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Note

Detection of oxidatively modified 2′-deoxyguanosine-3′-monophosphate, using ^{32}P-postlabelling and anion-exchange thin-layer chromatography

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When 2′-deoxyguanosine-3′-monophosphate is treated with L-ascorbic acid and hydrogen peroxide, followed by an enzyme-catalysed reaction with [^{32}P]ATP and anion-exchange thin-layer chromatography (TLC), an extra radioactive spot appears close to 2′-deoxyguanosine-3′,5′-diphosphate. It is postulated that this spot represents the C-8 hydroxy derivative of 2′-deoxyguanosine-3′,5′-diphosphate, a compound possibly involved in carcinogenesis induced by oxygen radicals.

Hydroxylation on C-8 in guanosine residues in DNA seems to be an important aspect of oxygen-radical-induced carcinogenesis [1]. Recently, an ultrasensitive analytical technique, called ^{32}P-postlabelling analysis, was described to detect minute amounts of modified base residues in DNA [2]. It consists of cleaving the DNA enzymically to 3′-monophosphate-2′-deoxynucleosides (dNp), incorporating a radioactive ^{32}P atom on the 5′-hydroxy group to produce 3′,5′-diphosphate-2′-deoxynucleosides (dpNp), chromatographing the mixture on an anion-exchange thin-layer and autoradiographing the TLC plate by an X-ray film. The picture obtained after development of the X-ray film shows the characteristic pattern of the normal DNA nucleotides plus extra spots representing the covalently modified nucleotides.

We applied this technique to search for oxidatively modified guanosine after treatment with L-ascorbic acid and hydrogen peroxide, a mixture known to hydroxylate in vitro 2′-deoxyguanosine to the C-8 hydroxy derivative [3]. The C-8 hydroxylation of guanosine base residues in a DNA template causes misreading at the modified base and at adjacent residues, and could be an important cause

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of mutation and/or carcinogenesis [1]. This paper shows the possible use of anion-exchange TLC to detect oxidatively damaged nucleotides.

EXPERIMENTAL

Reagents and instrumentation

Perhydrol (30% hydrogen peroxide), L-ascorbic acid, ammonium sulphate and polyethyleneimine cellulose TLC plates were purchased from Merck (Darmstadt, F.R.G.). Apyrase and bicine–sodium hydroxide were from Sigma (Deisenhofen, F.R.G.). Deoxynucleotides [2′-deoxyadenosine-3′-monophosphate (dAp), 2′-deoxycytidine-3′-monophosphate (dCp), 2′-deoxythymidine-3′-monophosphate (dTp) and 2′-deoxyguanosine-3′-monophosphate (dGp)] were from Pharmacia (Uppsala, Sweden). Adenosine triphosphate (ATP) and T4-polynucleotide kinase were from Boehringer (Mannheim, F.R.G.). [^{32}P]ATP (specific activity 5000 Ci/mmol; 250 μCi) was from Amersham International (Amersham, U.K.). Autoradiography cassettes equipped with intensifying screens were purchased from Kodak (Rochester, NY, U.S.A.). The X-ray films (Hyperfilm MP) were purchased from Amersham International.

Oxidative damage

To 2 μl of an aqueous solution of the four nucleotides, containing 600 pmol of each, were added 2 μl of an extemporaneously prepared solution of 0.6 mg of L-

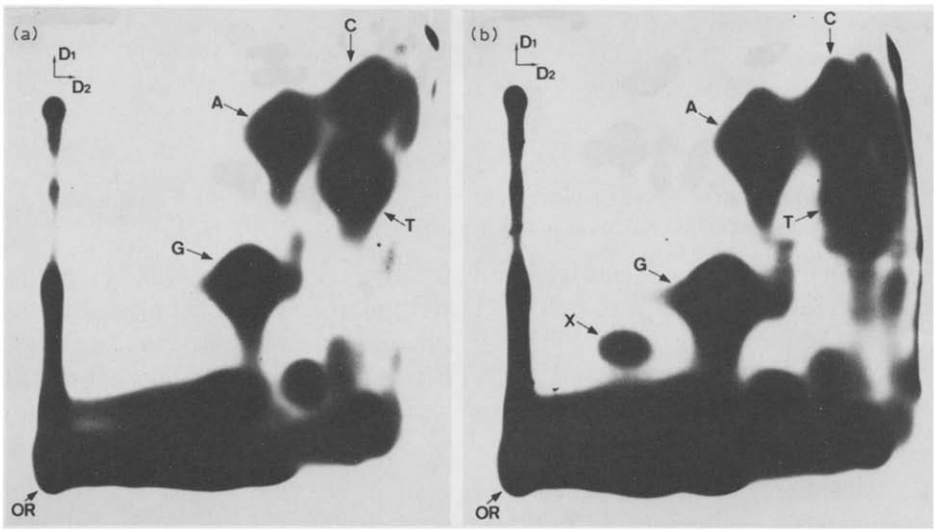

Fig. 1. Autoradiograms of the ^{32}P-postlabelling and anion-exchange TLC of (a) the 3′-monophosphate nucleosides dCp (C), dAp (A), dTp (T) and dGp (G), and (b) of the ^{32}P-postlabelling and anion-exchange TLC of the reaction mixture of these four 3′-monophosphate nucleosides with L-ascorbic acid and hydrogen peroxide. OR = origin; D1 = first development in 1.5 *M* ammonium formate buffer (pH 3.5); D2 = second development in 0.4 *M* ammonium sulphate; X = possibly the C-8 hydroxy guanosine derivative. The TLC plates were autoradiographed for 15 min at room temperature in autoradiography cassettes equipped with intensifying screens.

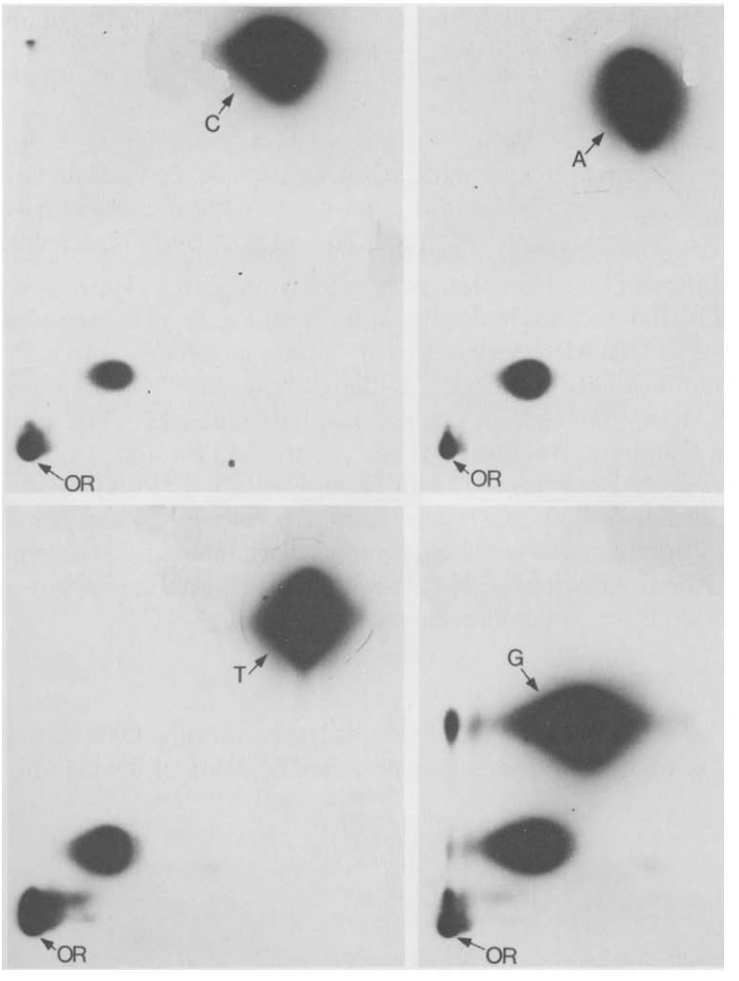

Fig. 2. Autoradiograms of the ^{32}P-postlabelling and anion-exchange TLC of the four individual nucleotides. Conditions and abbreviations as in Fig. 1.

ascorbic acid in 2 ml of deionized water and 2 μl of a solution of 1.2 μl of perhydrol in 2 ml of deionized water, also prepared extemporaneously. This mixture, kept at 37°C in the dark, was used for ^{32}P-postlabelling analysis. Apyrase treatment as a method for removing excess [^{32}P]ATP was performed by adding 5 μl of a solution of 3 mg of apyrase per 100 μl of deionized water.

^{32}P-Postlabelling

To the sample (volume 6 μl) to be analysed, 5.4 μl of bicine buffer (40 m*M* bicine–sodium hydroxide, 10 m*M* magnesium chloride, 10 m*M* dithiothreitol and 0.1 m*M* spermidine in deionized water and kept at −23°C between analyses), 1.5 μl of T4-polynucleotide kinase (49 000 U/mg, 8000 U/ml), 1.5 μl of [^{32}P]ATP and 1.0 μl of ATP (580 pmol ATP per μl deionized water) were added. The reaction mixture was kept at 37°C for 2 h. When needed, 5 μl of apyrase solution were added, and the mixture was kept at 37°C for another 30 min. A 10-μl aliquot

was applied on the TLC plate (10×10 cm), which previously was soaked in 0.1 *M* ammonium formate buffer (pH 3.5) for 15 min and dried in warm air. Development in the first direction was done with 1.5 *M* ammonium formate buffer (pH 3.5) to the top. After drying in warm air, the plate was soaked in a 10 m*M* Tris base solution for 10 min, then in deionized water for 5 min and finally dried. Development in the second direction was carried out with 0.4 *M* ammonium sulphate to the top. After drying in warm air, the plate was brought in contact with the X-ray film in an autoradiography cassette equipped with intensifying screens.

RESULTS AND DISCUSSION

Fig. 1 shows the autoradiograms of the ^{32}P-postlabelling of the solution of the 3′-monophosphate nucleosides (1a) and of the reaction mixture of these nucleo-

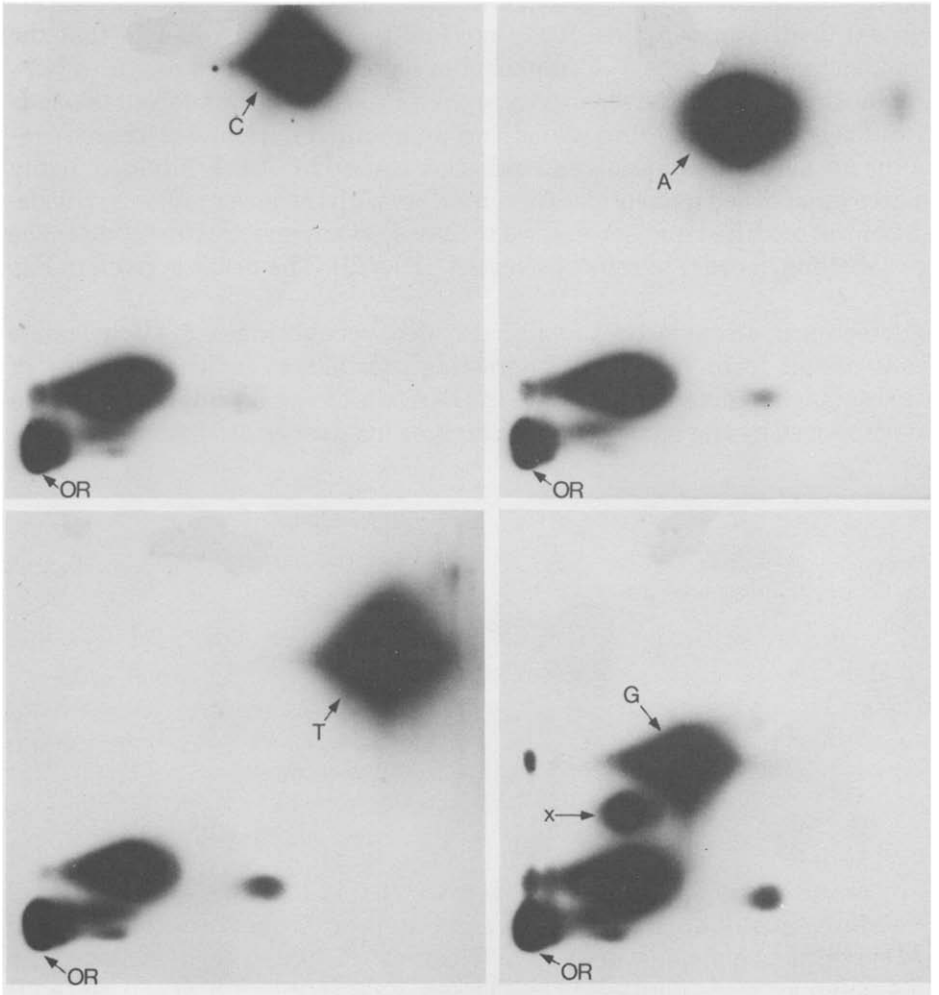

Fig. 3. Autoradiograms of the ^{32}P-postlabelling and anion-exchange TLC of the reaction mixtures of the four individual nucleotides with L-ascorbic acid and hydrogen peroxide. Conditions and abbreviations as in Fig. 1.

tides with L-ascorbic acid and hydrogen peroxide (1b). Fig. 1b illustrates the appearance of an extra radioactive spot (X) near the spot representing ^{32}P-labelled dpGp. Fig. 2 shows the autoradiograms of the 5′-^{32}P-labelled nucleotides (dpAp, dpCp, dpGp and dpTp).

Fig. 3 shows the autoradiograms of the ^{32}P-labelled reaction mixture of the 3′-phosphate nucleosides with L-ascorbic acid and hydrogen peroxide. In the presence of L-ascorbic acid and hydrogen peroxide an extra radioactive spot appears close to the spot representing 2′-deoxyguanosine-3′,5′-diphosphate. This extra spot does not appear when dGp is treated without L-ascorbic acid and hydrogen peroxide or when the three other nucleotides are treated with these oxidizing agents. This is in agreement with the high-performance liquid chromatographic (HPLC) results obtained by Kasai and Nishimura [3]. These authors also detected an extra peak in the HPLC chromatogram, which they identified as 8-hydroxy-2′-deoxyguanosine, after oxidation of 2′-deoxyguanosine with L-ascorbic acid and hydrogen peroxide. It is therefore tempting to speculate that the extra radioactive spot is the 3′,5′-diphosphorylated derivative of 8-hydroxy-2′-deoxyguanosine. Because the sensitivity of the ^{32}P-postlabelling analysis depends on the efficient separation of modified and unmodified nucleotides, thereby reducing the background radiation and radiation caused by closely situated highly radioactive unmodified nucleotides that interfere with the lower radioactivity derived from the modified nucleotides, we included an apyrase treatment after the ^{32}P-postlabelling in order to remove excess [^{32}P]ATP. The result is given in Fig. 4.

The detection of an oxidatively modified 2′-deoxyguanosine-3′,5′-diphosphate by means of this technique offers interesting possibilities for the detection of oxidative damage of guanosine residues in DNA, which can be induced by oxygen radicals produced by the metabolism of carcinogens such as diethylstilbestrol.

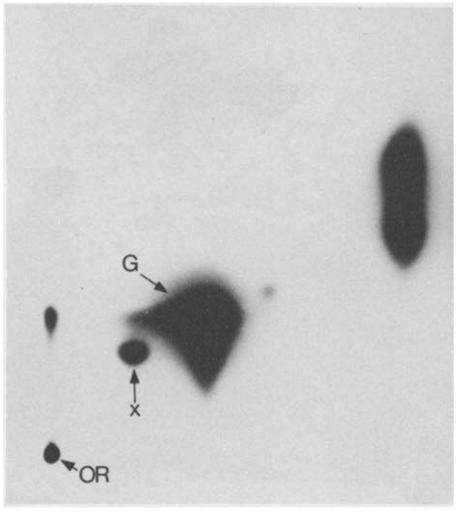

Fig. 4. Autoradiogram of the ^{32}P-postlabelling and anion-exchange TLC of the reaction mixture of 2′-deoxyguanosine with L-ascorbic acid and hydrogen peroxide, combined with an apyrase treatment in order to hydrolyse the excess [^{32}P]ATP. Conditions and abbreviations as in Fig. 1.

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