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Evaluation of ^{32}P-postlabelling as a Suitable Detection Technique for Biomonitoring of Human DNA Adducts

MARIE STIBOROVÁ[a*], MARTINA RUPERTOVÁ[a], PETR HODEK[a], EVA FREI[b] and HEINZ H SCHMEISER[b]
[a]*Department of Biochemistry, Faculty of Natural Sciences, Charles University, Albertov 2030, 128 40 Prague 2, The Czech Republic*
[b]*Department of Molecular Toxicology, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*

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DNA adducts generated by carcinogenic chemicals reflects human exposure and DNA adducts are directly related to tumour formation. For detection of DNA adducts the most widely used methods include mass spectroscopy, fluorescence spectroscopy, immunoassays and ^{32}P-postlabelling. Among them, the ^{32}P-postlabelling method meets most of the requirements needed to be applicable in human exposure settings, and, therefore, it seems to be the most appropriate for biomonitoring of human DNA adducts. This technique has emerged as a major method to detect DNA adducts induced by structurally diverse carcinogens and other DNA lesions (i.e. apurinic sites in DNA, oxidative damage to DNA, UV-induced photodimers). The method comprises enzymatic degradation of DNA to deoxynucleotides, enrichment of adducts, 5´-^{32}P-labelling, adduct separation by TLC (or HPLC), and detection and quantitation of adducts. The article presents the individual ^{32}P-postlabelling techniques (standard procedure, enrichment methods) and a critical evaluation of these assays, besides reviewing the applications of the method to different DNA modifications, and its utilization in human biomonitoring studies to detect damage from occupational exposure to carcinogens, and also from environmental (i.e. non-occupational) exposures.

Key Words: Carcinogen, DNA adducts, Risk assessment, Biomonitoring, ^{32}P-postlabelling assay

Introduction

Genetic damage that produces a heritable loss of growth control comprises a major mechanism in chemical carcinogenesis. Most chemical carcino-gens require activation to reactive intermediates that bind to nucleophilic centres in proteins and nucleic acids thereby forming covalent adducts (Miller & Miller 1981, Hemminki 1983). Now, the weight of evidence supports the notion that exposure to most carcinogens results in damage to the structural integrity of DNA, which most likely results from covalent binding of a reactive metabolite of the carcinogen to DNA, leading to the formation of DNA adducts (Hemminki et al. 1994). Approximately 90% of the chemicals considered carcinogenic for humans form covalent DNA adducts. Therefore, such DNA damage is generally considered to be causative and directly related to tumour formation (Lutz 1986, Hemminki 1993, Hemminki et al. 1994, Otteneder & Lutz 1999, Poirier et al. 2000, Wiencke 2000, Tank et al. 2001). Indeed, associations have been observed between DNA adduct formation, and mutagenesis (Hemminki 1993, Verna et al. 1996), and tumourigenesis (Lutz 1986, Otteneder & Lutz 1999, Poirier & Beland 1992); while reduction in DNA adduct levels have been associated with chemoprevention (Kensler et al. 1996, Poirier 1996). Nevertheless, molecular mechanisms for these and other biological consequences of carcinogen exposure remain obscure. It was found that some adducts are highly mutagenic and associated with carcinogenesis, while other adducts are not (Yuspa & Poirier 1988, Hemminki 1993). The majority of DNA adducts is eliminated by DNA repair processes, however, some persistent adducts often cause mutations in important growth-controlling genes or loci, resulting in aberrant cellular growth and cancer (Bishop 1991,

*Corresponding author: *E-mail:* stiborov@natur.cuni.cz; *Tel*: +420-221951285; *Fax*: +420-221951283

Hemminki 1995). Particularly notable have been studies in animal models that have demonstrated an association between mutation "hot-spots" in proto-oncogenes and tumour suppressor genes and specific DNA adducts. Mutations considered carcinogen specific have been observed in *p53*, *ras* and other reporter genes in humans (Lehman & Harris 1994, Brash et al. 1996, Denissenko et al. 1996, Kinzler & Vogelstein 1996, Arlt et al. 2002, Feng et al. 2002a, b); however caution must be used in the interpretation of such data because of the complex nature of human exposures (Poirier et al. 2000, Povey 2000). Furthermore, the relative roles of chemical reactivity in the formation of a lesion and the carcinogenic potency of a particular lesion in the establishment of clonal growth advantage remain enigmatic. Elucidation of these interactions might best be achieved by concomitant application of a spectrum of biomarker assays (Poirier et al. 2000). Of them, methods for the detection and characterization of DNA adducts are the most crucial. Therefore, during the last two decades the development of new sensitive and specific methods for DNA adduct detection was enormous. Knowledge of such methods has become extremely important not only for the above mentioned purposes, but also for their utilization in risk assessment of chemicals as well as in determinations of exposures to carcinogens for humans.

Detection Techniques for Biomonitoring of DNA Adducts in Humans

For an assay to be applicable in human exposure settings, it should (i) be sensitive enough to detect low levels of adducts; (ii) require only microgram quantities of DNA; (iii) produce results that can be quantitatively related to exposure; (iv) be applicable to unknown adducts that may be formed from complex mixtures, (v) be able to resolve, quantitate and identify adducts, (vi) be inexpensive, (vii) be rapid, (viii) be able to analyse large number of DNA samples and (ix) produce low risk to the person carrying out the procedure (Gupta 1993, Stiborová et al. 1998).

Until 1980, the detection of DNA adducts has usually required the use of highly radioactive labelled chemical carcinogens (labelled by ^{3}H or ^{4}C) prepared synthetically. Therefore these studies in humans were impossible. Substantial achievements, over the last two decades, in our understanding of carcinogen-DNA interactions, have resulted largely from the development of sensitive and specific methods for DNA adducts measurement (Hemminki et al. 1994, Phillips 1997, Stiborová et al. 1998, Phillips & Castegnaro 1999, Poirier et al. 2000). The most frequently used methods for carcinogen DNA adduct detection, which represent marked technological improvements in the field of adduct measurement and have extended the detection limits for carcinogen-DNA adducts to monitor human exposure, include immunoassays (Poirier 1981, 1993, Muller & Rajewsky 1981, Strickland & Boyle 1984), and immunohistochemistry (Poirier 1981, Poirier et al. 1993, Santella 1999) using adduct-specific antisera, fluorescence and phosphorescence assays (Weston 1993, Li et al. 1999), ^{32}P-postlabelling (Randerath et al. 1981, Randerath & Randerath 1994, Phillips 1997, Stiborová et al. 1998), electrochemical detection (Yamamoto & Ames 1987, Vasquez et al. 2001), and mass spectroscopy [i.e. electron spray ionization (ESI), matrix-assisted laser desorption ionization (MALDI) and accelerator mass spectrometry] (Marnett & Burcham 1993, Hecht et al. 1994, Vogel et al. 1995, Dingley et al. 1999, Koc & Swenberg 2002, Tretyakova et al. 2002). Recently, another method, capillary electrophoresis and laser-induced fluorescence monitoring, was developed and showed to be promising for DNA adduct measurement (Schmitz et al. 2002, Stach et al. 2003). Furthermore, the single cell gel eletrophoresis (comet) assay might be another of the methods suitable for DNA adduct detection. Although primarily developed to detect DNA strand breaks, this method is increasingly being used to detect certain types of DNA adducts such as DNA interstrand crosslinks (which are not easily detected by other methods, including ^{32}P-postlabelling) (Hellman et al. 1995, 1997, Vaghef & Hellman 1995, Vaghef et al. 1996, 1997, Abt et al. 1997). Each method has specific advantages and disadvantages and most have been successfully applied in experimental models where only one compound is administered. However, for human

samples where multiple diverse adducts are present, it is difficult to obtain either exact quantitation of individual adducts or chemical characterization of a specific adduct, unless combined with preparative techniques.

Physicochemical methods, including mass spectrometry (MS), offer the advantage of high chemical specificity. Nevertheless, this technique typically employs a preliminary derivatization before analysis (Hecht et al. 1994, Norwood 1999). Major improvements in sensitivity have allowed the measurement of increasingly smaller amounts of adducted species in biological matrices. Although ever improving hard-ware together with separation techniques may lower detection limits for human biomonitoring, this approach will continue to require expensive equipment and large quantities of DNA.

Quantitative immunoassays have the disadvantage that they usually require large amounts of DNA (>100 mg) (Poirier et al. 2002), but this difficulty can be overcome by immunohistochemical adduct localization. While immunohistochemical staining cannot, at present, be regarded as better than semi-quantitative, Poirier et al. (2000) postulated that recent advances in antigen retrieval, signal amplification, and the capacity to quantify nuclear staining vastly improved the capacity of immunohistochemical staining to provide quantitative comparisons between samples. This approach is likely to become widely applied in the future due to the availability of small amounts of exfoliated human cells and tissue biopsies (Poirier et al. 2000, Yang et al. 2000).

Detection of carcinogen-DNA adducts by fluorescence has been applied only to compounds that lead to either highly fluorescent products or adducts that can subsequently be derivatized to highly fluorescent chemical species. This approach has been useful for adducts of polycyclic aromatic hydrocarbons and aflatoxin B_1, but remains limited (Weston 1993). The method is sensitive in the range of 1 adduct/10^8 normal nucleotides. Increased sensitivity may ultimately be possible by concentration of large amounts of the fluorescent material for analysis, or the development of more intense fluorophores (Weston 1993).

The ^{32}P-postlabelling technique was introduced in 1981 by Randerath and coworkers, and meets most of the above mentioned requirements needed to be applicable in human exposure settings (Randerath et al. 1981, Gupta et al. 1982, Gupta 1985, Reddy & Randerath 1986, Beach & Gupta 1992). Since then ^{32}P-postlabelling has emerged as a major tool for the detection and quantitation of DNA adducts (Beach & Gupta 1992, Binková 1993, Phillips 1997, Stiborová et al. 1998, Phillips & Castegnaro 1999, Phillips et al. 2000, Reddy 2000). Table 1 shows a brief overview of the most sensitive methods for carcinogen DNA adduct detection to monitor human exposure. The sensitivities of individual methods vary and often depend on the amount of DNA that is available for analysis (table 1). Among them, the ^{32}P-postlabelling methods (enhancement versions), utilizing only 5 mg DNA and detecting as little as 1 adduct/10^{10} normal nucleotides are one of the most sensitive, and, therefore are highly appropriate for human biomonitoring.

The identification of the structure of adducts is achieved by a combination of several physicochemical methods such as liquid chromatography–electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS), fast atom bombardment (FAB) spectroscopy, UV-vis-spectroscopy, nuclear magnetic resonance (NMR). The limiting point is separation and isolation of sufficient amounts of individual adducts from DNA digests (hydrolysates). Frequently, comparison of physicochemical properties (i.e. several spectral and/or chromatographic properties) of adduct standards prepared synthetically with those of DNA adducts detected in human samples by several above mentioned methods including ^{32}P-postlabelling, is utilized for identification of structures of adducts (Yamazoe et al. 1986, 1988, Pfau et al. 1990, 1991, Weston 1993, Phillips et al. 1994, Stiborová et al. 1994, Zeisig & Möller 1995, Mourato et al. 1999, Shibutani et al. 1999, 2000, Turesky 2002, http://es.epa.gov/ncer/final/centers/trc/heiegiese93.html/http://www.mskcc.org/mskcc/html/10832.cfm, http://monographs.iarc.fr/, http://www.wrcsb.org.pbc/cgi/resultsBrowser.cgi).

^{32}P-Postlabelling Techniques

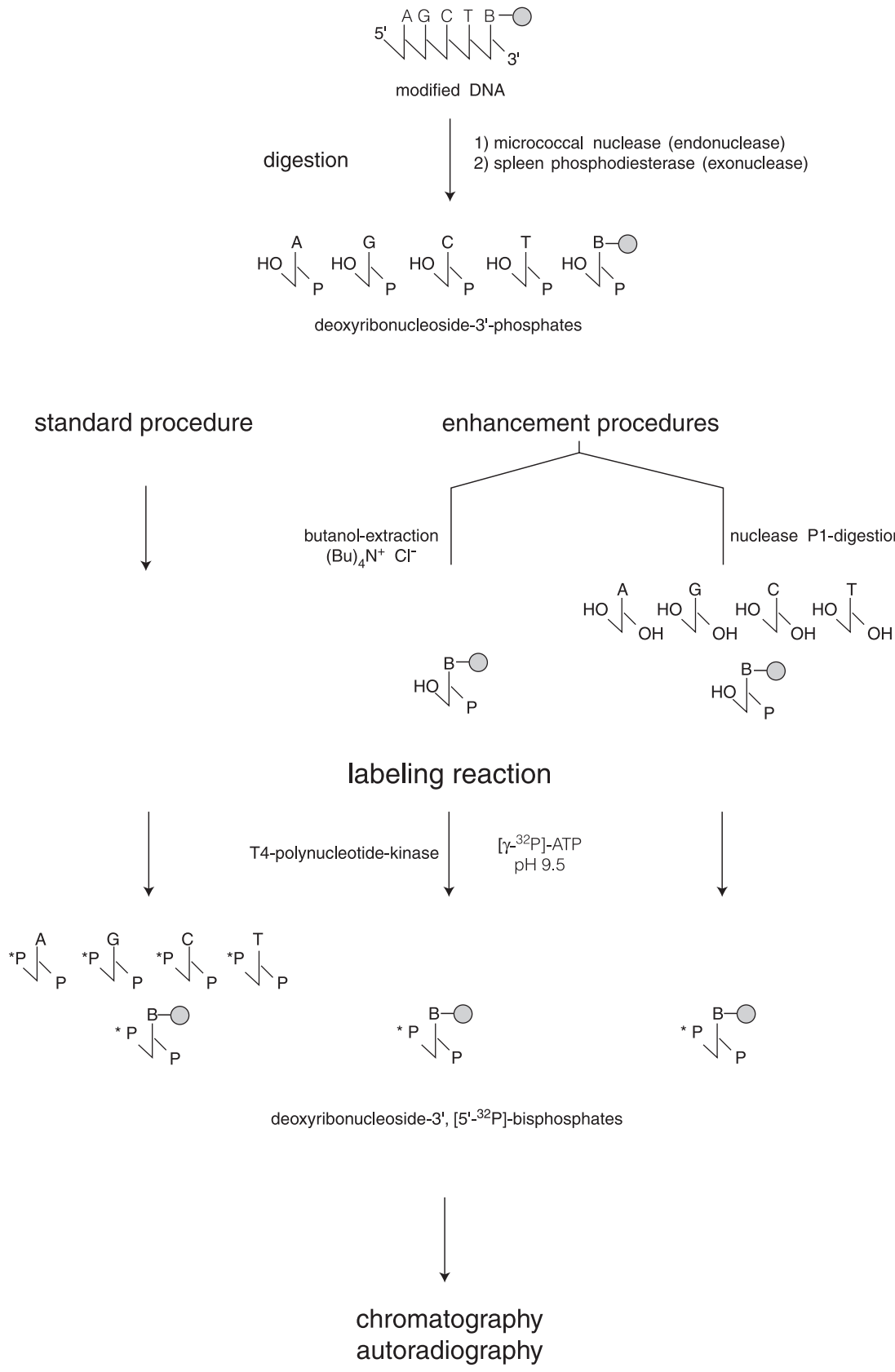
The ^{32}P-postlabelling method is based on the enzymatic hydrolysis of non-radioactive carcinogen-modified DNA to 3´-phos-pho-nucleosides, subsequent [^{32}P]phosphorylation

Table 1. *Detection methods for human DNA adduct biomonitoring*

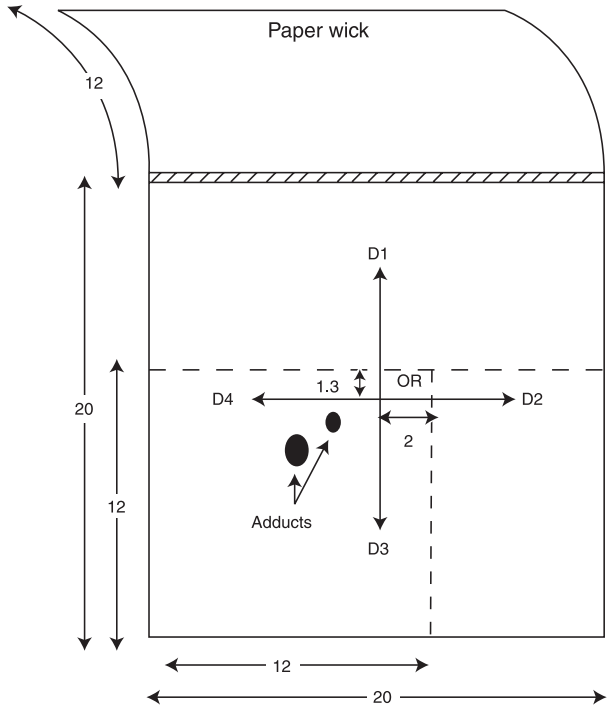
Method	Quantitation		Minimum quantity of DNA per assay	Advantages	Disadvantages
	Number of adducts per normal nucleotides	amol[+]/μg DNA			
HPLC with fluorescence detection	$1/10^7$	300	100 μg	rapidity	only for fluorescent adducts; amounts of DNA
Fluoresce labelling with CE[++] separation	$1/10^7$	300	10 μg	rapidity	only for some types of adducts
Immunoassay (RIA, ELISA)	$1/10^7$	300	>100 μg		preparation of antibodies; amounts of DNA
Fluorescence spectroscopy	$1/10^8$	30	100 μg	sensitivity	only for fluorescent adducts; amounts of DNA
HPLC-MS, GC/MS with ESI or MALDI	$1/10^9$	3	100 μg	high chemical specificity; sensitivity	derivatization before analysis; expensive equipment; amounts of DNA
^{32}P-postlabelling - standard procedure	$1/10^7$	300	1 μg	amounts of DNA;	risk to persons performing the assay; time-consuming analysis
-intensification procedure	$1/10^{8-9}$	3 -30	1 μg	amounts of DNA; sensitivity	risk to persons performing the assay; time-consuming analysis
- nuclease P1 version	$1/10^{10}$	0.3	5-10 μg	amounts of DNA; high sensitivity	risk to persons performing the assay; unsuitable for C8-arylmine dG adducts; time-consuming analysis
- extraction with n-butanol	$1/10^{10}$	0.3	5-10 μg	amounts of DNA; high sensitivity	risk to persons performing the assay; only for hydrophobic bulky adducts; time-consuming analysis
^{32}P-postlabelling/PAGE analysis	$1/10^{8-9}$	3-30	5 μg	amounts of DNA; high sensitivity;	risk to persons performing the assay; rapid separation of adducts

[+]amol = 10^{18} mol, [++]CE –capillary electrophoresis

at the free 5´-OH group, and chromatographic separation of carcinogen-nucleotide adducts from non-modified (normal) nucleotides (Scheme 1). In the early 1980s the original, "standard", ^{32}P-postlabelling protocol was developed (Randerath et al. 1981, Reddy et al. 1981, Gupta et al. 1982). In this technique, carcinogen-modified DNA is digested enzymatically to deoxyribonucleoside 3´-monophosphates with endonuclease (micrococcal nuclease) and exonuclease (spleen phosphodies-terase). Thereafter, DNA hydrolysates (normal and modified deoxyribo-nucleoside 3´-monophosphates) are converted to 5´-^{32}P-labelled 3´,5´-bisphosphates by incubation with [g-^{32}P]ATP in the presence of carrier ("cold") ATP and T4-polynucleotide kinase at *p*H 9.5 (Scheme 1). This alkaline *p*H is used in order to minimize the 3´-phosphatase activity of the polynucleotide kinase. ^{32}P-Labelled adducts are separated and resolved from the excess of labelled non-modified nucleotides in two dimensions by multidirectional anion-exchange thin layer chromatography (TLC) on polyethyleneimine (PEI) cellulose plates (Scheme 2). During the first elution (D1 and D2 directions) with aqueous electrolyte, labelled unmodified nucleotides and [^{32}P]phosphate are removed from the origin onto a paper wick while aromatic hydrophobic adducts are retained at the origin for subsequent resolution using different solvent systems (D3, D4 directions) (Scheme 2). Recently, polyacrylamide gel electrophoresis (PAGE) of DNA digests has also



Scheme 1. Scheme of the ^{32}P-postlabelling assay



Scheme 2. Elution pattern of PEI-cellulose TLC plates

proved useful for resolving the ^{32}P-postlabelled species (^{32}P-postlabelling/PAGE analysis) (Terashima et al. 2002). Location of the adducts is carried out by screen enhanced autoradiography and visualized as dark distinct spots on X-ray films. These areas are then excised for quantitation by liquid scintillation or Cerenkov counting. A technique known as storage phosphor imaging was recently adapted for mapping and quantitation of DNA adducts on chromatograms generated by the ^{32}P-postlabelling assay; Instant Imager is, therefore, now frequently utilised for scanning TLC maps for adducts (Reichert et al. 1992). This technique yields an about 10-fold improvement in sensitivity compared to screen enhanced autoradiography for the detection of ^{32}P (Chang et al. 1994).

Resolution of adducts depends not only on their physico-chemical properties but also on the presence of multiple adducts possessing similar chromatographic mobilities on PEI-cellulose thing layer plates. Figure 1 shows examples of autoradiographs of ^{32}P-postlabelling analysis of well-resolved adducts formed by the plant carcinogen aristolochic acid in human kidney DNA of patients suffering from Chinese herbs nephropathy (Schmeiser et al. 1996, Bieler et al. 1997, Arlt et al. 2002a, b) (figure 1A) and DNA adducts formed by the complex mixture of carcinogens in tobacco smoke in human kidney DNA, which migrate poorly resolved along a diagonal radioactive zone (DRZ) (figure 1B).

Adduct levels are calculated as relative adduct labelling (RAL) values according to:

$$RAL = \frac{\text{c.p.m. in adduct nucleotides}}{\text{specific activity (in c.p.m./pmol)} \times \text{pmol dNp}}$$

RAL values represent the ratio of count rates of adducted nucleotides over count rates of total (adducted and normal) nucleotides (Gupta et al. 1982, Reddy & Randerath 1986). The caution should, however, be taken into account; the adducts and normal nucleotides should be labelled with equaled efficiency (see Mourato et al. 1999, Gonçalves et al. 2001). The "standard" protocol of ^{32}P-postlabelling method is suitable for most DNA adducts (bulky and/or non-bulky adducts), but its

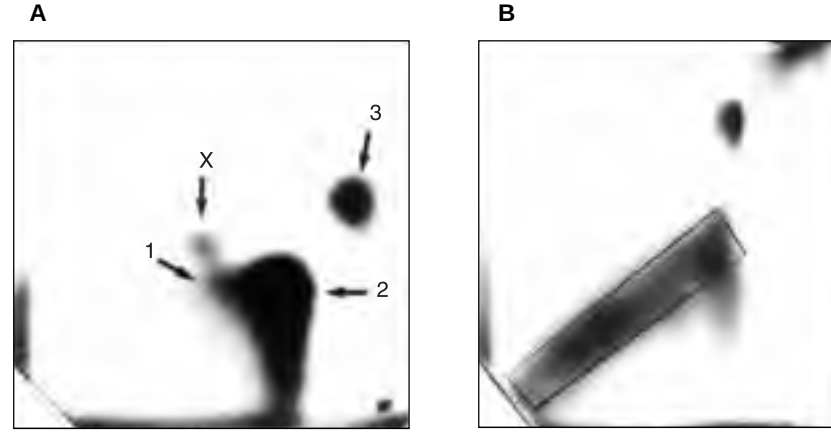


Figure 1. Autoradiograms of ^{32}P-postlabelling analyses (nuclease P1-enrichment) of DNA adducts in (**A**) renal cortical tissue from patient suffering from Chinese herbs nephropathy generated by aristolochic acid I, and (**B**) renal cortical tissue from a smoker. Spot 1: dG-AAI, 7-(deoxyguanosin-N^2-yl)-aristolactam I; spot 2: dA-AAI, 7-(deoxyadenosin-N^6-yl)-aristolactam I); spot 3: dA-AAII, 7-(deoxyadenosin-N^6-yl)-aristolactam II; spot X: unknown adduct.

sensitivity is not sufficient for detection of adducts present in lower levels in DNA; utilizing this protocol, DNA adducts present at levels of 1 adduct in 10^7 normal nucleotides (0.3 fmol adduct/ µg DNA) can be detected.

Several modifications of the standard assay have been employed in order to increase the sensitivity of the method. ^{32}P-labelling of adducts with limiting amounts of [g-^{32}P]ATP has been shown to enhance the method's sensitivity 10- to 100-fold for a number of adducts (the intensification procedure) (Randerath et al. 1985, Everson et al. 1986). The additional enhancement procedure uses an enzymatic postincubation of DNA digests with nuclease P1 (from *Penicillium citrinum*) (Reddy & Randerath 1986) (Scheme 1) to enrich adducts. Nuclease P1 preferentially dephosphorylate - sunmodifieddeoxyribonucleoside 3´-monophos- phates to deoxyribonucleosides and in most cases not the adducted nucleotides. Deoxyribonucleosides do not serve as substrates for T4-polynucleotide kinase for the transfer of [^{32}P]phosphate from [g-^{32}P] ATP. However, some adducted nucleotides are dephosphorylated by nuclease P1 (e.g., arylamine adducts substituted at C8 of deoxyguanosine), while others are not (primarily adducts substituted at N2 of deoxyguanosine). This version of the assay increases its sensitivity significantly (by three order of magnitude, see table 1). Adduct enrichment over normal nucleotides thus achieved before labelling, allows the use of larger amounts of DNA (5-10 µg) and of excess of carrier-free [g-^{32}P]ATP.

The additional enrichment procedure introduced by Gupta (1985) exploits the properties of hydrophobic carcinogen-adducted nucleotides to be extracted into n-butanol in the presence of a phase transfer agent tetrabutylammonium chloride (Scheme 1). Hydrophobic adduct nucleotides are preferentially extracted into the organic solvent prior to[^{32}P]phosphate labelling, while normal nucleotides are extracted only to some extent. Likewise, more polar adducts containing non-aromatic bulky residues or a small alkyl moiety are hardly extractable into n-butanol and cannot be analysed with this version of the ^{32}P-postlabelling technique.

The nuclease P1 and n-butanol extraction enrichment methods enhance the sensitivity of detection and quantitation of DNA adducts by several orders of magnitude, enabling the detection of one adduct per 10^{9-10} normal nucleotides (0.3 - 3 amol/µg DNA) depending on structures of adducts, which in turn, allows the measurement of 1-10 adducts per genome.

Additional improvements involve (i) the use of nuclease P1 and prostatic phosphatase for hydrolysis of DNA, before ^{32}P-postlabelling, followed by venom phosphodiesterase digestion (Randerath et al. 1993, Pfau et al. 1994) and (ii) the classical nuclease P1 version of the method followed by venom phosphodiesterase and/or a second nuclease P1 digestion (the dinucleotide version) (Pfau et al. 1994). These versions are suitable mainly for detection of adducts formed by heterocyclic aromatic amines, which cause the complex modification of DNA (not only the formation of simple covalent adducts) (Randerath et al. 1993, Pfau et al. 1994). Furthermore, another method, an alternative snake venom phosphodiesterase-based ^{32}P-postlabelling procedure (SVPD-postlabelling) was recently developed and found to be a suitable method for small DNA lesions, such as those resulting from oxidative damage of DNA (Jones et al. 1999). Because these methods are utilized only for some types of DNA adducts, they are used less frequently (Keith & Dirheimer 1995, Randerath et al. 1996, Stiborová et al 1998, Phillips et al. 2000). An increasingly popular method is the combination of enzymatic enrichment procedures of the ^{32}P-postlabelling assay with other methods [i.e. with n-butanol extraction (Reddy et al. 1990, Stiborová et al. 1990, 1992a,c, 1995, 1999c, Jones et al. 1993, Binková et al. 1995, Beach & Gupta 1994, Randerath & Randerath 1994, Randerath et al. 1999) or with HPLC (Pfau & Phillips 1991, Pfau et al. 1992, 1996, Försti et al. 1994, Stiborová et al. 1994, 1998, 1999b, 2001a,b,c, Savella et al. 1995, Zeisig & Möller 1995, Möller et al. 1996, Schmeiser et al. 1996, 1997, Bieler et al. 1997)].

HPLC can provide a more rapid resolution and quantitation of adducts than TLC. ^{32}P-Labelled DNA digests are resolved by C_{18} reverse-phase HPLC and quantitated by on-line radioactivity flow detection or by the counting of eluant fractions (Dunn & San 1988, Schmeiser et al. 1988, Pfau & Phillips 1991, Pfau et al. 1992, 1996, Försti et al. 1994, Hemminki et al. 1997). This method alone, however, often exhibits somewhat lower sensitivity than TLC and

autoradiography (Dietrich et al. 1987). Therefore, the HPLC method is attractive mainly for some special applications, i.e. resolution of complex DNA adduct mixtures poorly separated by TLC (Försti et al. 1994, Zeisig & Möller 1995, Möller et al. 1996, Carlberg et al. 2000, Akkineni et al. 2001) or identification of adducts by cochromatography (Pfau & Phillips 1991, Pfau et al. 1992, 1996, Stiborová et al. 1994, 1998, 1999, 2001b,c,d, Savella et al. 1995, Schmeiser et al. 1996, 1997, Bieler et al. 1997, Arlt et al. 2002b, Frei et al. 2002).

A promising enrichment method seems to be the immunoaffinity/^{32}P-postlabelling assay (King et al. 1993, Nair et al. 1995, Fernando et al. 1996). This method is based on immunoaffinity purification of adducts and subsequent ^{32}P-postlabelling followed by separation as 5´-monophosphates on PEI-cellulose thin layer plates or HPLC. It is suitable for detection mainly of etheno-DNA adducts, which are hardly detectable by other version of ^{32}P-postlabelling assay (Nair et al. 1995, Hagenlocher et al. 2001, Godschalk et al. 2002).

Critical Evaluation of the ^{32}P-Postlabelling Methods

The enrichment and separation procedures, but not the differences in the methods of the ^{32}P-postlabelling technique, allow the concentration of adducts in DNA digests prior to ^{32}P-postlabelling, improving their sensitivity. However, none of the above mentioned ^{32}P-postlabelling methods can be used indiscriminately for all kinds of adducts. More polar adducts containing a small alkyl moiety, non-aromatic bulky residues or residues with one aromatic ring, for instance, exhibit chromatographic properties that are too similar to those of normal nucleotides to allow complete removal of the latter from the chromatograms without loss of the former (Randerath et al. 1981, Reddy et al. 1990, Vodièka & Hemminki 1991, Stiborová et al. 1992b, 1995, 1998, 1999a,c, 2002c, Vodièka et al. 1993, Nair et al. 1995, Fang & Vaca 1997, Koivisto et al. 1997, Kumar et al. 1997). Additionally, the multistep ^{32}P-postlabelling process not only lends itself to potential modifications of several steps but also demands careful control of all these steps in order to obtain reliable qualitative and quantitative results. The incomplete digestion of carcinogen modified DNA (e.g. DNA modified by cross-links), different degrees of resistance of adducted nucleotides to dephosphorylation (e.g. low resistance of arylamine adducts substituted at C8 of deoxyguanosine), incomplete extraction into n-butanol (e.g. more polar adducts formed by carcinogens containing an alkyl moiety or only one benzene ring in their molecules), incomplete ^{32}P-postlabelling for several adducts [i.e. series of N-(deoxyguanosin-8-yl)arylamine 3' phosphate adducts, for which the substantially higher ATP concentrations are needed to be used than for typical ^{32}P-postlabelling assays (Mourato et al. 1999)], losses of material during the experimental manipulations or retaining of compounds at the origin of the PEI-cellulose TLCs can cause underestimation of DNA adducts (Beach and Gupta 1992, Stiborová et al. 1990, 1992a,c, 1998, Randerath & Randerath 1994, Keith & Dirheimer 1995, Phillips 1997). The relatively low efficiency of the DNA digestion of highly adducted DNA and/or DNA modified by DNA-DNA and/or DNA-protein cross-linking (Yamazoe et al. 1986, 1988, Stiborová et al. 1990, 1992a,c, 1998, 2001e, 2002d, Fukutone et al. 1994, Pfau et al. 1994) is a limiting factor. The formation of multiple adducts from one carcinogen (Stiborová et al. 1990, 1992a,c, 2002d) or the presence of multiple adducts from different carcinogens [such as environmental pollutants or carcinogens in tobacco smoke (see also figure 1)] also extremely complicate the evaluation of adducts. Hence, even when digestion of DNA occurs at high efficiency, the combination of several modifications of the ^{32}P-postlabelling method (standard procedure, nuclease P1 or n-butanol extraction-enrichment, ^{32}P-HPLC etc.) as well as different chromatographic procedures to separate adducts of different structures (Reddy et al. 1990, Stiborová et al. 1995, Phillips et al. 2000, Hemminki et al 2000, 2001) should be used for the exact determination and quantitation of adducts.

There is also an additional disadvantage of ^{32}P-postlabelling for human biomonitoring. This technique measures total DNA-adduct levels, but it can rarely identify specific adducts accurately. The lack of adduct standards has, in many cases, limited the interpretation of data to a demonstration of higher adducts levels in exposed groups compared with unexposed groups (Phillips 1997, Reddy 2000). Nevertheless, the exceptions of this disadvantage exist; for example,

using specific adduct standards formed by benzo(a)pyrene (Peluso et al. 2001, Besaratinia et al. 2002), heterocyclic aromatic amines (Turesky 2002), anticancer drug tamoxifen (Hemminki et al. 1996, Shibutani et al. 1999, 2000, Philips 2001) or aristolochic acids (Schmeiser et al. 1996, Bieler et al. 1997, Stiborova et al. 1999, Arlt et al. 2002, see also figure 1A) specific DNA adducts were identified and quantified in human tissues. The preparation of further DNA standards modified by carcinogens and elucidation of the structures of other DNA lesions whose existence has been revealed by the technique might overcome this disadvantage (Phillips & Castegnaro 1993, 1999, Hemminki et al. 2001).

Another problem with promoting the ^{32}P-postlabelling technique for biomonitoring is lack of detection of large amounts of adducts, which are lost by depurination from the DNA. It was described previously than the stable adducts detectable by ^{32}P-postlabelling assay seem to represent only a minor portion of DNA adducts formed by several carcinogens (e.g., at least the bulky adducts formed by polycyclic aromatic hydrocarbons or by estrogens such as estradiol-3,4-quinone) (Chen et al. 1996, Devanesan et al. 1996, Chakravarti et al. 2001).

Application of ^{32}P-Postlabelling to Different DNA Modifications

Even though the ^{32}P-postlabelling assay has the above mentioned disadvantages, it is a very sensitive method for the detection of various different DNA adducts (table 1), and, therefore, utilized for many DNA lesions. The increasing popularity of the ^{32}P-postlabelling assay for the determination of modified DNA evolved from the ability of this method to detect and characterize DNA lesions such as covalent carcinogen DNA adducts formed from both bulky aromatic chemicals and from small molecules, oxidative DNA lesions formed by radical oxygen species, apurinic sites and radiation-induced DNA damage, cyclic DNA adducts formed from a wide range of bifunctional genotoxic chemicals, and UV-induced photodimers (Gupta 1985, Reddy et al. 1990, Vodièka & Hemminki 1991, Beach & Gupta 1992, 1994, Weinfeld & Buchko 1993, Randerath & Randerath 1994, Keith & Dirheimer 1995, Vodièka et al. 1995, Randerath et al. 1996, Kumar et al. 1997, Fang & Vaca 1997, Phillips 1997, Bykov et al. 1998, Stiborová et al. 1998, Phillips & Castegnaro 1999, Xu et al. 2000, Hemminki et al. 2001). Therefore, this method is applicable to detect DNA modifications, which are caused by different multiple exposures. Nevertheless, the method is the most often used for the detection and the characterization of covalent adducts from chemicals being classified as mutagens and carcinogens. This is convenient not only for the screening of the genotoxicity of many chemicals but also to confirm their toxic (carcinogenic) mechanisms. Carcinogen adduct characterization, moreover, helps to resolve the molecular mechanisms of carcinogenesis. From this point of view, ^{32}P-postlabelling analyses of DNA adducts are used to assess the risk to humans of compounds in our diet such as food-borne carcinogens [i.e. chemicals found in fried meat, cooked food (heterocyclic aromatic amines) (Pfau et al. 1994, 1996, Randerath & Randerath 1994, Garner 1998, Dingley et al. 1999, Turesky 2002) or plant (vegetable) products such as safrole and related alkenylbenzene derivatives (Gupta et al. 1993, Randerath et al. 1993), aristolochic acids (Stiborová et al. 1994, 1999, 2002, Schmeiser et al. 1996, 1997, Bieler et al. 1997, Arlt et al. 2001, 2002a,b), quaternary benzo[c] phenanthridine alkaloids sanguinarine and chelerythrine (Stiborová et al. 2002e), pyrollizidine alkaloids (Yang et al. 2001a,b) or compounds responsible for DNA adduct formation by cola drinking (Randerath et al. 1993b, Randerath & Randerath. 1994)], or food additives and mycotoxins in food [i.e. aflatoxins (Randerath & Randerath 1994), ochratoxin A (Pfohl-Leszkowicz et al. 1993, Arlt et al. 2001, 2002b)]. On the other hand, the ^{32}P-postlabelling assay was used to study some dietary constituents for their ability to decrease DNA adduct formation or other DNA lesions induced by carcinogens and might be candidate drugs for cancer prevention [e.g. chemicals present in green and black tea (Keith & Dirheimer 1995), natural or synthetic flavonoids (Stiborová et al. 2001a,c, Hodek et al. 2002)].

Another group of chemicals often analyzed are compounds known as environmental pollutants. Single compounds or the natural mixtures are analyzed. Polluted urban air arising from several industrial productions or other sources (i.e. chemical or pharmaceutical industries, extensive use of the pesticides in agriculture), from heating systems or from the incomplete combustion in vehicle engines are analyzed for their potential to form DNA adducts (Beach & Gupta 1992, Vodièka et al. 1993, Randerath & Randerath 1994, Keith & Dirheimer 1995, Hemminki 1995, Savela et al. 1995, Munnia et al. 1999, Randerath et al. 1999). In addition, cigarette smoke is another highly studied pollutant. A direct correlation of the levels of DNA adducts in both surrogate and target (lung) tissues with smoking habits was shown by ^{32}P-postlabelling (Phillips et al. 1988, Jones et al. 1993, Besaratinia et al. 2002). Furthermore, using ^{32}P-postlabelling methods carcinogen DNA adducts, oxidative DNA damage generated by various agents including redox-cycling chemicals, nonmutagenic carcinogens/tumour promoters or chemical mixtures containing or producing radicals are determined (Randerath & Randerath 1994). In these studies, animal experiments are frequently performed, but analysis of DNA from human white blood cells (WBC) is also performed (Grzybowska et al. 1993, Vodièka et al. 1995, Möller et al. 1996, Fang & Vaca 1997, Hagenlocher et al. 2001). DNA adducts of these cells were monitored by ^{32}P-postlabelling in populations living in polluted or unpolluted regions of several countries (Chang et al. 1994, Binková et al. 1995, Keith & Dirheimer 1995, Möller et al. 1996, Munnia et al. 1999, Hemminki et al., 2000, 2001, Peluso et al. 2001, Tang et al. 2001) and the levels of adducts found in cells correlated with the amount of air pollution. Therefore, these cells are suggested to be suitable for noninvasive human biomonitoring studies. Many studies have also used tissues collected at the time of biopsy, surgery or autopsy (Poirier et al. 2000). In addition, urinary excretion of exfoliated bladder cells containing DNA adducts, including alkylation products, oxidized bases and bulky adducts, are excellent markers of exposure (Hemminki et al. 2001, Vermeulen et al. 2002).

^{32}P-Postlabelling is increasingly used to analyze DNA adducts formed from pharmaceutical drugs or their additives in human therapy (e.g. mitomycin, *cis*-platin, cyclophosphamide, cyproterone acetate, daunorubicin, tamoxifen, ellipticine) (Beach & Gupta 1992, Purewal & Liehr 1993, Topinka et al. 1993, Försti et al. 1994, Phillips et al. 1994, Keith & Dirheimer 1995, Schmeiser et al. 1996, Shibutani et al. 1999, 2000, Stiborová et al. 2001a, 2002b, 2003, Frei et al. 2002). Not only *in vivo* studies to assess the risk of drugs used in human medicine are carried out but also *in vitro* experiments can contribute to identify the enzymatic systems responsible for the activation of these compounds (or other toxicologically important chemicals mentioned above) (Lévay et al. 1993, Pathak & Roy 1993, Stiborová et al. 2001a,b,c,d, 2002a,b,c).

^{32}P-Postlabelling is also utilized to analyze DNA modifications formed by endogenous compounds (or endogenous factors). Randerath and coworkers (Randerath et al. 1993c, 1996, Vulimiri et al. 1998) found DNA adducts even in experimental animals unexposed to any chemicals. These adducts [I-(indigenous) compounds] are found also in humans and are derived from endogenous electrophiles formed in the course of normal metabolism of nutrients and other natural dietary components and accumulate in an age-dependent manner (Marnett & Burcham 1993, Randerath et al. 1993c, 1996, Randerath & Randerath 1994, Keith & Dirheimer 1995, Stiborová et al. 1998, Phillips 1997). It is questionable whether I-compounds represent functional modifications that are necessary for normal growth, or are promutagenic lesions, or whether they play both roles. Studies of Randerath and coworkers show that both roles are possible (Randerath et al. 1993c, 1996). There are two classes of I-compound, type I and type II. While many type I I-compounds may not reflect DNA damage, type II I-compounds have been identified as oxidative DNA lesions some of which can be produced *in vitro* under Fenton reaction conditions (Vulimiri et al. 1998).

Conclusions and Future Directions

DNA adduct formation by carcinogenic chemicals (or their reactive metabolites) is considered to be the first important step during the multistage process of chemical carcinogenesis. The results of multiple different tumour/adduct comparisons have demonstrated that although DNA adducts form in the absence of tumours, tumours do not form in the absence of DNA adducts (Poirier et al. 2000). In the preamble to the International Agency for Research on Cancer Monographs, DNA adducts are mentioned among "other data relevant to an evaluation of carcinogenicity and its mechanisms" (Hemminki et al. 2001). The large interindividual variability in DNA adduct formation, observed in individuals experiencing similar exposures, suggest that genetic differences in carcinogen metabolism, DNA repair and cell-cycle control modulate the individual response to exposure (Poirier et al. 2000, Hemminki et al. 2000, 2001, Brockstedt et al. 2002, Wiencke 2002). The relationship between formation of DNA adducts and polymorphisms in some carcinogen metabolizing genes involved indicates that large scale studies will be needed to understand the complex nature of such gene-environment interactions. Elucidation of underlying mechanisms will be necessary to support interpretation of DNA adduct data currently being collected in epidemiologic studies (Hecht 1997, Kensler & Groopman 1997, Santella 1997). The research activity in this area, promising to resolve this very important factor for a cancer development, should therefore be enlarged.

The detection of DNA adducts in tissues of organisms (including humans) exposed to chemicals represents a highly sensitive rapid *in vivo* assay for genotoxicity testing of potential carcinogens. The increasing use of the ^{32}P-postlabelling assay for the detection (and/or identification) of DNA adducts in animals and humans exposed to chemicals is attributable to its extremely high sensitivity, without the need to administer radioactive xenobiotics to experimental animals. The ^{32}P-postlabelling method can be used in prospective studies to assess the risk for humans due to their exposure to industrial pollutants, environmental contaminants, food contaminants and drugs (Tang et al. 2001). Nevertheless, human biomonitoring by ^{32}P-postlabelling has some limitations; for ^{32}P-postlabelling, the adduct recovery, including labelling efficiencies, depend on the type of DNA adduct, thus preventing exact quantitation of unknown adducts. Moreover, the assay can give an estimate of total adduct burden, but it is only rarely possible to identify specific adducts accurately in human samples. Nevertheless, DNA adducts formed from several carcinogens [e.g. benzo(a)pyrene, heteroxyclic aromatic amines, tamoxifen, aristolochic acids] were successfully identified by ^{32}P-postlabelling in human samples. Advances may lie in preparation and the use of additional chemical standards, more advanced preparative techniques, standard curves and use of corroborate assays. Precautions should also be taken when using individual enhancement procedures of the ^{32}P-postlabelling assay alone. An underestimation or even an overestimation (Keith & Dirheimer 1995) of the number of different adducts present in DNA can occur. The combination of several modifications of the technique or that of this technique with other methods useful for DNA adduct determination (Hemminki et al. 2001) leads to more exact determination and quantification of DNA adducts. Indeed, the recent trend is to combine the best of several techniques to be both specific and sensitive. Having said this, it is important to project ^{32}P-postlabelling, as a very suitable technique that could be combined with other techniques. Furthermore, considerable scope exists for improvement of this method. A unification of the ^{32}P-postlabelling assay procedures and use of the same adduct standards in individual laboratories over the world should be established (Phillips & Castegnaro 1999). Reliable testing procedures and a standardized set of protocols are necessary. They will help to improve the reproducibility and specificity of ^{32}P-postlabelling assays as well as the comparability of results. Finally, all this might help to achieve improvements in cancer epidemiology and in the prevention of cancer.

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