cytosine, adenine and guanine respectively. Peak 5 corresponds to the trimethylsilyl derivative of 5-methylcytosine, for which authentic material was available. Calf thymus DNA is known to have a 5-methylcytosine content of about 1.3% (Adams et al., 1981). Peak 7 also represents cytosine but with an additional trimethylsilyl group attached to its amino group, as revealed both by comparison with authentic material and by its mass spectrum. Peaks marked with ‘x’ were also present in control samples. Peak 10, corresponding to the trimethylsilyl derivative of cis-thymine glycol, was verified by the use of authentic material and by comparison of its mass spectrum with previously published data (Dizdaroglu, 1984). trans-Thymine glycol was also observed (peak 12 in Fig. 1); however, its presence was due to the partial isomerization of cis-thymine glycol by acidic treatment (Iida & Hayatsu, 1970). The isomerization was confirmed in the present work by g.c.-m.s. analysis of formic acid-treated cis-thymine glycol. Peak 8 appeared to correspond to a sugar derivative; however, its origin could not be defined.

Peaks 6, 9 and 11 in Fig. 1 represent the trimethylsilyl derivatives of 5-hydroxyuracil (isobarbituric acid), 5-hydroxycytosine and 5,6-dihydroxyuracil (isodialuric acid or dialuric acid) respectively. Of these three, authentic materials were available for isobarbituric acid and isodialuric acid (for the mass spectra of their trimethylsilyl derivatives see Dizdaroglu, 1983). The trimethylsilyl derivative of dialuric acid is known to be identical with that of isodialuric acid as a consequence of enolization (Schuchmann & von Sonntag, 1983). 5-Hydroxycytosine was identified on the basis of the known mass spectrum and gas-chromatographic behaviour of its trimethylsilyl derivative (Dizdaroglu, 1984). The two uracil derivatives discussed above are believed to be derived from some OsO₄-induced products of the cytosine moiety of DNA by acid treatment. For example, it is well known that deamination and dehydration readily occur with the cytosine derivatives saturated at the C-5–C-6 double bond (Green & Cohen, 1957; Evans et al., 1975; Taguchi et al., 1977; Teoule & Cadet, 1978). It is therefore suggested that 5-hydroxyuracil was formed
by formic acid-induced deamination and dehydration of cytosine glycol, which was the actual oxidation product of the cytosine moiety of DNA. 5-Hydroxycytosine was most probably the result of the dehydration of the same compound (Scheme 1).

The presence of 5,6-dihydroxyuracil (peak 11 in Fig. 1) can be explained analogously to the formation of 5-hydroxy-5-methylbarbituric acid from thymine on permanganate oxidation (Iida & Hayatsu, 1970, 1971) (Scheme 2). The actual oxidation products of cytosine, i.e., structures (I) and (II), were not observed in DNA. As the calf thymus DNA used in this work did not contain uracil, the uracil derivatives discussed above could not have been formed from uracil in DNA.

In order to support the suggestion that 5-hydroxuracil and 5,6-dihydroxuracil were derived from the cytosine moiety of DNA, cytosine was also treated with OsO₄ and analysed by g.c.-m.s. and h.p.l.c. before and after formic acid treatment. Fig. 2 shows a total-ion chromatogram obtained from a sample of OsO₄-treated cytosine after trimethylsilylation. Peaks 1 and 3 represent cytosine, and correspond to peaks 4 and 7 in Fig. 1. Peak 2 represents the trimethylsilyl derivative of 5-hydroxuracil (as peak 6 in Fig. 1). A mass spectrum taken from peak 4 is illustrated in Fig. 3. A molecular ion (M⁺⁺) and a characteristic M−CH₃ ion were observed at m/z 434 and 419 respectively. This mass spectrum was assigned to the trimethylsilyl derivative of 5,6-dihydroxy-5,6-dihydro-uracil (uracil glycol), which shows a fragmentation pattern similar to that of the trimethylsilyl derivative of thymine glycol (for a comparison of the mass spectra see Dizdaroglu, 1984). Authentic uracil glycol was also available, obtained from OsO₄ oxidation of uracil. Since cytosine used here did not contain uracil, the presence of uracil glycol in OsO₄-treated cytosine can only be explained by deamination of cytosine glycol, which was the actual oxidation product of cytosine. However, it is not known whether the deamination occurred spontaneously in aqueous solution or was induced by trimethylsilylation.

Peak 5 in Fig. 2 represents the trimethylsilyl derivative of 5,6-dihydroxyuracil, also observed in OsO₄-treated DNA, as explained above (peak 11 in Fig. 1). Although the g.c.-m.s. analysis of OsO₄-treated cytosine after
formic acid treatment clearly showed the absence of uracil glycol (Fig. 4), 5-hydroxyuracil and 5,6-dihydroxyuracil were present (peaks 2 and 4 in Fig. 4 respectively). The increased amount of 5-hydroxyuracil (compare peaks 2 in Figs. 2 and 4) indicates its formation from uracil glycol by dehydration. In confirmation, formic acid treatment completely converted authentic uracil glycol into 5-hydroxyuracil.

OsO₄-treated cytosine was also analysed by reversed-phase h.p.l.c., as illustrated in Fig. 5. Cytosine gave a broad and tailing peak under these conditions (peak 5). Compounds represented by peaks 1–4 were collected by the use of a semi-preparative column and subsequently analysed by g.c.–m.s. Peaks 2 and 3 were found to correspond to uracil glycol and 5,6-dihydroxyuracil respectively. Fraction 2 also contained trace amounts of 5-hydroxyuracil. Fractions 1 and 4 contained no uracil or cytosine derivatives.

It is noteworthy that 5,6-dihydrothymine, which had been tentatively identified in OsO₄-treated DNA in
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previous work (Demple & Linn, 1980), was not observed in the present paper. This compound is easily detectable by g.c.–m.s. as demonstrated previously (Dizdaroglu, 1984, 1985), and authentic material was also available. The absence of 5,6-dihydrothymine from OsO₄-treated DNA confirms the assumption by Demple & Linn (1980) that this compound was most probably not formed in DNA by OsO₄ treatment.

The yields of conversion of thymine and cytosine into the products identified on OsO₄ treatment of DNA under the conditions described in the Materials and methods section were determined by g.c. with a flame ionization detector. Uracil was used as an internal standard and added to the samples before formic acid hydrolysis. The relative molar response factors versus uracil of thymine, thymine glycol, cytosine, 5-hydroxycytosine and 5,6-
dihydroxyuracil were determined to be 0.95, 0.75, 1.1, 0.9 and 0.8 respectively. The relative molar response factor of 5,6-dihydroxycytosine versus uracil was assumed to be 1.0. The results showed that approx. 73% of thymine and 20% of cytosine were converted into products. The ratio of cytosine glycol (in terms of the sum of 5-hydroxyuracil and 5-hydroxycytosine) to 5,6-dihydroxycytosine (in terms of 5,6-dihydroxyuracil) was approx. 4:1.

CONCLUSIONS

The data presented here clearly suggest that, in addition to thymine glycol, cytosine glycol and 5,6-dihydroxycytosine are formed in DNA on OsO₄ treatment, and their subsequent deamination and dehydration give rise to formation of uracil glycol, 5-hydroxycytosine, 5-hydroxyuracil and 5,6-dihydroxyuracil. These results may be of importance to DNA repair studies that use OsO₄-treated DNA as a substrate for base-excision repair enzymes such as glycosylases.

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REFERENCES


Fig. 4. Total-ion chromatogram obtained from a sample of OsO₄-treated cytosine after formic acid treatment and trimethylsilylation

Column details were as given in Fig. 1 legend.

Fig. 5. H.p.l.c. profile obtained from a sample of OsO₄-treated cytosine

The column was microbore Hypersil ODS (10 cm × 0.21 cm; particle size 5 μm). The flow rate was 0.1 ml/min. The eluent was prepared from water (solvent A) and acetonitrile (solvent B); isocratic elution was with solvent A for 5 min, then gradient elution at 0.5% solvent B/min, at ambient temperature.

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