RADIATION-INDUCED DAMAGE TO NUCLEIC ACIDS

DNA, the molecule that contains all the information necessary for the organism to function and reproduce itself, is the main target in the cell that is liable to be damaged by ionizing radiation. The damage can be direct, through energy transfer or electron loss, which breaks or distorts DNA segments. It can also be indirect, through the effects of these same processes on the surrounding water molecules, which make up more than two thirds of the organism's mass. After identifying the different types of lesion that radiation can inflict on nucleic acids (DNA and RNA), researchers are continuing to develop their knowledge of the mechanisms involved, of the extent of the damage that can be properly attributed to ionizing radiation and on the role this damage plays in tissues and organs, and in the whole organism.

Reading off an autoradiogram. Each group of four dark bands corresponds to the nucleotide sequence of the four bases (adenine, guanine, cytosine and thymine) of a DNA fragment. Damage to the nucleobases is one of the main classes of modifications that ionizing radiation can cause to the organism's cell DNA.

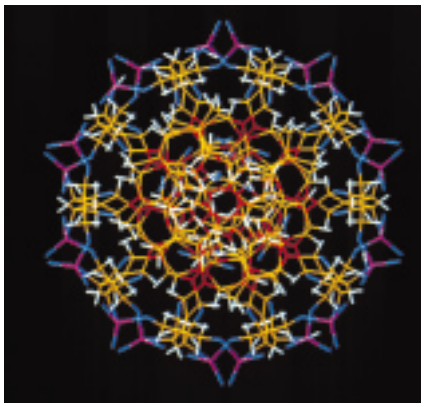
S. Stammers/SPL/COSMOS

Two main mechanisms

Deoxyribonucleic acid (**DNA**) is the main target in the **cell** for the biological action of **ionizing radiation** (**X rays, gamma rays, heavy ions**). The effects of such radiation on DNA can trigger a sequence of events that may end in the death of the cell (**lethality**) or that modify its «program» by **mutagenesis** and (or) **carcinogenesis**. Two main processes are implicated in radiation-induced modifications to cell DNA.

The first of these processes involves a direct or near-direct effect. It results from the excitation (energy transfer) and ionization (loss of electrons) of the DNA and of water molecules bound to it. The ejected electrons react preferentially with the **pyrimidine** (**thymine** and **cytosine**) and **purine** (**adenine** and **guanine**) bases that make up the «rungs» of the DNA «ladder» (box A: ***DNA molecule, heredity vector***) to form anion radicals. Electric charge transfer reactions occur in particular along the DNA chain, with a preferential localization on the guanine-type bases, where they open «positive holes» in the form of radical cations.

The second process of radiation-induced DNA damage is essentially *indirect*.



J-L Martin/J-C Lambry/INSERM

Cross-section of a DNA molecule

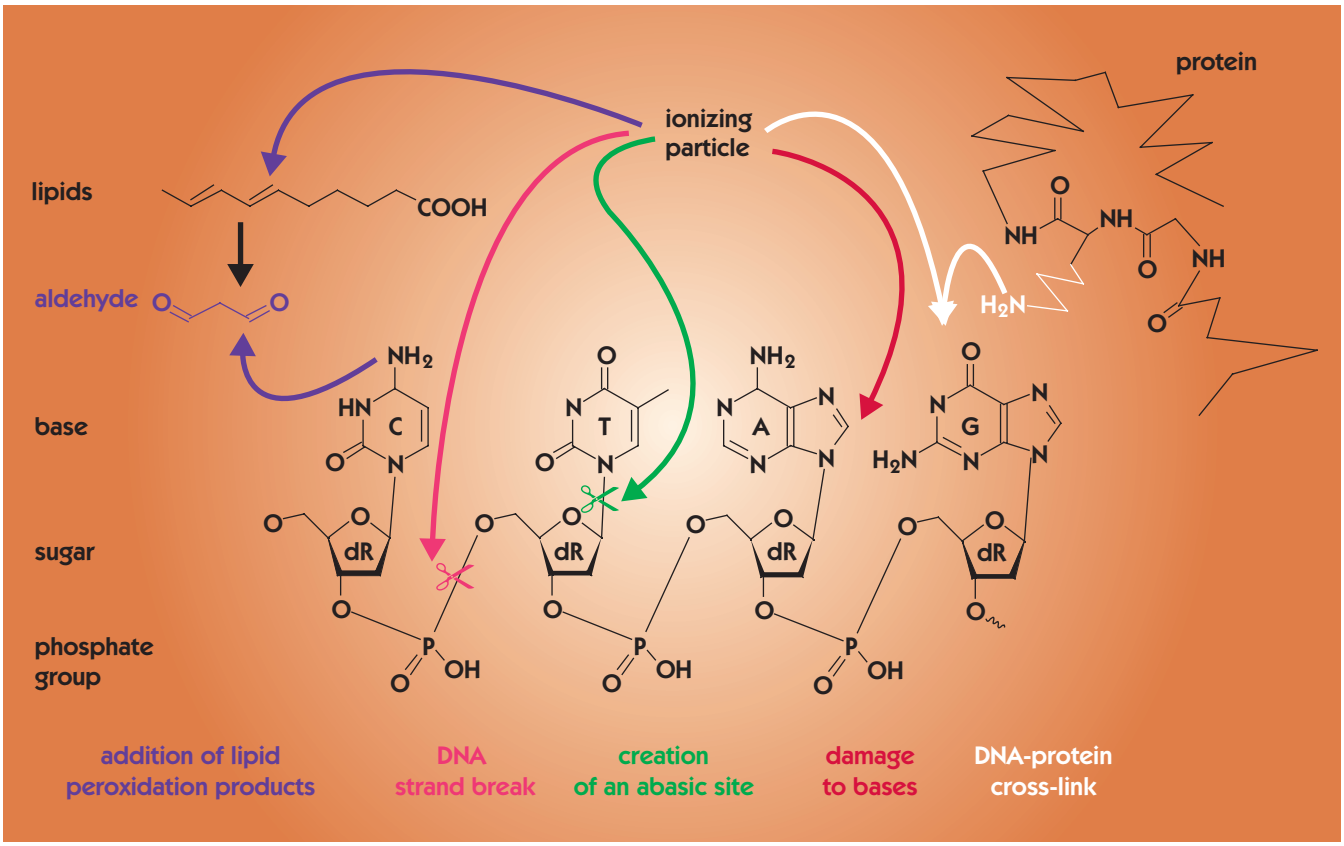
It involves reactive species derived from the radiolysis of water molecules that are some distance away from the DNA (**radiolysis**). These reactive species are essentially hydroxyl radicals (•OH), the reactivity of which is predominant, hydrogen atoms (H•) and hydrated electrons ($e^{-\bullet}_{aq}$) (*see Radiolysis of water*). The radical base intermediates and sugar fragments (2-deoxyribose) generated by ionization or as a result of the reaction of electrons and products of the radiolysis of water are very rapidly converted into final decomposition products (in less than a millisecond). Oxygen, through its sensitizing effect, is involved in most of these radical damage reactions.

Five main types of modification

The modifications resulting from these two principal damage mechanisms fall into five main categories (Figure 1):

- **DNA chain breaks**, single (one **strand**) and double (both strands) resulting from radical reactions affecting sugars (2-deoxyribose) that form the «rungs» of the double DNA «ladder».
- **Damage** to purine and pyrimidine bases.
- **Creation of abasic sites** (disappearance of bases), resulting from either the radiation-induced elimination of an initially normal base or the spontaneous release of a modified base.
- **DNA-protein cross-links**, involving the formation of a chemical bond between a base and an amino acid, one of the «building blocks» (box F, ***Amino acids, the chemical alphabet of proteins***) of a protein close to the DNA molecule.
- **Addition to DNA bases** of lipid per-

Figure 1. The five main types of DNA damage depicted on the four vulnerable bases, the pyrimidines (cytosine C and thymine T) and purines (adenine A and guanine G). DNA-protein cross-links in particular is illustrated by an example that has been thoroughly studied at the CEA's Nucleic Acid Lesions Laboratory. First of all an electron is lost from the guanine base. Then the primary amine group NH_2 of the amino acid (here a lysine) binds to the ionized guanine.



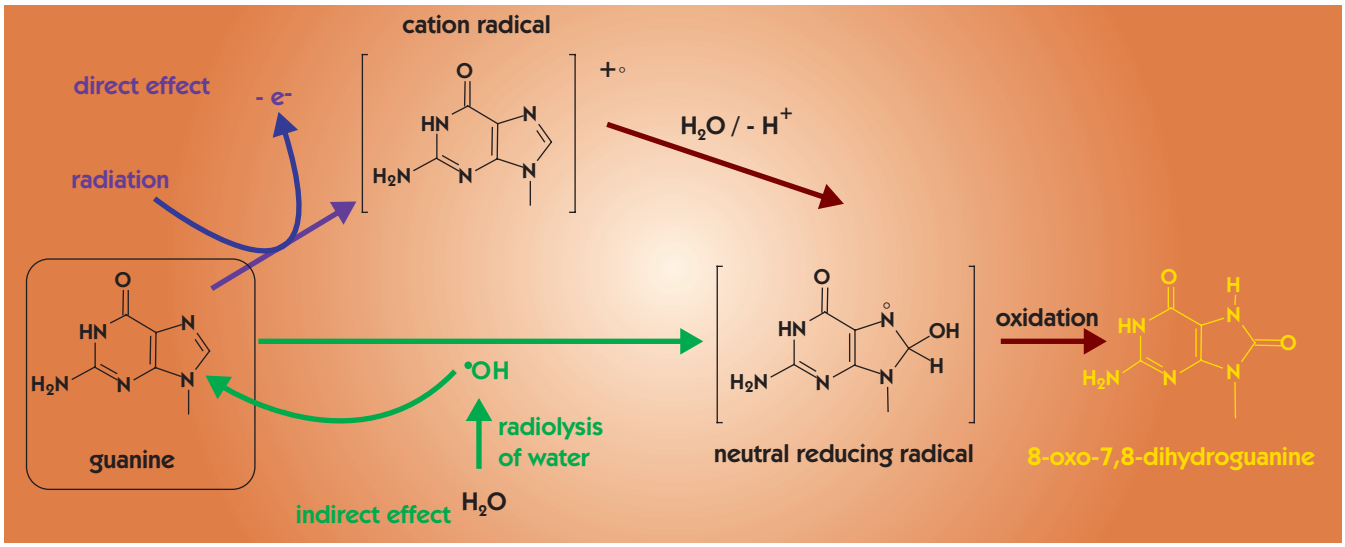


Figure 2. Simplified mechanism of the radical oxidation of a guanine base yielding 8-oxoGua: top: direct effect by electron loss; bottom: indirect effect via radiolysis of water.

oxidation breakdown products.
The addition products, here reactive aldehydes, derive from the decomposition of radiation-induced membrane lipid peroxidation products.

The possibility of multiple damage formation (several bases modified on one or both DNA strands, perhaps with strand breaks) further complicates the picture.

Several modifying events are liable to occur in the immediate vicinity of the DNA molecule when the energy of the ionizing radiation is transferred. Double-strand breaks are currently the only known example of radiation-induced multiple damage in cell DNA. This type of lesion is characteristic of ionizing radiation damage.

Three avenues of research

The research strategy of the French Atomic Energy Commission (CEA) for the study of the effects of ionizing radiation on DNA is in three parts with complementary aims. The first part focuses on achieving increasingly precise determination of the mechanisms of formation of radiation-induced lesions. The second concerns the measurement of damage in cell DNA after exposure to gamma radiation, while the third addresses the evaluation of the biological role of the radiation-induced damage, the repair of the lesions caused and the study of the attendant mutagenic effects.

Research on the physical and chemical mechanisms responsible for the lesions in particular are conducted at the Nucleic Acid Lesions Laboratory of the CEA's Physical Sciences Division at Grenoble, jointly with researchers of the Life Sciences Division and the National Scientific Research Center (CNRS).

Mechanisms of formation of radiation-induced lesions

The first series of studies concerns the mechanistic aspects of the radiation-induced reactions of the purine and pyrimidine bases of isolated DNA and *model systems*. These systems are nucleosides (base + sugar) and **oligonucleotides** (short segments of DNA each composed of one purine or pyrimidine base, one sugar and one phosphate group). The studies are based on the isolation and characterization of the final decomposition products of the constitutive elements (bases, nucleosides) and oligonucleotides. Consistent mechanistic descriptions have been proposed based on structural information and certain kinetic data, e.g., concerning radiation-induced radicals appearing transiently in these reactions, which are already published in the literature. These descriptions account for the reactions of modification of the purine and pyrimidine bases after oxidation by electron loss (ionization) and by addition of a hydroxyl radical. For example, Figure 2 shows the (simplified) mechanism of radical oxidation of the guanine base leading to the formation of 8-oxo-7,8-dihydroguanine (8-oxoGua). It was thus shown that this modification of DNA can result indifferently from either an indirect effect, by addition of the hydroxyl radical at position 8 of guanine, or a direct effect after loss of an electron from the purine nucleus. This modification of the DNA causes mutations that correspond to the replacement of a guanine-cytosine base pair by a thymine-adenine base pair.

Theoretical studies using the methods of quantum chemistry(1) are increasingly important here. In particular they make it possible to determine both conformational and electronic properties of radiation-induced nucleoside degradation products.

(1) See also *Clefs CEA* n°42, p 14.

DNA molecule, heredity vector

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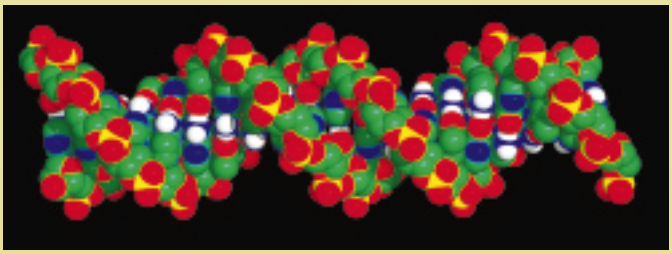


Seddon & Evans/SPL/COSMOS

Depiction of a DNA molecule showing the double helix (green, red and yellow) and base pairs (blue and white).

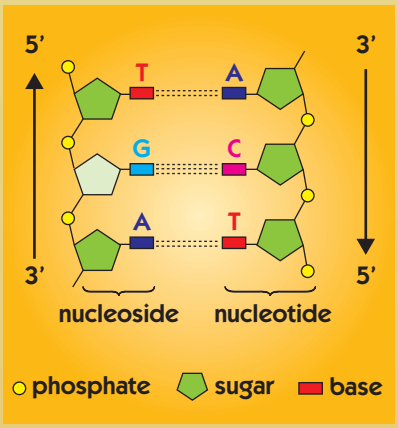
All of us carry, in the **nucleus** of each of our **cells**, a series of very long molecules of **DNA** (deoxyribonucleic acid) all nestled together. Tens of thousands of messages are inscribed in them in a code that biologists can now decipher. All life on earth uses the same genetic code. Human cells contain about 700 times more DNA than the bacterium *Escherichia coli*, but 30 times less than certain cells of amphibians and plants.

Each message, each **gene**, orders the building of one of the innumerable components necessary for the proper functioning of the organism, in particular **proteins**. The set of all these messages, the **genome**, thus forms our genetic heredity. It defines most of our physical characteristics. The code it uses, written in the form of «words» called **codons**, which each determine an **amino acid**, nicely solves the problem of how to store a large amount of genetic information in a restricted space.

DNA, like **RNA** (ribonucleic acid), the other essential nucleic acid, which is involved in the **replication** of the information in the DNA and in the transmission of that information to the protein to be built, is made up of millions of elementary units called **nucleotides**. A nucleotide is composed of a **sugar** (a five-carbon glucid) linked on one side to one or more groups comprising a phosphorus atom surrounded by four oxygen atoms (**phosphate group**) and on the other side to a molecule called a **base** (nitrogen-containing here). In DNA, the elementary sugar is 2-deoxyribose. The base in a DNA nucleotide is one of the following four: adenine (A), thymine (T), guanine (G) and cytosine (C). The DNA molecule is made up of two chains of nucleotides or **strands** organized in a **double helix** that resembles a spiral stairway. The RNA molecule is made of a single such strand. Its sugar is ribose. Its bases are the same except for thymine (T), which is replaced by another base, uracil (U).

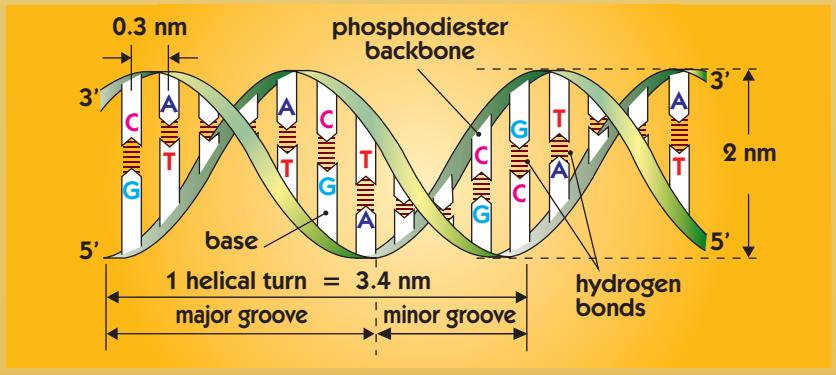
Sugars and phosphates form the rails of the DNA «ladder» and the bonds linking the bases form the rungs. This structure comprises two sorts of bonds; those linking together the sugars and phosphates along each chain, and those that pair up opposing bases across each chain, forming about 10 nucleotide pairs per turn of the helix. A codon is composed of three nucleotides.

The nucleotides are linked by **phosphodiester** bridges that join the 5'-carbon of one 2-deoxyribose group to the 3'-carbon of the next one along (Figures). The bases are attached to this repeating **2-deoxyribose-phosphate** chain, also called **phosphodiester backbone**. All the bases of the DNA molecule lie inside the double helix, whereas the 2-deoxyribose-phosphates are located on the outside. This means that all the bases on one chain have to be extremely close to those on the other chain, which requires specific pairing of a large base, called a **purine** (A or G, each possessing a double ring), on one chain, with a small base, called a **pyrimidine** (T or C, each with a single ring), on the other chain. The complementary base pairs thus formed between A and T and between G and C



are called **Watson-Crick** base pairs. The number of useful **hydrogen bonds** that can form between G and C or between A and T is greater than for all the other combinations.

Because each chain contains a **nucleotide sequence** that is exactly complementary to the one it is associated with on the opposite chain, the two strands in fact bear the same genetic information.



Another very promising domain of application of these theoretical approaches is the study of the reactivity of the •OH radical and the H• atom with DNA bases.

Measuring radiation-induced damage to DNA

The work on measuring radiation-induced damage to DNA focuses essentially on the lesions caused to purine and pyrimidine bases. Its main objective is the identification and quantitative measurement of the main lesions liable to form in cell DNA. One particular aim is to validate results obtained in isolated DNA and in model systems in the cell environment. The determination of the kinetics of repair of lesions caused in the cell DNA after exposure to a high dose of radiation - more than 10 **grays** (Gy) - is another purpose of these measurements.

Two main experimental approaches are currently being used to make these measurements. Among their many stringent specifications is a very high sensitivity of detection. The threshold required is of the order of one modified base per million, or better, one per ten million normal bases, from only a few micrograms of DNA! In addition, the risks of spurious oxidation of the normal bases during the processes of DNA extraction and analysis of induced damage have to be minimized (Figure 3).

A first approach to the study of the damage consists in optimizing the methods of **liquid**- or **gas-phase chromatography** that are used to separate the molecules according to one of their characteristics (size, charge) (box 1). The second method, applied to analysis in isolated cells, makes use of a modified version of what is known as the **comet assay** (box 2), a classical and highly sensitive method, but which does not allow the determination of particular sorts of damage. So far it affords an overall measurement of a heterogeneous set of radiation-induced damage in isolated cells (DNA single- and double-strand breaks and various lesions sensitive to the alkali treatment that characterizes the method) after exposure to doses as low as 20 cGy.

It is now possible to measure separately two great classes of lesions, namely modified pyrimidine bases, and the appearance of 8-oxo-7,8-dihydroguanine (8-oxoGua), by causing supplementary DNA breaks where these lesions are located by incubation with repair **enzymes** (see *The caretakers of the genome*). It is thereby possible to discern radiation-induced damage to bases in the DNA of isolated cells after exposure to a gamma-ray dose as low as 2 Gy.

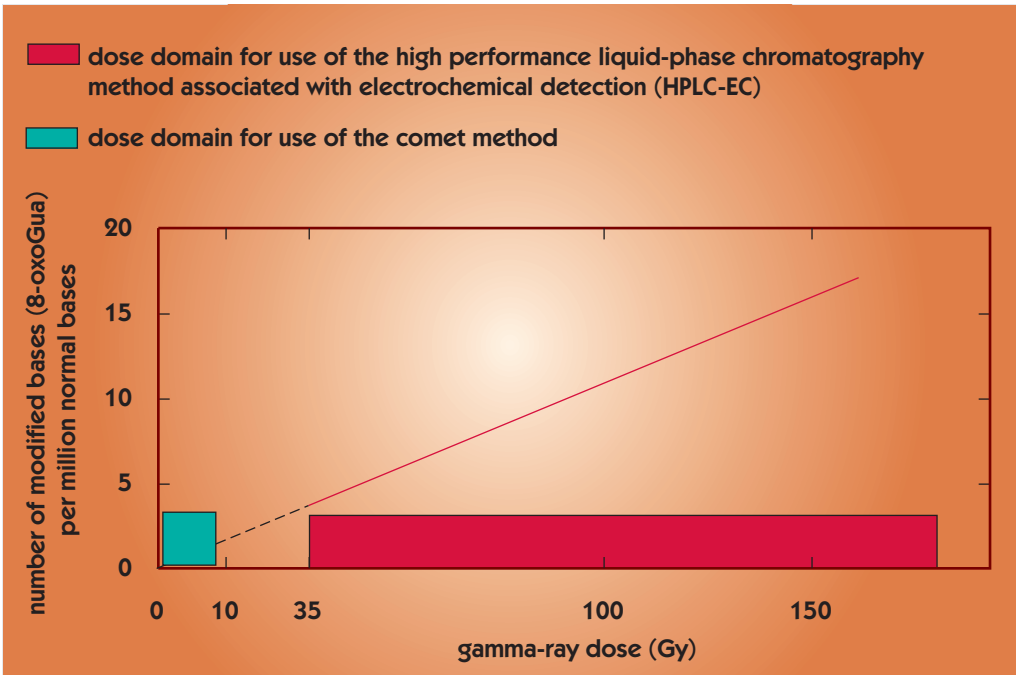
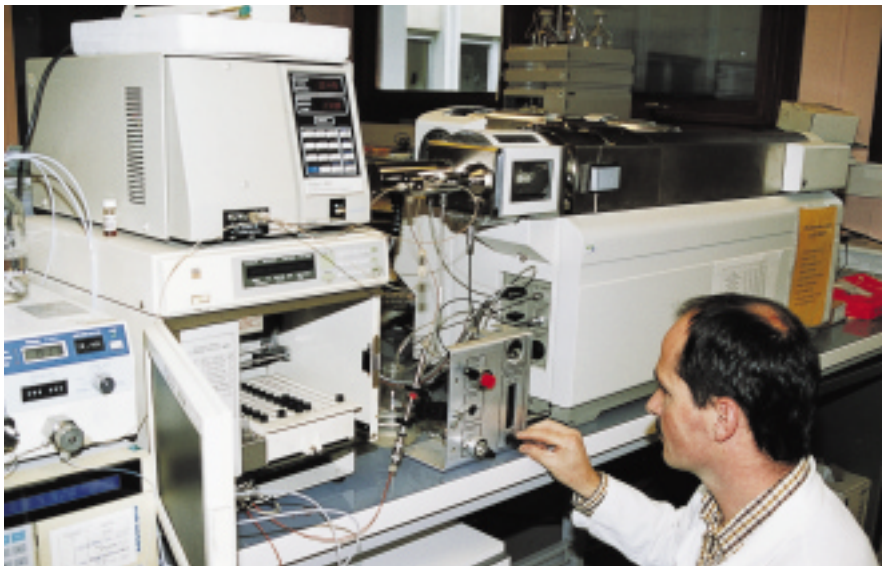


Figure 3. Domains of applicability of methods for the measurement of radiation-induced damage to DNA according to the radiation dose and the number of modified bases relative to the number of normal ones.



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Measurement of radiation-induced damage to cell DNA in the form of nucleosides by high performance liquid-phase chromatography associated with detection by mass spectrometry in tandem mode (ionization by electrospray).

Optimized chromatography methods

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The liquid- or gas-phase chromatography methods used to measure the damage inflicted on DNA allow the separation of trace amounts of modified bases from complex mixtures. The measurement of the damage is carried out directly at the output from the chromatography column using various detection techniques (e.g., electrochemical, mass spectrometric). These measurement methods, especially those using gas-phase chromatography coupled with mass spectrometry, have had to overcome numerous difficulties. The results initially obtained using a non-optimized application of this technique gave values that were overestimated by a factor of more than 20 before the spurious reactions responsible were identified. To date only the measurement of 8-oxoGua has been achieved using this method in cell DNA, at doses greater than 30 Gy, using high performance liquid-phase chromatography associated with electrochemical detection. Coupling of mass spectrometry in tandem mode (ionization by electrospray) with analysis by high performance liquid-phase chromatography has recently yielded excellent results. This method has proved to be both highly sensitive and applicable over a very broad domain.

Evaluation of the biological role of radiation-induced damage to DNA

The ultimate aim of this research work is to determine the biological role of radiation-induced damage to DNA. For this purpose it is necessary to quantify the occurrence of this damage in the cell (*see above*). Three main objectives are being pursued, in close cooperation with biochemists and biologists of CEA and other research bodies.

The first of these objectives is to determine the damage-induced conformational changes in the oligonucleotide molecule. To do this, researchers are using nuclear magnetic resonance analysis (see *Structural analysis of radio-induced lesions of DNA*). The second objective is to study the specificity of damage recognition by repair enzymes (see *The caretakers of the genome*). The third is to evaluate the mutagenic power of damage to bases and their repair after introduction into human cells of DNA fragments containing one or more modifications at defined sites. This work is being conducted jointly with Alain Sarasin of the Federative Cancer Institute (IFC1) of the CNRS at Villejuif.

For these different studies modified DNA fragments with defined sequences are prepared, 20 to 50 bases in length. When these modifications are chemically stable, they are inserted specifically at one or more sites selected in the chosen sequence (Figure 4). Otherwise the targeted base is modified in a relatively specific way in the oligonucleotide itself.

All this work is helping us both to develop our knowledge of the

The modified comet assay

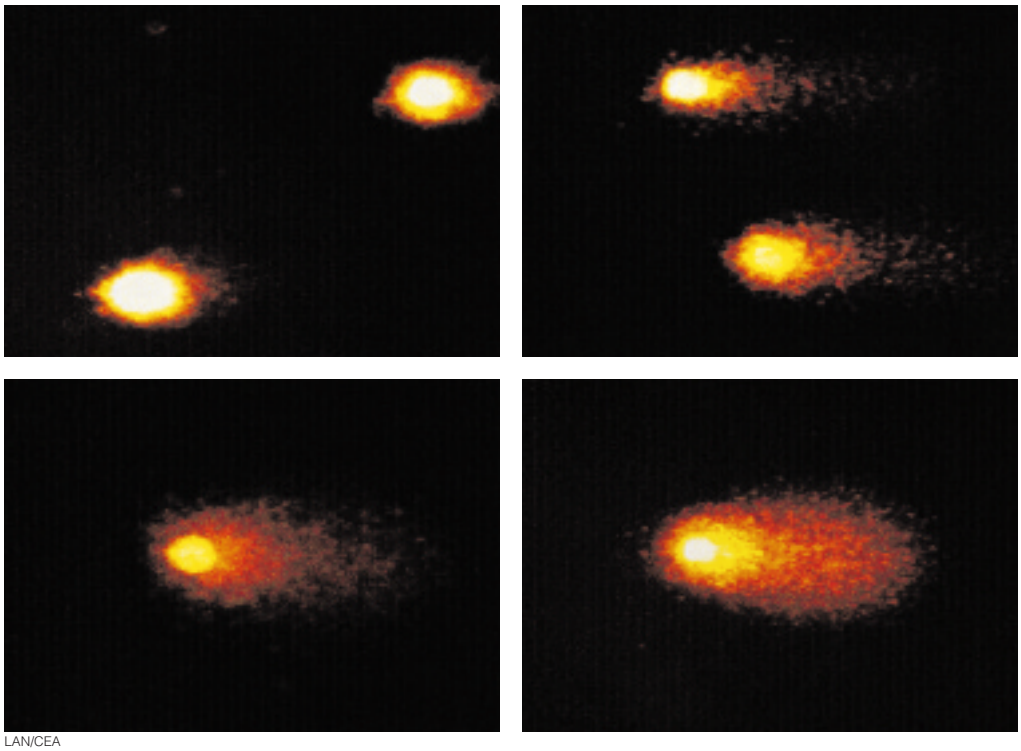
2

In its classic version the comet assay was designed to measure, in isolated cells, strand breaks and heterogeneous damage to the DNA molecule (e.g., modified bases and/or unstable sugar residues sensitive to alkali treatment) and evaluate how efficiently they were repaired. Some of this damage is converted into supplementary breaks in the test analysis conditions in alkaline medium. The cells are incorporated into a gel spread on a microscope slide, and the membranes are eliminated. The DNA is then subjected to an electric field that stretches it and draws the cleaved strands out of the nucleus. Under a microscope, after development with an added dye, the DNA presents a comet-like shape with a tail the length of which depends on the extent of fragmentation.

To extend the scope of this method an additional step is introduced during the procedure. In this step the DNA is «digested» with a repair enzyme specific to a particular type of base damage. This step creates new breaks after the first steps in the repair process, the excision of the base being followed by partial or total elimination of the sugar. These new breaks are visualized after migration of the DNA in the electric field. The comet tail is thus lengthened according to the amount of damage in the cell DNA that is recognised by the enzyme. The table below gives the number of damage sites in the cell DNA caused by exposure to ionizing radiation.

To create these breaks two enzymes are currently used: endonuclease III (endo III), and formamidopyrimidine DNA-glycosylase (FPG) of bacterial origin. The formation of spurious oxidation products from the very largely predominant normal bases is minimized or even suppressed during analysis by electrophoresis which spreads out the DNA fragments along the gel according to their length.

	reference level for 1 million normal base pairs	number of modifications per cell (baseline)	formation per Gy for 1 million normal base pairs	formation per Gy per cell
single - and double - strand breaks and damage sensitive to alkaline treatment	0.56	3,360	0.260	1,600
sites recognized by FPG	0.42	2,520	0.095	570
sites recognized by endo III	0.38	2,280	0.105	635



Comet method. Top: control cells (right: treated with FPG). Bottom: cells irradiated with gamma radiation of 8 Gy emitted from a cobalt-60 source (right: treated with FPG).

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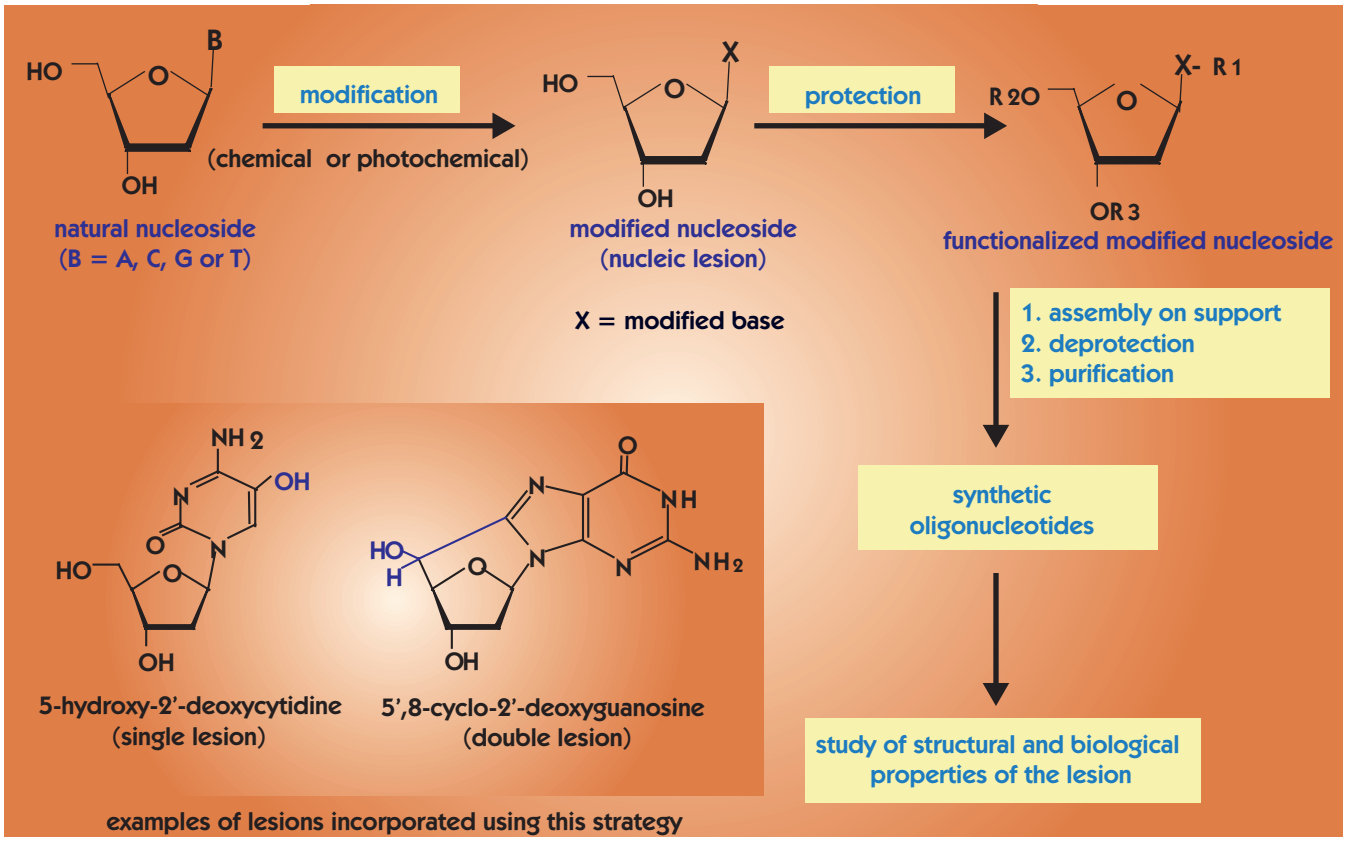

biochemical properties of modified DNA bases, and to keep on improving the sensitivity of the methods used for the detection of lesions.

Jean Cadet
and the Nucleic Acid Lesions
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Figure 4. Strategy for the synthesis of modified oligonucleotides for the study of modified DNA bases. The assembly is carried out on a solid support (glass beads). The protection is intended to prevent chemical reactions taking place other than the one being studied. R1, R2 and R3 are protective groups that mask the reactivity of the groups which are stable in acid or basic medium.

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Insertion of a lesion in the form of a nucleotide in a defined site of a fragment of DNA using an oligonucleotide synthesizer.