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Review

Methods used for analyses of "environmentally" damaged nucleic acids

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ABSTRACT

In this review, we present various techniques, currently applied in many laboratories, which are useful in the detection of "environmentally"-induced damage to DNA. These techniques include: (a) chromatographic methods, which allow determination of chemical changes within DNA, be they formation of adducts with or oxidation of bases in DNA; (b) electrophoretic methods, which facilitate finding the site(s) in DNA where that chemical modification occurred; and (c) immunological assays, which help to detect DNA damage using externally produced antibodies that recognize the specific chemical changes in DNA or its fragments, as well as by detection of autoantibodies that develop in response to environmental exposures of animals and humans.

CONTENTS

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LIST OF ABBREVIATIONS

Ab	Antibodies
A.S.A.P.	"As soon as possible"
ASO	Allele-specific oligonucleotide
B(a)P	Benzo[*a*]pyrene
bp	Base pairs
CAPE	Caffeic acid phenethyl ester
DGGE	Denaturing gradient gel electrophoresis
DMBA	7,12-Dimethylbenz[*a*]anthracene
DNase	Deoxyribonuclease
dTG	Thymidine glycol (5,6-dihydroxy-5,6-dihydrothymidine)
ED	Electrochemical detection
EDTA	Ethylenediamine tetraacetate
EGCG	(−)-Epigallocatechin gallate
ELISA	Enzyme-linked immunosorbent assay
FAPY	Formamido-substituted pyrimidine ring
FdU	5-Formyl-2′-deoxyuridine
GC	Gas chromatography
HMdU	5-Hydroxymethyl-2′-deoxyuridine
HMdU–BSA	HMdU coupled to bovine serum albumin
HMU	5-Hydroxymethyluracil
HPLC	High-performance liquid chromatography
HPMdU	5-Hydroperoxymethyl-2′-deoxyuridine
HRPO	Horseradish peroxidase
Ig	Immunoglobulin
kb	Kilo base pairs
Mb	Mega base pairs
MS	Mass spectrometry
NNK	4-(Methylnitrosamine)-1-(3-pyridyl)-1-butanone
8-OHdG	8-Hydroxyl-2′-deoxyguanosine
PAHs	Polycyclic aromatic hydrocarbons
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RFLP	Restriction fragment length polymorphisms
RNase	Ribonuclease
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate

SSCP	Single-strand conformational polymorphism analysis
TG	Thymine glycol (5,6-dihydroxy-5,6-dihydrothymine)
TLC	Thin-layer chromatography
TPA	12-O-Tetradecanoylphorbol-13-acetate
UV	Ultraviolet

1. INTRODUCTION

Nucleic acids, which have been exposed *in vitro* or *in vivo* to different environmental contaminants, sustain various types of damage that may lead to structural and/or functional changes. These contaminants usually are thought of as exposures to harmful agents in the work place, and indoor and outdoor air and water. However, we also can include damaging substances that are present in the foods we eat and medicines we take, both over-the-counter and medically prescribed pharmaceuticals. The terms "damage" and "damaging" are very broad, and scientists and physicians who invoke them often give them different meanings. The most common include "damaging agents" that are cytotoxic and cytostatic, mutagenic, carcinogenic and teratogenic. Each of these terms encompasses various cellular processes that ultimately can lead to alterations in the structure, conformation, as well as function of the genetic material. The term "DNA base modification" also encompasses many types of DNA damage [1]. These include alkylated, arylated and arylalkylated adducts created by interactions between the site(s) on a DNA (or RNA) base and the chemical to which the cell or organism is being exposed. A chemical may directly interact with the DNA base or it may require metabolic (or chemical) activation before it can modify those bases. Physical carcinogens, such as γ and UV radiations, also cause chemical changes within the bases of nucleic acids. In addition to the classical adducts, compelling evidence is emerging showing that toxic agents, mutagens, and initiating and promoting carcinogens can induce formation of reactive oxygen species (ROS) (reviewed in refs. 2–4), some of which can oxidatively modify bases in DNA and RNA, both *in vitro* and *in vivo* [4].

As there are different meanings to the term "damage", there are also a variety of techniques that allow us to establish its nature. Depending on the goal, those techniques can be divided into chemical and physicochemical methods, *i.e.* thin-layer chromatographic (TLC), high-performance liquid chromatographic (HPLC) or gas chromatographic (GC) separation of the modified base derivatives often coupled with microderivatization and/or postlabeling with ^{32}P or ^{3}H. One can also use biological end-points, such as enzyme or antibody recognition of the damage, chromosomal aberrations, as well as mutagenic responses in bacterial tester strains or in specific genes, including oncogenes, in mammalian or transgenic cell lines. Sometimes, a combination of several techniques provides the best analysis. Since many chemicals cause various types of lesions in nucleic acids, model systems are often studied to establish which lesions may have biological consequences.

The damage can be detected by analysis of enzymatic or acid hydrolysates of DNA, be it as modified bases, nucleosides, nucleotides, or oligonucleotides. Detection also may be achieved by analyzing body fluids such as urine for the presence of modified bases or nucleosides. Another approach currently being utilized is the detection of DNA damage by analysis of human sera for the presence of antibodies that recognize modified DNA bases, including adducts as well as base oxidation products.

In this review, we initially concentrate on the description of techniques currently in use or emerging for the analyses of chemical modification of DNA, with the understanding that some of the same methods also can be utilized or adapted for analysis of RNA. In addition, we will give examples of the applications of some of the techniques described. Since this is a rapidly growing field, this review will not be all inclusive, but only selected works will be presented.

2. METHODS

2.1. Preparation of samples for analysis

2.1.1. Isolation and purification of DNA

Currently, there are several preferred methods used for the isolation of DNA from biological sources and for its purification. Even commercially available DNA often has to be repurified because it still may be contaminated with proteins and RNA, and may also contain low levels of oxidized bases. The following methods of DNA isolation from animal organs are often used.

DNA can be extracted from cells or nuclei using an automated DNA extractor [5]. Organs, such as liver, kidney and brain, are placed in 10 volumes of buffer (10 m*M* Tris–HCl, 150 m*M* NaCl, 1 m*M* EDTA, pH 7.4), homogenized, the nuclei isolated by centrifugation at 1100 *g* for 15 min, and the DNA isolated by an extractor.

The liver also can be placed in buffer (50 m*M* Tris–HCl, pH 7.0) containing 0.25 *M* sucrose, minced, homogenized by Polytron for 20 s, and centrifuged at 800 *g* at 4°C for 10 min [6]. The pellet is suspended in Tris–sucrose buffer containing 0.05% Triton-X, centrifuged, and washed twice. The final pellet is resuspended in 10 ml of Tris–sucrose buffer, 10% SDS (1.5 ml) and 1 *M* NaCl (3.5 ml) are added, and the proteins removed from the aqueous phase with 15 ml of chloroform–isoamyl alcohol (24:1). The phases are separated by centrifugation at 2500 *g* for 10 min, and extraction is repeated until there is no white interphase between the phases. After treatment with DNase-free RNase A and subsequent extraction with chloroform–isoamyl alcohol (24:1), the DNA can be precipitated from the aqueous layer with 2 volumes of cold ethanol. This method was used for determination of the presence of aflatoxin B_1 adducts in DNA [6]. However, if the goal is to analyze the DNA for the presence of oxidized bases, care should be taken to chelate the Fe ions that can be released from the liver during the process of DNA isolation.

In some cases, upon removal, the liver is immediately frozen in liquid nitrogen and stored at −70°C. To extract the DNA, the frozen livers are ground into small pieces, thawed in buffer (50 m*M* Tris HCl, 150 m*M* NaCl, 50 m*M* EDTA, pH 8.0), homogenized, and centrifuged at 600 *g* for 15 min. The pellet is suspended in 10 ml of the same buffer with 10% N-lauroylsarcosine and RNase A (100 μg/ml), incubated at 37°C for 4 h, followed by addition of Proteinase K (100 μg/ml) and overnight incubation [7].

To isolate the epidermis from a topically treated mouse or other animal, the shaven skin is excised and immediately dipped into ice-cold Dulbecco's phosphate-buffered saline (PBS). Subcutaneous and connective tissues are scraped off with a razor blade, the skin cut into about 2 × 1 cm pieces that are floated (fur side up) on 0.5% trypsin–Hank's balanced salt solution in a Petri dish, and incubated at 37°C for 0.5 h. Instead of trypsinization, an alternative way to isolate the epidermis is to dip the mouse skin into hot water (55°C) for 30 s, than rapidly immerse it in ice-cold water [8]. The epidermis is scraped off with a scalpel, minced with scissors, and washed twice with PBS. Cells are lysed and A.S.A.P. DNA isolation kits are used to isolate the DNA according to the manufacturer's protocol (Boehringer-Mannheim Biochemicals). This procedure yields about 0.2–0.5 mg DNA from the epidermis of two mice [9]. This yield is somewhat higher than described by the vendor because of an additional washing of the column with the DNA eluting buffer. However, each batch of the A.S.A.P. columns should be calibrated with a known amount of DNA because, at times, these columns may differ and thus DNA recovery may vary as well (unpublished observations).

The vendor's recommended procedure includes incubation of epidermis with Proteinase K at 37°C for 4 h and RNase for 0.5 h, followed by chromatography on the DNA isolation columns, and DNA precipitation with isopropyl alcohol. After centrifugation and washing of the DNA pellet twice with 70% ethyl alcohol and drying, the pellet is dissolved in the buffer needed for enzymatic digestion of DNA to nucleotides or nucleosides or, if desired, in concentrated formic acid for release of bases from DNA.

There are many variations on protocols for DNA isolation from the epidermis. For example, in some cases, the epidermis is quickly frozen in liquid nitrogen and stored at −70°C until ready to proceed with the analysis. Frozen epidermis is allowed to thaw in a solution containing 1% SDS and 1 m*M* EDTA (100 mg tissue per ml), and homogenized with a Polytron [10]. DNA can be isolated and purified according to the procedures best suited to the objective of DNA analysis.

To isolate DNA from tissues or cultured cells, many of the same procedures can be utilized. After cell lysis, which depends on the source of the cells, the proteins frequently are removed by incubation with Proteinase K, followed by extraction of proteins with phenol–chloroform saturated with buffer or phenol–choroform–isoamyl alcohol (25:24:1). However, the presence of phenol can cause oxidation of some bases in DNA [11], and this may interfere with the subsequent hydrolysis and/or biological activity that is being assessed. Furthermore, it may provide an unacceptably high level of oxidized bases in DNA isolated from control animals or cells, and this could preclude determination of the effect of a particular treatment on the formation of oxidized bases in DNA.

There are two preferred DNA extraction methods that avoid utilization of phenol. One is based on the use of a high concentration of NaCl for dissociation of proteins, followed by digestion with Proteinase K and RNase A, and extraction of proteins with chloroform–isoamyl alcohol (24:1), as originally described by Marmur in 1961 [12]. The other method is based on separation on a proprietary A.S.A.P. column from Boehringer-Mannheim Biochemicals, after treatment with Proteinase K and RNase A [9,13], as described above. The remaining proteins and RNA elute first from this column, followed by elution of DNA, with a purity of 1.7–1.9 A_{260}/A_{280}. This purity allows efficient enzymatic digestion of linear DNA to nucleosides [13]. The eluate is collected in a polypropylene tube, precipitated with isopropyl alcohol and centrifuged, and the pellet is washed twice with 70% ethanol and dried under nitrogen. One should avoid using glass tubes for DNA precipitation because the DNA is difficult to remove from the glass. The DNA pellet is either stored dry, or dissolved in a proper buffer for enzymatic digestion to nucleosides or nucleotides, or is suspended in 88 or 98% formic acid to release DNA bases [14,15]. However, A.S.A.P. columns are not appropriate for purification of supercoiled circular DNA because nicks are introduced into the DNA during purification (G. Teebor, personal communication). Supercoiled circular DNA can be reliably purified on a CsCl gradient with only a small percentage of nicks leading to the appearance of the circular relaxed form. Alternatively, commercially available plasmids can be used for the *in vitro* experiments.

Quantitation of DNA yields should always be carried out. When in solution, the absorbance of DNA should be determined at 260 and 280 nm, and their ratio calculated. It is assumed that for double-stranded DNA 1 $A_{260} = 50$ μg DNA per ml, while due to the hypochromic effect for the single-stranded DNA or RNA 1 $A_{260} = 40$ μg/ml. The ratio of A_{260}/A_{280} can be used as a measure of DNA purity; if it equals 2.0 or higher this indicates contamination with RNA, if it is less than 1.85, proteins, which could interfere with enzymatic digestion of DNA, were not completely removed [16]. However, DNA isolated through an A.S.A.P. column is an appropriate substrate for digestive enzymes even at ratios between 1.7 and 1.9 [9]. DNA also can be quantitated by use of fluorescent reagents, such as Hoechst 33258 [17].

2.1.2. Chemical and enzymatic hydrolysis of DNA to bases, nucleosides, nucleotides, and small oligonucleotides

2.1.2.1. Acid hydrolysis. Even under physiological conditions, purines are released from DNA leaving apurinic sites which if not repaired can be mutagenic [18]. Certain types of purine modification, particularly those occurring at the N-7 or C-8 position of the imidazole ring, cause either an imidazole ring opening [with formation of a formamido-substituted pyrimidine ring (FAPY derivatives)], which remains on the sugar–phosphate backbone, or lead to a release of

modified purines. All depurination processes are enhanced under acidic conditions. For this reason, the most common acid-mediated depurination is carried out in dilute (*i.e.*, 50–100 μM) HCl at temperatures varying from 37 to 70°C during a 20–60 min incubation [19]. For example, this type of procedure was used to isolate the guanine–aflatoxin B_1 adduct.

Since spontaneously released base–carcinogen adducts can be easily missed, it is advisable to always analyze the alcohol supernatant obtained by precipitation of modified DNA for the presence of such adducts. The detection of released guanine (adenine)–propane sultone [20] and benzo[*a*]pyrene [B(a)P]–6-N7-guanine [21] adducts can serve as examples of modified base formation, which could have been overlooked because of their spontaneous release from DNA.

Acid hydrolysis also can be used when the modified base(s) of interest are more stable under acidic conditions than all other modified or natural bases. Analysis for UV-induced cyclobutane pyrimidine dimers can serve as an example. In this case, the DNA is hydrolyzed with 88 or 98% formic acid in sealed ampules or tubes at a very high temperature (180°C) [22,23]. Subsequently, UV pyrimidine dimers are extracted from the mixture and analyzed by paper chromatography [22], or, as more recently carried out, by HPLC [23]. Another use of formic acid hydrolysis is in the determination of oxidative DNA damage by GC coupled with mass spectroscopy (MS). Dried samples of DNA are dissolved in 88 or 98% formic acid, and heated in evacuated and sealed tubes at 150°C for 40 min. After cooling, hydrolyzates are lyophilized, derivatized and analyzed by GC MS [14,15,24,25]. This method allows concomitant determination of a number of oxidized bases.

However, some of those oxidized bases can undergo chemical changes during concentrated formic acid treatment. Unless one knows what those changes are, this type of analysis may lead to an underestimation of the formation of some and an overestimation of other oxidized DNA bases. For example, formic acid release of thymine glycol from the sugar–phosphodiester backbone of DNA results in formation of some 5-hydroxy-5-methylhydantoin, which is a five-membered ring, as well as some 5-methylene hydantoin (Fig. 1), the dehydration product of the former compound [26]. Also, formic acid-mediated release of 5-hydroxymethyluracil (Fig. 1) from DNA results in an apparent loss of a substantial amount of this oxidized base, as recently shown by Djuric *et al.* [15]. Since 5-hydroxymethyluracil is chemically quite stable, it is unlikely that it is degraded by formic acid. It is more probable that under the acid hydrolysis conditions used (concentrated formic acid, high temperature), the

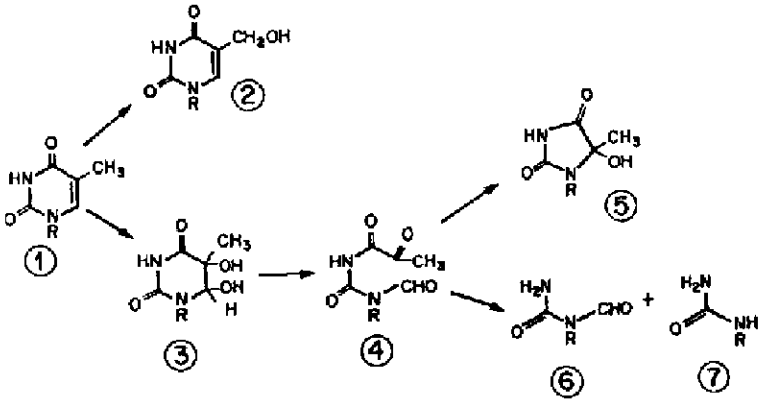

Fig. 1. Formation and decomposition of oxidized thymine moiety derivatives. Structures constitute bases when R = H and 2′-deoxyribonucleosides when R = 2′-deoxyribose. (1) Thymine; (2) 5-hydroxymethyluracil; (3) thymine glycol; (4) N′-formyl-N-pyruvylurea; (5) 5-hydroxyl-5-methylhydantoin; (6) N-formylurea; (7) urea. (From ref. 47.)

hydroxyl group of the 5-hydroxymethyl moiety would be esterified with formic acid. The formate derivative would elute at a different retention time from the GC or HPLC column than would the expected 5-hydroxymethyluracil. This could explain why for many years very little or no formation of this oxidized base was detected in DNA by GC MS [25], whereas enzymatic digestion to its nucleoside has shown its quite abundant presence in oxidatively damaged DNA [2,15, 27,28].

2.1.2.2. Enzymatic hydrolysis to nucleotides. There are many variants of enzymatic hydrolysis of DNA depending on the objective, *i.e.* whether hydrolyzates will be analyzed by ^{32}P-postlabeling of nucleotides or nucleosides, ^{3}H-postlabeling of nucleosides, or radiochemical, fluorometric or electrochemical detection of nucleosides. Each of those methods have certain advantages and disadvantages.

To obtain the mononucleotides needed for HPLC analysis of DNA adducts or as a substrate for ^{32}P-postlabeling, the DNA is dissolved in a buffer that differs depending on the laboratory carrying out those experiments. Recently, Floyd [29] compared the efficacy of digestion of DNA dissolved in three of the most commonly used buffers, as measured by 8-hydroxyl-2′-deoxyguanosine (8-OHdG) introduced to DNA by UV light and H_2O_2 treatment. The following buffers were used: 20 m*M* sodium succinate (pH 6.0) and 8 m*M* $CaCl_2$ (according to Randerath *et al.* [30]), 5 m*M* BisTris (pH 7) and 0.1 m*M* EDTA (according to Beland *et al.* [31]), and 20 m*M* sodium acetate (pH 4.8; Kasai *et al.* [32]). Although all three DNA solutions were incubated with the same enzymes and under the same conditions, the latter two buffers allowed release of more than twice as much 8-OHdG from the oxidized DNA than did the succinate–calcium buffer. These findings point to the necessity for optimization of the hydrolytic reaction conditions.

DNA solutions are either frozen in liquid nitrogen and stored at −70°C, or immediately hydrolyzed to nucleoside 3′-monophosphates. DNA is incubated with spleen phosphodiesterase (2.1 U/mg DNA) and micrococcal nuclease (5 U/mg DNA) at 37°C for 3 h [10,33]. Often, this is followed by digestion with nuclease P1 at pH 5 (125 m*M* sodium acetate buffer) in the presence of 0.09 m*M* Zn^{2+} ions [34], prior to or right after ^{32}P-postlabeling. Alternatively, the DNA itself is subjected to nuclease P1 digestion (generating 5′-monophosphates) followed by acid phosphatase, which should yield nucleosides to be used as substrates for ^{32}P-postlabeling [35].

However, recent results show that the presence of certain fragments derived from oxidized bases [*i.e.*, the formamido remnant of the thymine glycol (TG) moiety formed by oxidation of thymine] can inhibit the hydrolytic action of nuclease P1 [36]. Hence, caution should be exercised when using P1 digestion of DNA. This technique is most frequently used for determination of DNA adducts induced by carcinogens. However, many of these carcinogens also mediate formation of oxidized bases in DNA [2,4,37] and the presence of these oxidized bases might impair P1-mediated release of free nucleoside adducts, leading to inaccurate answers. Conversely, the inhibition of nuclease P1 by the formamido moiety is proposed as an assay system to detect this particular oxidation product, which cannot be detected by any other method [36].

Other examples of modified base inhibition of enzymatic hydrolysis are UV-induced dimers, both the cyclobutane type as well as the 6,4-photoproduct [38]. It was shown that snake venom phosphodiesterase is not able to cleave either the intradimer phosphodiester bond or the phosphodiester bond located immediately 5′ to the dimer. The inability of the phosphodiesterase to hydrolyze these particular bonds results in a release of pyrimidine dimers in trinucleoside diphosphates with the dimer at the 3′ end, when followed by a treatment with alkaline phosphates. Since unaffected DNA is hydrolyzed to mononucleosides, only the trimers can be ^{32}P-postlabeled by [γ-^{32}P]ATP and T4 polynucleotide kinase. The authors of this work propose to use this assay as a sensitive and accurate measure of the UV-mediated dimer formation. Weinfeld and Soderlind [39] used a similar assay to detect γ-radiation-mediat-

ed formation of thymine glycols and phosphoglycolate termini by analysis of ^{32}P-postlabeled dinucleoside monophosphates obtained by enzymatic hydrolysis of DNA.

To assure the most complete hydrolysis of DNA thought to contain a variety of modified bases (*i.e.*, carcinogen–base adducts as well as oxidized bases), it probably would be advisable to use a combination of enzymes. For example by using DNase I, snake venom phosphodiesterase as well as spleen phosphodiesterase, one can be assured that even if one of the phosphodiesterases (*i.e.*, snake venom) cannot hydrolyze the phosphodiester bond, because of inhibition due to a modified base (*i.e.*, UV dimer), the second enzyme, which proceeds in the opposite direction (5′ → 3′), should be able to hydrolyze this bond. This has been shown to be possible in the case of DNA that was exposed to B(a)P-treated rat liver microsomes. That DNA contained the oxidized bases 5-hydroxymethyluracil and TG (Fig. 2) [40], in addition to the DNA base–carcinogen adducts that are known to be formed under such conditions. That DNA was completely hydrolyzed to nucleosides using the aforementioned combination of enzymes followed by alkaline phosphatase. DNA, isolated from cells exposed to B(a)P or other polycyclic aromatic hydrocarbons (PAHs), was shown to be difficult to digest enzymatically when only one of the diesterases or nuclease P1 was used [41,42].

2.1.2.3. Enzymatic hydrolysis to nucleosides. In addition to nuclease P1-mediated digestion followed by alkaline or acid phosphatase treatment (described above), enzymatic hydrolysis of DNA to nucleosides has been used quite extensively and in a variety of enzyme combinations (see above). Again, depending on the objective of the analysis, different enzymatic digestion DNA conditions should be used. As mentioned above, nuclease P1 is used frequently as an exonuclease to release 5′-monophosphates. Although its optimal activity requires an acidic pH of 5.0–5.5 and the presence of Zn^{2+} cations, the literature is full of procedures that do not apply these conditions. Obviously, optimal conditions should be used whenever possible. However, if an adduct or oxidized base is not stable under acidic pH, or Zn^{2+} interferes with the subsequent analysis, then the DNA can be incubated with nuclease P1 at neutral pH, by using either higher amounts of the

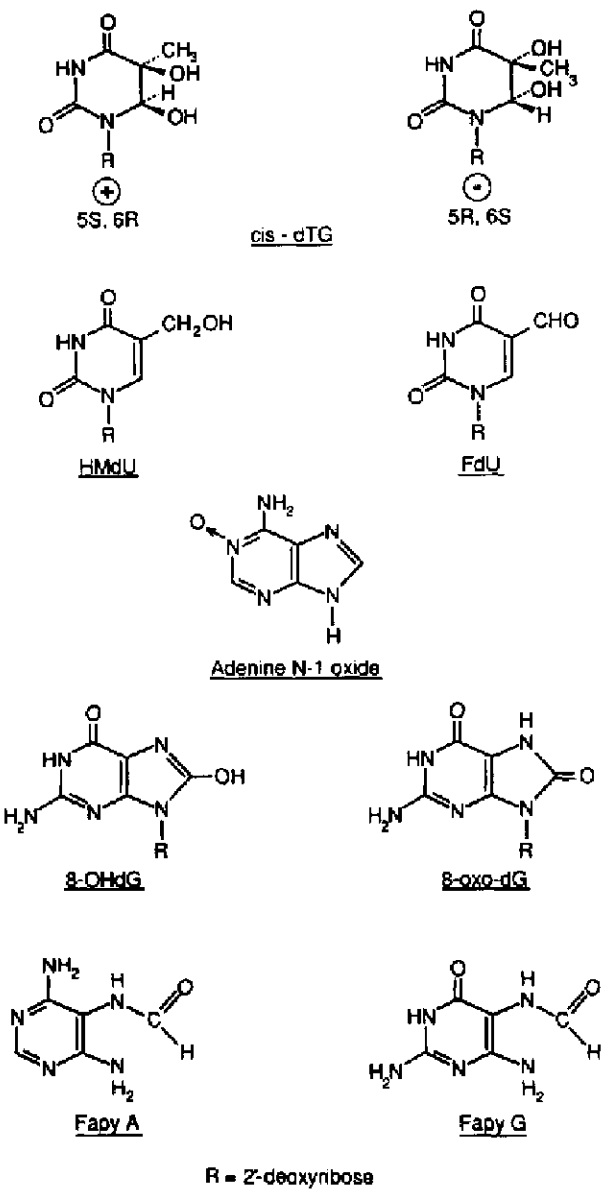

Fig. 2. Structures of selected oxidized derivatives of DNA bases. When R = 2′-deoxyribose: *cis*-dTG: (+) and (−) diastereoisomers of *cis*-thymidine glycol; HMdU: 5-hydroxymethyl-2′-deoxyuridine; FdU: 5-formyl-2′-deoxyuridine; 8-OHdG and 8-oxo-dG: 8-hydroxyl(oxo)-2′-deoxyguanosine; Fapy A: 5-formamido-4,6-diaminopyrimidine; Fapy G: 2,6-diamino-4-hydroxy-5-formamidopyrimidine.

enzyme or extending the digestion time. To obtain nucleosides, treatment with nuclease should be followed by alkaline or acid phosphatase, depending on the pH of the incubation mixture.

To analyze DNA for the presence of intact oxidized nucleosides, we found that the following are the best conditions (Fig. 3) [9,13]. DNA (100 μg), dissolved in 10 m*M* Tris–HCl and 100 m*M* NaCl (200 μl) buffer, pH 7.0, first has to be sheared by passing through consecutively smaller gauge [18–25] needles or by vortex-mixing. This facilitates digestion of undamaged control DNA, which, in particular, cannot be fully digested when at the high molecular mass [16]. After shearing, DNA should be digested with DNase I (100 U in 40 μl of 10 m*M* Tris–HCl) in the presence of Mg^{2+} (20 m*M* final concentration). Mg^{2+} is absolutely required for the activity of DNase I. The reaction mixture is incubated at 37°C for 1 h, the pH is lowered to 5.1 with 0.5 *M* sodium acetate (15 μl), 5 U of nuclease P1 (10 μl) and Zn^{2+} (1 m*M* final concentration) are added, and the mixture is incubated for one additional hour. Finally, the pH is readjusted with 0.4 *M* Tris–HCl (pH 7.8, 100 μl), 3 U of alkaline phosphatase (20 μl) are added, and the mixture is incubated for 0.5 h. DNA hydrolysis is terminated by the addition of 5 ml (or no less than 5 volumes) of pure (HPLC-grade) acetone, which precipitates the enzymes. After 0.5 h at −20°C, the precipitates are removed by centrifugation, and the supernatant is evaporated to dryness under reduced pressure and temperature. The dry residue can be phosphorylated with [γ-^{32}P]ATP, acetylated with [^{3}H]acetic anhydride or dissolved in a small volume of HPLC-grade water and analyzed by an HPLC system equipped with the appropriately sensitive detector(s).

These described conditions of enzymatic DNA hydrolysis were used for concurrent analyses of at least four types of oxidized nucleosides [13]. These are 5-hydroxymethyl-2′-deoxyuridine (HMdU), the (+) and (−) isomers of *cis*-thymidine glycol (dTG), 5-formyl-2′-deoxyuridine (FdU) and 8-OHdG (Fig. 2). However, it appears that under these conditions about half of the 8-OHdG is lost [13]. At this point, it seems that the acidic digestion conditions required for the optimal activity of nuclease P1 may be responsible for that loss, perhaps leading to the release of the free base. When the digestion is carried out with the same enzymes under neutral pH, 8-OHdG seems to be fully recovered, however, recovery of dTG is suboptimal. It is known that dTG is the most stable at acidic pH. The more alkaline conditions allow TG ring opening [43], which may lead to the

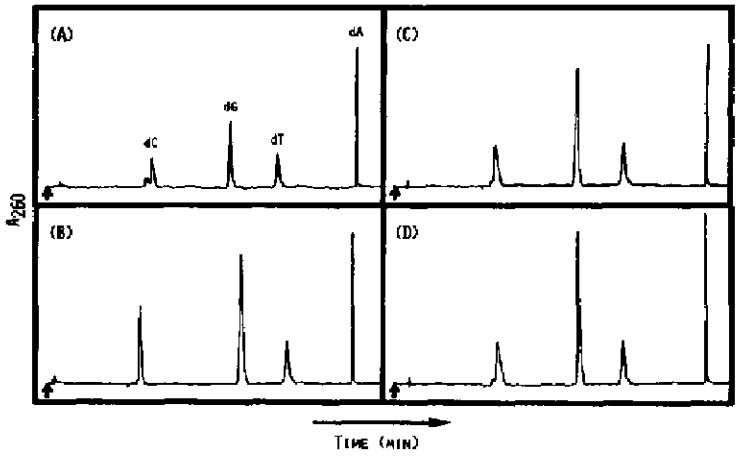

Fig. 3. Optimization of enzymatic DNA digestion to nucleosides. HPLC separation of enzymatic hydrolyzates containing dC, dG, dT, and dA on an ODS column (25 cm × 1 cm I.D.; 5 μm). DNA was hydrolyzed with (A) nuclease P1 and alkaline phosphatase, (B) same as (A) but predigested with DNase I (+Mg^{2+}), (C) same as (A) but in the presence of Zn^{2+}, (D) predigestion with DNase I (+Mg^{2+}) followed by nuclease P1 in the presence of Zn^{2+}, and then by alkaline phosphatase. (From ref. 13.)

mistaken conclusion that its formation in DNA was lower. Hence, depending on the experimental design, one or the other of the oxidized nucleosides cannot be fully recovered. However, due to DNA digestion conditions, this loss can be determined *a priori* and taken into account when calculating the levels of oxidized nucleosides formed due to a particular treatment.

The recovery of nucleosides may also depend on the glassware or plasticware used in their isolation. Floyd [29] showed that the recovery of 8-OHdG can be enhanced over two-fold when plastic microfuge tubes are prewashed with ethanol. Apparently, some tubes are contaminated with a substance or substances that interfere with enzymatic digestion. Routine prewashing of tubes also increases the reproducibility of the assays [29].

Occasionally some digestive enzymes, including DNase I and phosphodiesterase, are contaminated with other degradative enzymes, such as pyrimidine or purine phosphorylases. The latter enzymes release free pyrimidines or purines from the sugar–phosphate backbone, and therefore analyses for the presence of nucleosides and nucleotides cannot be carried out. Although a DNA adduct itself might not be a good substrate for phosphorylase, some or all of the natural bases could be released from the polymer. When the presence of phosphorylase is suspected, its inhibitor should be added to the reaction mixture prior to treatment with other digestive enzyme. For example, use of dA in excess allowed total recovery of normal and oxidized thymine nucleosides, whereas in the absence of dA only free bases could be recovered [44].

2.1.2.4. Enzymatic hydrolysis to oligonucleotides. As opposed to the use of digestive enzymes, this method utilizes a large number of restriction enzymes, which hydrolyze the DNA bonds in regions of specific DNA sequences. The oligonucleotide products of this enzymatic hydrolysis are usually separated electrophoretically. Chemical modification of certain DNA bases either inhibits the normal hydrolysis at those sites or, after several rounds of replication, new enzyme-sensitive sites are formed. These techniques will be described in more detail in Section 2.3.

2.2. Chromatographic analysis of modified bases, nucleosides, nucleotides, and small oligonucleotides

2.2.1. HPLC with various detection systems

The development of HPLC and, in particular, of the columns packed with reversed-phase adsorbents, has made HPLC a very versitile and common chromatographic method used for the identification and purification of modified base derivatives in DNA and RNA. The reversed-phase adsorbents usually consist of silica gel spheres with chemically attached alkyl or phenyl groups. The alkyl moiety may have variable numbers of carbons, from C_2 to C_{18} (octadecylsilane, ODS). To assure complete neutralization of the silica gel's free hydroxyl groups in C_8 and C_{18} adsorbents, those free hydroxyls are end-capped with C_2 alkyl groups.

Reversed-phase adsorbents can be used also for the separation of ionizable solutes, which include nucleoside mono-, di- and triphosphates. Instead of water ± organic solvent, buffers of different ionic strengths and pH values are used. The most commonly used are phosphate buffers, however, care should be taken to prevent their precipitation within the pumps or column. For this reason, whenever possible, salts with the highest solubility should be used. For example, potassium phosphate is preferable to sodium phosphate. Also ion-pairing buffers [45] can be used to override the reversed-phase properties of the columns, and make them cationic or anionic columns. These HPLC-grade buffers are commercially available and HPLC-grade reagents should be utilized whenever possible.

HPLC can be used as an endpoint for analysis, if the modified base derivatives of interest are radioactively prelabeled [6,16,19,28,40,46,47], or if appropriately sensitive detectors are available. For example, if the analysis is for an adduct (*i.e.*, aflatoxin B_1–guanine derivative [19]) or an oxidized nucleoside (*i.e.*, 8-OHdG [48]), a fluorescence or electrochemical detector, respectively, can be used to quantitate the levels of modified bases. However, it is advisable to use each of them in tandem with either a UV or photodiode-array

spectral detector [6,13]. This tandem use of detectors allows quantitation of modified bases with respect to the normal bases present in the DNA being analyzed. When modified base derivatives cannot be detected this way, they are usually postlabeled using radioactive reagents that contain a ^{3}H, ^{32}P or recently utilized ^{35}S label [13,49,50]. Commonly, modified base derivatives are isolated first by a variety of means, and then derivatized and analyzed by HPLC and/or TLC. The HPLC system, in addition to the spectral detector needed for quantitation of normal bases, should be equipped with an on-line radioactive detector. Alternatively, fractions monitored by a spectral detector are collected and, after addition of a scintillant, the radioactive content is determined using an external scintillation counter. The quenching properties of each of the scintillation-counting systems should be determined prior to analysis, using control samples containing expected amounts of radioactive material.

Exposure of DNA to mutagenic and carcinogenic agents often results in binding of those agents to cellular DNA either directly or after metabolic activation. Most agents cause formation of a variety of adducts with DNA bases [1]. It is beyond the scope of this review to discuss in detail what is known about most of those adducts. However, the following will provide examples.

2.2.1.1. Use of radioactive carcinogens. PAHs generally have to be metabolized before they can covalently interact with DNA or RNA. Treatment of cells grown in tissue culture, or of animals by various routes of exposure, with a radioactively labeled [^{3}H]carcinogen [such as 7,12-dimethylbenz[*a*]anthracene (DMBA) and B(a)P] or its synthetically prepared metabolites, frequently results in the formation of labeled DNA base–PAH metabolite adducts. Then, DNA and/or RNA are isolated and hydrolyzed to nucleosides, which are analyzed by HPLC in the presence of non-tritiated marker adducts [46,51,52].

Using co-chromatography with marker compounds on an analytical ODS HPLC column, Jeffrey *et al.* [51] established that the DNA of human and bovine bronchial explants that were exposed to B(a)P contained certain B(a)P–diol-epoxide adducts because some of the markers co-eluted with the ^{3}H-containing 2′-deoxynucleosides. Frenkel *et al.* [46] showed that treatment of cultured rat liver cells with [^{3}H]DMBA caused formation of novel adducts in RNA, since the nucleosides co-eluted with the same marker compounds that were obtained by reacting guanosine with DMBA 5,6-oxide. Guanosine was modified at the C-8 position of the guanine or at the 2′-hydroxyl group of the ribose moiety. Recently, it was shown by Sayer *et al.* [53] that B(a)P 7,8-diol 9,10-epoxide can modify the ribose moiety of adenosine in a similar manner. Ashurst and Cohen [54] and Dipple *et al.* [52] also used a similar approach to determine whether the susceptibilities of different stocks of mice to B(a)P or DMBA carcinogenesis, respectively, are due to the differences in metabolic activation of PAHs and subsequent binding to DNA. Dipple *et al.* [52] demonstrated that, although carcinogenesis is significantly decreased in the presence of antioxidants, DNA adduct levels are comparable to those obtained in the absence of antioxidants. Those results suggest that the formation of adducts, although necessary, is not sufficient for carcinogenesis to occur. It is likely that antioxidants, by inhibiting tumor promotion and/or progression, also suppress tumor development (reviewed in ref. 4). Recently, it was shown that the prooxidant state modulates *in vivo* metabolism of B(a)P, its metabolites, as well as their binding to DNA [55,56], which suggests that ROS also can affect initiation of carcinogenesis (reviewed in refs. 3 and 4).

2.2.1.2. Use of electrochemical detectors in HPLC analysis. In the late 1980s, methods utilizing HPLC analysis and some very sensitive detectors also were developed. Goda and Marnett [57] showed that a pyrimidopurinone adduct, formed by the interaction of a guanine moiety in DNA with malondialdehyde, can be quantitated using an electrochemical detector. Modified DNA was reduced with sodium borohydride, hydrolyzed with 0.1 *M* HCl to bases and separated on an ODS column. The detection limit was 100–200 fmol, using the applied potential of 700 mV *versus* Ag/AgCl.

The availability of electrochemical detection (ED) allowed analysis of DNA isolated from various sources for the presence of 8-OHdG, as is illustrated by Fig. 4. This method was first developed by Floyd *et al.* in 1986 [48], but it rapidly became the method of choice of many investigators. Since then, 8-OHdG has been used as a measure of oxidative DNA damage mediated by a variety of oxidative stress-inducing treatments. It also allowed determination of the background levels of steady-state oxidative DNA damage in humans and animals [58]. The determination of 8-OHdG formation allowed the demonstration that carcinogen (acetoxime, 2-nitropropane) treatment causes different extents of DNA damage, depending on the sex and organ of the rats being analyzed. It also showed that RNA sustains more extensive oxidative damage than does DNA [59]. Moreover, this technique allowed comparisons among various nitroalkanes and oximes (industrial solvents and byproducts) as to their abilities to induce oxidative DNA and RNA damage and to determine their potential for hepatocarcinogenicity [60]. Roy *et al.* [61] found that chronic treatment of male Syrian hamsters with diethylstilbestrol (a drug that caused cancer in the daughters of women who were given it during their pregnancies) induced significantly higher levels of 8-OHdG in their kidneys (the tumor site in this animal model) than in their livers (which are not common tumor sites). Although it has been known for some time that cigarette smoke causes DNA strand breaks [62], only recently, was it shown that it also causes 8-OHdG formation in cellular DNA of human peripheral leukocytes [63] and in cultured cells [64]. Chung and Xu [65] found that one of the carcinogenic tobacco-specific nitrosamines formed from nicotine [4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone (NNK)] induces formation of 8-OHdG in the lung DNA of mice and rats, the site of tobacco carcinogenesis. The same laboratory also showed that chemopreventive agents present in green tea extract and one of its major polyphenols [(−)-epigallocatechin gallate (EGCG)] prevent NNK-mediated oxidative DNA damage [66].

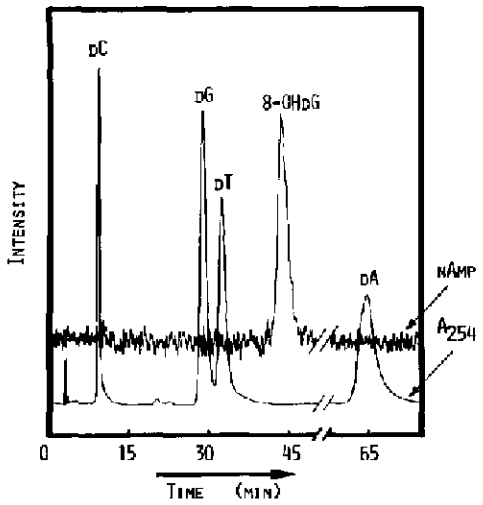

Fig. 4. HPLC separation of 8-OHdG from normal nucleosides dC, dG, dT, and dA. Enzymatic DNA hydrolyzate was separated by HPLC on ODS column (25 cm × 0.46 cm I.D., 5 μm) and monitored by ED (0.5 nA full scale) and by UV detection at 254 nm. Citrate (10 m*M*) + acetate buffer (20 m*M*), pH 5.1, + 5% methanol solution was used as an eluent at a 1 ml/min flow-rate.

Electrochemical detectors were also utilized in the analyses of a number of other oxidized base derivatives and endogenously alkylated bases [5,58]. The oxidized bases include 4,6-diamino-5-N-formamidopyrimidine (FAPY derived by ring opening of 8-OHdA), 5-hydroxymethylcytosine and 5-hydroxy-2′-deoxycytidine, 5-hydroxyuracil and 5-hydroxy-2′-deoxyuridine, the latter two being deamination products of the cytosine derivatives. The ED-detectable alkylated bases also include O^6-methyl-, 3-methyl-, 7-methyl- and 7-ethylguanine, as well as several deoxyguanosine–acrolein products. Hence, HPLC–ED provides a very sensitive measure of a number of alkylated and oxidized DNA products induced by endogenous and exogenous oxidative stress, as well as by alkylating agents.

2.2.2. Postlabeling techniques

2.2.2.1. ^{32}P-Postlabeling with [γ-^{32}P]ATP. In the past decade postlabeling of nucleosides and nucleotides with ^{32}P became a popular method for detection of modified DNA base derivatives with a sensitivity of one modified base per 10^7–

10^{10} normal bases. This technique is applicable to structurally diverse classes of DNA adducts, which are mostly bulky in nature. Some of the smaller (alkyl) adducts [67,68] or oxidized base derivatives also can be postlabeled with ^{32}P but often require different conditions [69]. This method was first introduced in 1981 [70] and takes advantage of the ability of T_4 polynucleotide kinase to phosphorylate the 5′-hydroxyl end of modified 3′-mononucleotides. Other kinases, *i.e.* Epstein–Barr-derived thymidine kinase, also have been utilized [71]. The sensitivity is conferred by use of the high-specific-activity [γ-^{32}P]ATP. The details of the basic assay and modifications thereof are given in a recent review by Beach and Gupta [49] and, therefore, will not be reviewed here. We will just give some examples of its recent applications.

This technique showed that cigarette smokers contain significantly higher levels (> twenty-fold) of DNA adducts per 10^8 nucleotides in the non-cancerous surgical lung parenchymal samples than do ex-smokers [72]. Moreover, the authors found that the levels of aryl hydrocarbon hydroxylase activity (enzyme system involved in the metabolism of PAH present in tobacco smoke) positively correlated with the DNA adducts analyzed in the same lung samples. Although inducibility of this enzyme system has been used as a crude measure of long-term exposures to various PAHs, this work [72] actually showed the connection between the two processes. Using ^{32}P-postlabeling, other investigators suggested that PAH–DNA adducts in human white blood cells cannot be used as a surrogate measure for the PAH–DNA adducts in the lung [73]. However, once the lymphocytes were separated from the granulocytes, adduct levels were found to be significantly higher in the lymphocytes of smokers than of non-smokers [74].

Use of ^{32}P-postlabeling also allowed identification of dGMP substituted at the N-2 position with styrene 7,8-oxide as a major product [75]. This oxide is a carcinogenic metabolite of styrene, a chemical used widely in plastic production. Glycidaldehyde, a reactive metabolite obtained by oxidative dealkylation of glycidyl esters (industrial chemicals), was shown to form an adduct with dA in mouse skin and *in vitro* at pH 7 [10], rather than an adduct with dG which was predominantly formed at pH 10 [76]. Again, this was accomplished because of the sensitivity afforded by ^{32}P-postlabeling. Cyclophosphamide is used as an anti-cancer drug that requires metabolic activation before its activity becomes apparent. That activation results in the formation of several intermediates, including phosphoramide mustard which is considered to be therapeutic, while acrolein, the byproduct of the metabolism, is a mutagen. The use of ^{32}P allowed identification of some of the adducts produced by acrolein and other metabolites that were present in the DNA of the livers of mice treated with cyclophosphamide. Those products included a phosphoester adduct between phosphoramide mustard and dGMP [34,77].

Only recently has oxidative DNA modification begun to be analyzed by the ^{32}P-postlabeling assay. Oxidative stress-induced damage, mediated by either endogenous or exogenous sources, has become of general interest because of a new awareness of its widespread occurrence (reviewed in refs. 2, 4 and 77). 8-OHdG and dTG (Fig. 2), two of the many of oxidized base derivatives, were first analyzed by the ^{32}P-postlabeling technique [39,69,78], followed by identification of the N-1-oxide adenine (Fig. 2) derivative formed by H_2O_2 treatment [79]. Some of the more complex products including γ-radiation oxidized dinucleotides [39], as well as intrastrand crosslinks between A-A and A-G [80] also were analyzed by this technique. ^{32}P-Postlabeling also was applied to the analysis of malondialdehyde-modified dGMP formed in intraperitoneally treated mice [81], and of adducts formed by treatment of human fibroblasts with fecapentaene-12 [82]. Malondialdehyde is of interest as an end-product of chemical- or radiation-induced oxidative degradation of unsaturated fatty acids. Fecapentaene-12 is representative of a family of compounds that are highly genotoxic in human cells, and is thought to contribute to colon cancer. Fecapentaene-12 was shown to produce DNA adducts as well as 8-OHdG, indicating that more than one mecha-

nism may be contributing to its biological effects.

2.2.2.2. ^{3}H Postlabeling with [^{3}H]acetic anhydride. Despite the utility and sensitivity of the ^{32}P-postlabeling technique, it has some disadvantages. The protocol is quite elaborate and depends on the specificity (or rather the lack of it) of enzymes used for the postlabeling. The requirement for an extremely high specific activity of [γ-^{32}P]ATP, which has a short half-life, also makes this method less desirable because usually the background levels are also quite high. As an alternative to this technique, we developed another postlabeling assay [13] that is more appropriate for small adducts and for oxidation products of nucleic acids. It can be used for analysis of various types of nucleosides, since the basis for this radioactive labeling assay is acetylation of hydroxyl groups with [^{3}H]acetic anhydride. The presence of more hydroxyl groups that can be acetylated in a nucleoside renders the assay even more sensitive. In brief, DNA isolated from any source (both *in vitro* and *in vivo*) is enzymatically digested to nucleosides, as described in Section 2.1.2. The hydrolyzate is separated by HPLC on the reversed-phase column (ODS; 5 μm particle size, 25 cm × 1 cm I.D.) in the absence of any marker compounds (Fig. 5). All fractions that elute after 15 min (except those containing normal nucleosides) are combined, dried and acetylated in acetonitrile with [^{3}H]acetic anhydride in the presence of the catalyst 4-dimethylaminopyridine and triethylamine. Unreacted [^{3}H]acetic anhydride is decomposed with methanol. The products are concentrated, and after addition of non-tritiated marker acetates, analyzed by HPLC on the ODS column (Fig. 6). The products are quantitated by relating the amount of radioactive material coeluting with the marker acetate on the second HPLC to the peak areas of normal nucleosides obtained by their integration on the first chromatogram. The quantitation is carried out according to the following formula:

$$\text{pmol nucleoside} = \frac{\text{net cpm of nucleoside acetate}}{0.3 \times \text{conversion coefficient} \times \text{number of acetyl groups}}$$

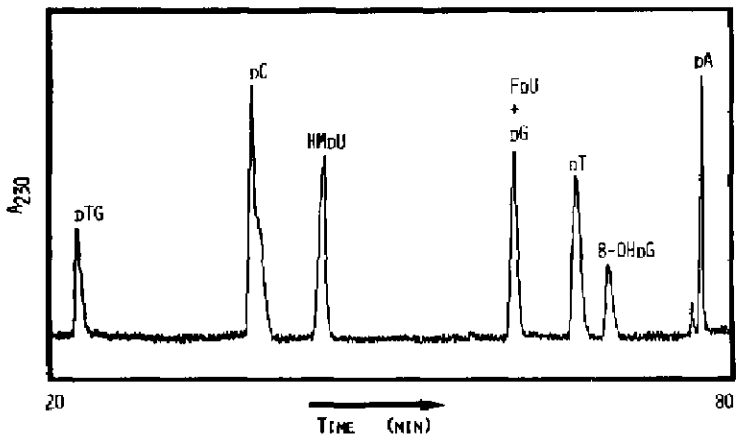

Fig. 5. HPLC separation of normal and oxidized DNA nucleosides. Enzymatic DNA hydrolyzate was spiked with oxidized nucleosides and separated by HPLC on ODS column (25 cm × 1.0 cm I.D., 5 μm) with water (20 min), 2% acetonitrile in water (20 min), 4% (25 min) and 10% (10 min) as eluent, at a 2 ml/min flow-rate. (From ref. 9.)

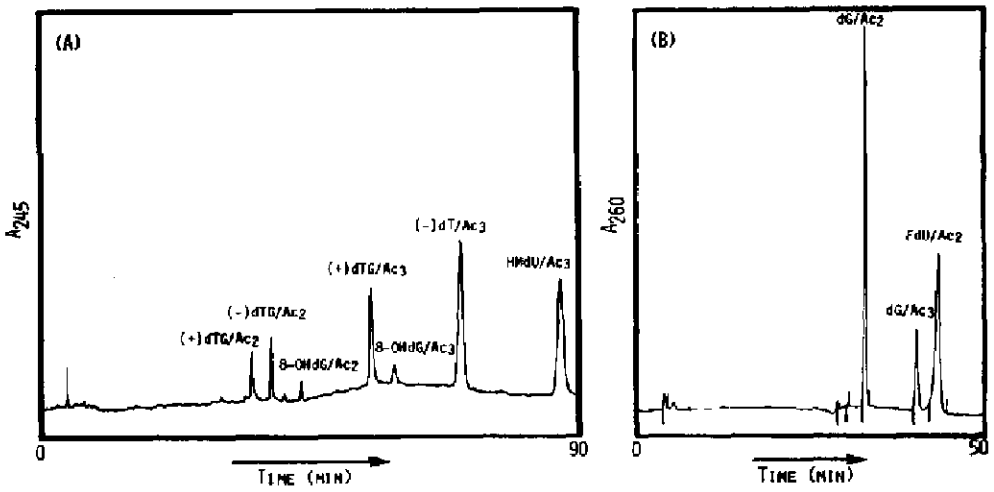

Fig. 6. HPLC separation of nucleoside di (Ac_2) and tri (Ac_3) acetates of (+) and (−) *cis*-dTG, HMdU and 8-OHdG (A) and dG and FdU (B). Fractions known to contain the oxidized 2'-deoxyribonucleosides dTG, HMdU and 8-OHdG, or FdU and dG (collected as described in the legend to Fig. 5) were combined, dried and acetylated. Acetates were separated by HPLC on an ODS column (25 cm × 1.0 cm I.D., 5 μm) at a 2 ml/min with (A) 10% acetonitrile in water (10 min), gradient to 17% (20 min) and held at 17% (60 min) or (B) 10% acetonitrile (20 min) followed by 19.5% (30 min). (From ref. 13.)

In this formula, 0.3 is the efficiency of the scintillation counter (30%). The conversion coefficient depends on the specific activity of the [^{3}H]acetic anhydride used, and is 22.2 or 11.1 when [^{3}H]acetic anhydride (50 mCi/mmol) is diluted with non-radioactive acetic anhydride 1:5 or 1:10, respectively.

Using this assay, we showed that the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) induces *in vivo* formation of oxidized bases HMdU, dTG and 8-OHdG in the epidermal DNA of TPA-treated SENCAR mice [9]. Their formation was TPA dose- and time-dependent. It was inhibited by chemopreventive agents [4,83], including EGCG (from green tea), sarcophytol A (from marine soft coral) and caffeic acid phenethyl ester (CAPE; from the propolis of honeybee hives), as well as the anticancer drug [84] tamoxifen [85,86]. Moreover, we showed that TPA evokes formation of these oxidized bases in SENCAR mice at much lower doses than it does in C57BL/6J mice, which correlates well with the responsiveness of these two mouse strains to TPA-mediated tumor promotion [87]. Treatment of SENCAR mice with other tumor promoters of the TPA type also caused formation of HMdU and 8-OHdG, the levels of which correlated with the potencies of these agents as tumor promoters [86]. Recently, we found that DMBA, which is a PAH requiring metabolic activation to express its carcinogenic properties, also induces oxidative DNA base damage (HMdU and 8-OHdG) at the same time as its DNA base adducts are formed [88,89]. It appears then that formation of oxidative DNA base damage may be necessary for tumor-promoting properties. Using ^{3}H-postlabeling with [^{3}H]acetic anhydride, we also showed that the hydroperoxide moiety of 5-hydroperoxymethyl-2'-deoxyuridine (HPMdU), which is a product of ionizing radiation, is capable of oxidizing bases in bacterial DNA [90]. This finding may explain some of the protracted effects of γ radiation.

2.3. Electrophoretic analyses

The chromatographic techniques described above allow identification and quantitation of specific modified bases and adducts. However, electrophoretic methods are invaluable for determination of the genomic sites of those modifications and the structural consequences. To acquire

sufficient quantities of DNA needed for those techniques, polymerase chain reaction (PCR) DNA amplification provides a necessary tool.

2.3.1. Polymerase chain reaction

Rather than analyzing total genomic DNA, PCR allows the selective study of any gene or subgene region, provided that appropriate PCR primers are available. The PCR techniques, which were first introduced in the late 1980s [91–93], have revolutionized the field of molecular biology. The original PCR protocols, as well as optimization, automation procedures, and experimental applications have been summarized in detail in several review articles [94,95] and laboratory manuals [96,97]. In brief, the repetitive temperature cycling allows DNA denaturation, primer annealing, and primer extension by a thermally stable polymerase (taq, Vent™, pfu), such that exponential synthesis of the desired DNA fragment occurs during an average of thirty amplification cycles. Various modifications of the original protocols have evolved, so that in addition to DNA amplification, also RNA amplification, mRNA quantitation, DNA sequencing, gene cloning and labeled probe preparation are among the frequent current uses of PCR technology. Additionally, multiple primers can be combined in a multiplex reaction to simultaneously amplify several DNA fragments. This is particularly useful for the detection of DNA damage among the multiple exons of large mammalian genes, such as *hprt* [98,99] or the Duchene muscular dystrophy gene [100], yielding both experimental and clinical information. PCR protocols are relatively straightforward, however, extreme caution must be taken to avoid the cross contamination of DNA samples leading to the amplification of undesired DNA. The sensitivity of the amplification procedure is so great that even 1 pg of spurious DNA can be a suitable template for this process [101].

2.3.2. Gel electrophoresis

Gel electrophoresis can be employed when separation of different sizes of DNA fragments is required. The methodology is based on the acquisition of an electric charge by the nucleic acid molecules, such that those with the highest charge (the longest molecules) will be retarded in the gel, while shorter molecules (lesser charge) will migrate further. Although agarose gels (0.5–1.5%) effectively separate DNA fragments ranging from 30 to 0.2 kilo base pairs (kb), acrylamide gels are often utilized for better separation of smaller DNA fragments (< 1 kb). Vertical denaturing urea–acrylamide gels are used for high-voltage separation of DNA sequencing products. The DNA migration distance is dependent upon several parameters, including the composition (% gel) and pH (neutral or alkaline) of the gel, and the electric field components (voltage, current, duration). Although both Tris–acetate and Tris–borate are commonly used buffers, Tris–borate is often recommended for long, overnight electrophoresis protocols.

Horizontal gel electrophoresis is an essential molecular biology technique for numerous preparative and analytical purposes. Gels are typically used to purify plasmids and to recover other small DNA fragments including probes. They can be useful for estimating DNA amounts when known quantities of DNA are included for comparison, since double-stranded DNA is readily observed in the gels by ethidium bromide staining and UV visualization. Accurate estimates of the size of small DNA molecules or fragments (< 10 kb) can also be obtained from either agarose or acrylamide gels when appropriate low-molecular-mass DNA size standards are run simultaneously. Agarose gels are routinely used to monitor the expected size of PCR-generated products (as illustrated by Fig. 7). Alterations such as small deletions and insertions can often be detected by the recovery of smaller or larger than expected PCR products, respectively. For detection of DNA damage, restriction enzyme-digested or otherwise fragmented DNA is separated on agarose gels, which are subsequently processed for gene- or sequence-specific probe hybridization.

2.3.3. Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis (PFGE) allows

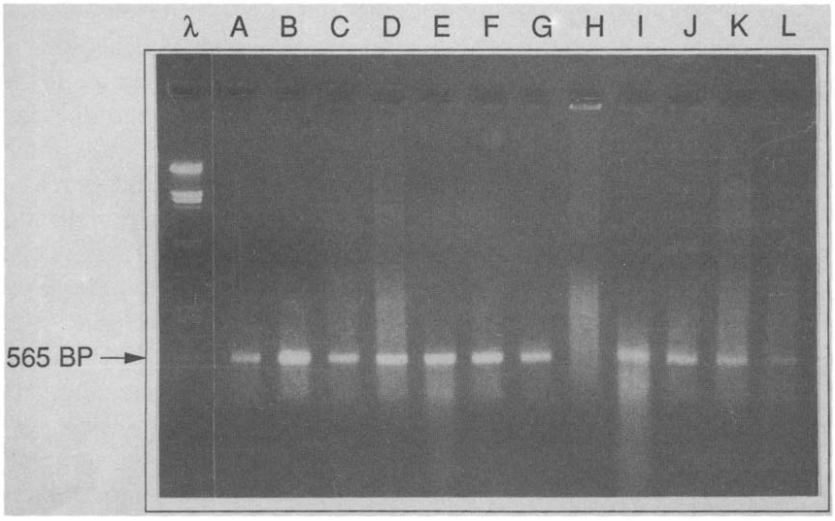

Fig. 7. Agarose gel electrophoresis of PCR-amplified DNA. X-Ray-induced mutants of transgenic *gpt*$^+$ Chinese hamster V79 cells are screened by PCR for loss of the integrated sequence. The figure shows ten mutants (lanes B–K), of which all but one (lane H) have retained the gene. Lane A is the PCR amplified *gpt* sequence from non-mutant transgenic cells, and lane L shows the lack of this sequence in parental V79 cells.

fractionation of large DNA fragments in the range of 50 kb to 2 Mega base pairs (Mb) [102]. Unlike standard horizontal gel electrophoresis units, which utilize generally low-voltage (20–100 V) unidirectional electric fields, PFGE units employ rapidly alternating bidirectional electric fields of higher voltage. Using PFGE, electrophoretic separation of intact yeast chromosomes has been accomplished [102]. When combined with the use of the rare cutter restriction enzymes, pulsed-field gels facilitate genome mapping and allow detection of large-scale DNA damage, such as multilocus deletions. PFGE also is useful for the detection of double-strand DNA breaks [103], and has been applied recently to studies of the induction and repair of ionizing radiation-induced double-strand breaks [104]. Detection of single-strand DNA breaks generated by metal-mediated oxidative processes has also been accomplished using these methods [105,106]. Additionally, PFGE methods have been useful in studies of Wilm's tumor gene methylation patterns [107].

2.3.4. Southern blot analysis

The Southern blot protocol for DNA transfer and hybridization [108] represents one of the first universally adopted methodologies for the detection of DNA damage and genetic alterations within defined regions of the genome. These protocols have been well defined [109] and genome analysis generally relies upon the permanent transfer of denatured DNA fragments to a solid filter matrix, which can be nitrocellulose, neutral or positively charged nylon, or other synthetic materials such as polysulphone. The DNA transfers occur by capillary action in salt or alkaline solutions, or can be accomplished much faster with the aid of a vacuum transfer system. The DNA is linked to the supporting filter by baking (80°C in a vacuum oven) or more recently by UV linkage, hybridized with radiolabeled gene- or sequence-specific probes, and analyzed on autoradiograms. Alternatively, recently developed chemiluminescent detection systems [110,111] can be used. Large-scale genetic damage is determined by analyses of variations between the experimental restriction enzye patterns *versus* those of the wild-type DNA. However, single DNA base pair (bp) changes, as well as rearrangements and small deletions involving less than 50 bp, are not readily detectable on Southern blots. Thus, these blots

are most useful in identifying gene deletions (>50 bp), insertions, or other major genetic rearrangements. In lieu of the blotting procedures, hybridization of the desired DNA probes can be accomplished directly on dried agarose gels [112], bypassing the often inefficient DNA transfer step. Southern blot protocols have been modified to accommodate RNA studies (Northern blots) [113] and protein studies (Western blots) [114]. Dot blot and colony blot protocols take advantage of DNA transfer and probe hybridization technology in procedures where gene probing but not DNA fragment separation is required.

2.3.5. Restriction fragment length polymorphisms

Restriction fragment length polymorphism (RFLP) analysis [115,116], in which differential patterns of gene-specific or DNA sequence-specific restriction enzyme digestions are visualized on Southern blots, reflects both the natural variation in the human genome and disease-related or environmentally induced genome rearrangements, which specifically affect the recognition sequences of the restriction enzymes [117]. RFLP analysis is being applied to studies of environmental exposures to DNA damaging agents, and to screen populations for potential susceptibilities to environmentally-induced diseases [118, 119]. These methods have been made even more sensitive by the use of allele-specific oligonucleotide (ASO) probes, which can be used to screen for specific DNA sequence changes. The use of ASO probes can identify disease-related genome alterations (base changes) at predetermined genetic hotspots, as well as mutations relevant to the development of cancer. Specific mutations in the *ras* oncogene codon 61, for example, can be readily identified and quantitated with ASO probes [120].

2.3.6. Denaturing gradient gel electrophoresis

Although electrophoresis of fragmented genomic DNA generally permits detection of large-scale perturbations, urea formamide denaturing gradient polyacrylamide gels [121,122] are useful for visualizing DNA sequences harboring smaller genetic alterations including single base mutations. This method is based on the fact that a single base change in any given sequence alters the melting properties of that sequence, and thus can be detected. The denaturing gradient gels, which are defined as parallel when the gradient increases toward the bottom of the gel and perpendicular when the gradient increases from left to right, can distinguish between DNA:DNA homoduplexes *versus* heteroduplexes. Denaturing gradient gel electrophoresis (DGGE) has been utilized to monitor the induction of DNA damage in many human genes, including *hprt* [123]. Using synthetic matrix gels rather than denaturing acrylamide gels, a variable migration of homoduplexes *versus* heteroduplexes also can be seen, as reported in a recent study of human tumor-derived p53 mutations [124]. DGGE can be applied also to the analysis of PCR products, and is more effective when 5′GC clamps are engineered into the PCR fragments by addition of a 40 nucleotide GC-rich sequence to the 5′ end of the sense PCR primer [125]. DGGE is not limited to the detection of small changes, but also separates double-stranded DNA that differs from the wild type by small deletions and insertions.

2.3.7. Single-strand conformational polymorphism analysis

Non-denaturing gels can also be utilized for analysis of single base alterations in DNA sequences by single-strand conformational polymorphism (SSCP) analysis [126,127]. The protocol is based on differential electrophoresis of denatured single-stranded PCR products through glycerol-modified polyacrylamide gels, or through some novel polymer gels. Although SSCP analysis can visualize altered migration of mutant *versus* wild type DNA samples, this method seems to be more accurate for shorter (<200 bp) rather than longer (>350 bp) PCR products [128]. All electrophoresis procedures, which identify specific gene regions that have sustained small-scale damage, are invaluable DNA-sequencing prerequisites that narrow the scope of the subsequent sequencing task.

2.3.8. Heteroduplex mismatch cleavage analysis

Another method by which small-scale specific DNA base damage can be detected within large mammalian genes is *via* cleavage of single-stranded regions or single-base mismatches in RNA:RNA or RNA:DNA heteroduplexes [121, 129], when the heteroduplexes are subjected to RNase A or chemical cleavage then run on non-gradient denaturing polyacrylamide gels. Although RNase A cleavage may only recognize 30–50% of the possible single-base mismatches [129], longer regions of non-homology (due to deletions or rearrangements and insertions) offer an excellent opportunity for additional RNase cleavage. This method was applied to detection of a specific deletion in Lesch–Nyhan syndrome patients [130]. Cleavage of specific DNA base mismatches has been accomplished by chemical modification of those mismatches followed by piperidine treatment. Hydroxylamine and osmium tetroxide modify mismatched or unmatched cytosines and thymines, respectively, which can be cleaved by piperidine [131]. Carboiodomide can be used to stall polymerases at the unpaired G**T residues [132]. Chemical cleavage protocols, which have been utilized to analyze many human genes, have recently been optimized to allow complete detection of all possible base changes in DNA fragments up to 2 kb, and to identify these as well as small insertions and deletions by non-radioactive detection methods [133].

2.3.9. DNA sequencing

DNA sequence analysis, *via* base-specific chemical cleavage [134,135] or dideoxy nucleotide chain termination [136] protocols, allows direct identification of the DNA base sequence of a given gene or DNA fragment when the end-labeled fragments are run onto acrylamide gels. These methods have been described in detail elsewhere [137,138] and, therefore, will not be extensively reviewed here. It should be noted, however, that DNA sequencing technology has undergone many revisions since those original protocols, to allow sequencing through genomic regions of difficult secondary structures, to utilize several different polymerases, and to facilitate automated sequence analysis and multicolor fluorescent nucleotide identification [98,139]. Sequencing technology has been highly commercialized, such that numerous ready-made reagent kits are available to suit all research and diagnostic purposes. Most recently, DNA sequencing protocols have been modified to take advantage of PCR technology and *taq* polymerase for copying DNA templates and incorporating dideoxynucleotides [140,141]. For this purpose, it may be preferable to utilize other polymerases [142], which are more faithful than *taq* [143].

Several methods can be followed to recover the DNA required for sequencing. PCR amplification of a known sequence can be useful, when specific PCR primers are available, however, sequencing of the resulting double-stranded PCR product is sometimes problematic. Alternatively, asymmetric PCR [144], in which the two sequence-flanking primers are present at a 1:100 ratio during amplification, allows generation of a single-stranded sequencing template. In order to produce readable sequencing gels, however, the PCR-generated templates must first be thoroughly purified to remove all traces of PCR reagents. Any residual dNTPs will interfere with the incorporation kinetics of the dideoxynucleotides, resulting in ambiguous sequencing bands [145]. Template purification can be accomplished by the use of specifically designed microcentrifuge columns or gels [103,146]. Single-stranded DNA sequencing templates can also be generated by cloning the required DNA fragments into an appropriate sequencing vector such as M13.

Although DNA sequence information is useful to identify sequence changes resulting from base alterations, which may have occurred by exposure to DNA-damaging agents, the analysis requires comparison of the experimentally derived sequence with a known gene sequence. For many applications this is very useful, however, not all DNA base adducts ultimately yield sequence alterations. There also may be some ambiguities in both the experimental and known sequence, due to the inherent infidelity of the polymerases used to generate the data, particularly when

PCR-generated templates are being used [147]. DNA sequence analysis is useful primarily for the identification of DNA damage induced by agents that have the capacity to interact with DNA and alter its chemical structure. For agents which may alter gene expression *via* transcriptional regulation, post-transcriptional modification or gene amplification, DNA sequence analysis of the coding region of a given gene would not yield useful information. Additionally, DNA sequencing protocols are quite laborious and the required effort is compounded by the generally large size of most mammalian genes. Detailed sequence analysis is therefore often intentionally restricted to several exons of a particular gene, or to previously identified hot-spot regions in order to limit the scope of the analysis.

2.4. Immunological techniques

In addition to chemical, chromatographic, and electrophoretic methods of detection, immunological techniques are used in analysis of environmentally-induced DNA damage. These include: (1) analysis of DNA with polyclonal and monoclonal antibodies (Ab), which recognize either a specific base modification or a group of related DNA products; (2) detection of fragments of modified base derivatives excreted into the urine; and (3) detection of Ab that recognize DNA damage in the sera of individuals, who were or were suspected of being exposed to the particular damaging agents.

2.4.1. Detection of modified bases in DNA

Occupational exposure to a variety of genotoxic and carcinogenic agents often results in accumulation of damage in the DNA. That damage can be detected by chemical and physicochemical means. However, those techniques often are not sensitive or specific enough. As early as the late 1970s, there were reports showing that Ab can be used to detect DNA modified by the carcinogen N-acetoxy-N-acetyl-2-aminofluorene [148]. Alkylated dG was quantitated (by a high affinity Ab) in DNA exposed to N-ethyl-N-nitrosourea and other alkylating agents [149,150], while B(a)P-modified dG was detected by radioimmunoassay [151]. Since then, the immunological methods have become streamlined as well as more sophisticated, and have been quite widely used in monitoring environmental exposures. For example, B(a)P diol epoxide adducts in DNA have been detected in animal and human tissues [152], and in peripheral blood lymphocytes of coke oven workers [153,154]. Monoclonal Ab were used for quantitation of carcinogen–DNA adducts [155], and for assessing DNA damage and repair following exposure to aflatoxin B_1 [156].

Those methods utilized the competitive enzyme-linked immunosorbent assay (ELISA). This assay is based on attaching appropriate antigens (*i.e.*, modified and unmodified DNA, or a protein carrying a particular modified base) to the wells of polyvinylchloride microtiter plates, followed by incubation with polyclonal or monoclonal Ab. Those Ab can be preincubated with DNA isolated from a biological source or with modified nucleosides obtained from DNA prior to their application to the wells. If DNA samples contain relevant modification(s) recognized by those Ab, the specific binding to the wells would decline. Next, an Ab (with a covalently-bound enzyme, *i.e.*, horseradish peroxidase or alkaline phosphatase) that recognizes the primary Ab bound to the plate is used, followed by addition of the substrate of that enzyme. The extent of the enzyme-mediated reaction with the substrate is proportional to the amount of enzyme bound through a secondary Ab to the primary Ab, which interacted with the antigen attached to wells. These interactions can be measured colorimetrically, fluorometrically or by detection of radioactive products.

Similar assays also have been applied to the detection of oxidized bases in DNA. As early as 1982, a radioimmunoassay was used to detect TG in DNA using rabbit antiserum [157]. Shortly thereafter, a monoclonal anti-TG Ab was prepared [158]. This Ab was utilized for detection of TG in DNA exposed to ionizing or near-UV (actinic) radiations, and DNA oxidized by OsO_4 or H_2O_2. ELISA, utilizing this monoclonal Ab, allowed monitoring of the formation and repair of TG in γ-irradiated cultures of African green

monkey cells. Using anti-TG rabbit polyclonal Ab, Hubbard *et al.* [159] studied radiogenic and OsO_4-mediated formation of TG, and compared those results with ones obtained by using chemical and enzymatic methods. More recently, monoclonal anti-TG Ab were applied to the analysis of DNA of human mammary epithelial cells that were exposed to B(a)P [160]. That DNA contained increasing amounts of TG with increased exposure time. Moreover, the results indicated that it is the arachidonic acid cascade that is involved in formation of the B(a)P-induced oxidative DNA damage. These findings are very important because the presence of an oxidized base in DNA was shown in cells exposed to a PAH. Prior to that, our laboratory showed (using chemical methods) that treatment of hepatic microsomes with B(a)P causes formation of TG and 5-hydroxymethyluracil (HMU) in the coincubated DNA [40]. Recently, we showed that oxidative damage in DNA also occurs *in vivo* in epidermis of SENCAR mice treated with DMBA [88,89]. That DNA contained HMdU as well as 8-OHdG. Anti-8-OHdG rabbit polyclonal Ab were used with a sensitivity approaching that of ED but were much less susceptible to interference by other components present in the DNA or excreted into the urine [161].

2.4.2. Detection of modified DNA base derivatives in urine

This subject and its applicability to biomonitoring were recently reviewed [162] and, therefore, it will not be extensively discussed. Briefly, DNA modified by a variety of agents is subject to repair, which is either chemical or enzymatic in nature [163–165]. Many of the removed DNA base adducts or oxidized bases are excreted in the urine, from which they can be isolated and analyzed. Since urine contains a variety of components, it is not easy to isolate and quantitate a particular chemical. Immunological methods hold great promise in this respect because they can utilize Ab-containing immunoaffinity columns for isolation and purification of an antigen from urine. Preferably, those Ab should not be too avid to allow elution of that antigen from the column. Then, high-affinity Ab can be used for that antigen's quantitation. These methods (immunoaffinity chromatography and ELISA) were utilized for quantitation of urinary 3-methyladenine and the effect of a diet on its levels [166], and for quantitation of several 4-aminobiphenyl–DNA adducts [167]. 4-Aminobiphenyl, a human bladder carcinogen, is an occupational hazard (although decreased because of governmental regulation), and is present also in tobacco smoke. Additionally, these techniques were applied to the quantitation of aflatoxin-N^7-guanine, which is a product of DNA interaction with hepatocarcinogen aflatoxin B_1, a contaminant in foodstuffs such as peanuts [168]. This assay also was used to show that 1,2-dithiole-3-thione is chemoprotective in rats exposed to aflatoxin B_1. As mentioned above, 8-OHdG can be isolated from urine by immunoaffinity chromatography [161], and used as a biomarker of *in vivo* oxidative stress [169].

2.4.3. Recognition of modified DNA bases by antibodies present in human sera

Coke-oven workers are exposed to a number of PAHs (51–162 $\mu g/m^3$ inside a protective respiratory mask), including B(a)P (7–10 $\mu g/m^3$) [153, 154]. Uncompetitive ELISA [using goat anti-human immunoglobulin (Ig) reagents and the avidin–biotin horseradish peroxidase (HRPO) detection system] showed that 11 (about 27%) of 41 workers whose sera were analyzed elaborated Ab that recognize B(a)P diol epoxide–DNA adducts [153]. Cigarette smoking did not influence the frequency of those Ab present in the workers' sera. Those Ab were predominantly of the IgG isotype, but sera of two workers also contained IgM Ab.

Recently, our laboratory looked for Ab that recognize oxidized DNA base derivatives in human sera [170–172]. We also used ELISA as the assay system but with different detection reagents. Microtiter plates were coated with HMdU coupled to bovine serum albumin (HMdU–BSA) or mock-coupled BSA for detection of non-specific binding, which usually was very low. After incubating human sera (dilutions of $2.5 \cdot 10^3$–$1 \cdot 10^5$) in the precoated wells, goat anti-human IgM with

bound HRPO was used as the secondary Ab, since these Ab were exclusively of the IgM isotype. Addition of the substrate, *o*-phenylenediamine, and H_2O_2 resulted in the development of a yellow color (at acidic pH), which was proportional to the amount of human Ab bound to wells, that was measured at 492 nm in an ELISA reader. We found that even healthy people develop anti-HMdU Ab. However, those suffering from a variety of inflammatory diseases contained significantly elevated titers of this type of Ab. The avidity of binding seems to depend on the type of inflammatory condition, being the lowest for systemic lupus erythematosus < psoriasis < immune complex diseases < patients with a history of neoplasia. Ab titers in the sera of patients with some non-inflammatory conditions (*i.e.*, bulous pemphigoid, venous leg ulcers) were even lower than those in healthy controls. Interestingly, treatment with cytotoxic and anti-inflammatory drugs decreased the elevated Ab titers virtually to control levels [171]. In contrast, treatment of psoriasis with UVB enhanced those Ab titers to even higher levels [172]. Recently, we analyzed the sera of workers exposed to carcinogenic metal derivatives. These Ni–Cd battery workers were exposed to Cd and Ni oxides. We found that both metals enhance anti-HMdU Ab titers, with Ni being more potent than Cd (Fig. 8) [173]. Since a number of metals have been shown to induce oxidative processes [174–176], the presence of anti-HMdU Ab in human sera may be a measure of exposure to those metals as well as to the cumulative effects of oxidative stress, which is a hallmark of chronic inflammation known to exert cocarcinogenic actions [4]. Interestingly, the sera of workers exposed to Ni and/or Cd also contained Ab that recognize brain glial fibrillary acidic protein as well as neuron-specific neurofilament proteins (H. Evans, personal communication).

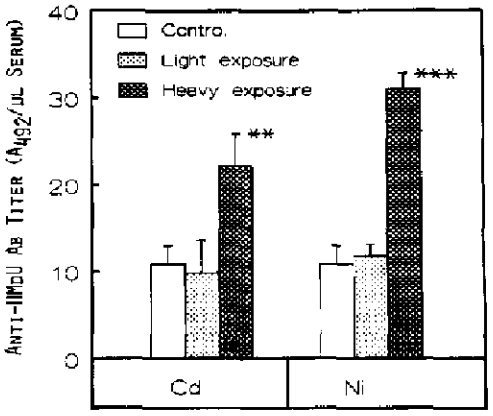

Fig. 8. Anti-HMdU Ab titers in sera of workers monitored for exposure to Cd and Ni. Paired experiments; exposures of Ni–Cd battery factory workers measured by individual monitors. $^{**}p < 0.01$; $^{***}p < 0.001$. Occupational controls are included for comparison. Monitored light exposures [20.2 ± 8.6 μg/m^3 (n = 7) for Cd, and 49.3 + 11.9 μg/m^3 (n = 10) for Ni], and heavy exposures [1034 ± 236 μg/m^3 (n = 5) for Cd, and 1140 ± 830 μg/m^3 (n = 2) for Ni]. (From ref. 173.)

3. CONCLUSIONS

In summary, we have presented a number of techniques that are currently being used in the analysis of "environmentally" damaged DNA. These typically include chromatographic and chemical methods, as well as electrophoretic and immunological techniques. These methods rapidly become more sophisticated and new types of assays continually become available. There are many other analytical methods that take advantage of biological end-points, which are beyond the scope of this review. These may include the use of various repair enzymes or detection of mutagenic and/or carcinogenic potential, as well as cytogenetic analysis of chromosomal exchanges and aberrations. In conclusion, regardless of the scientific background there is an appropriate method that can be applied to the analysis of "damaged DNA".

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