MONITORING OF DNA ADDUCTS IN HUMANS AND ^{32}P-POSTLABELLING METHODS. A REVIEW

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Dedicated to the 50th anniversary of the foundation of the Department of Biochemistry, the first biochemical department in Czechoslovakia.

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DNA adduct formation in humans is a promising biomarker for elucidating the molecular epidemiology of cancer. 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 appears to meet best the criteria of sensitivity and amount of DNA needed, and, therefore, is one of the most appropriate methods for biomonitoring of human DNA adducts. Most classes of carcinogens have been subjected to ^{32}P-postlabelling analysis, ranging from bulky and/or aromatic compounds to small and/or aliphatic compounds; it has also been used, with modifications, to detect apurinic sites in DNA, oxidative damage to DNA, UV-induced photodimers and, to a lesser extent, DNA damage caused by cytotoxic drugs. It has been used in human biomonitoring studies to detect DNA damage from occupational exposure to carcinogens, and also from environmental (i.e. non-occupational) exposures. It has also led to the discovery of the presence of numerous modifications in DNA arising from endogenous processes. The principle of the method is the enzymatic digestion of DNA to nucleotides, 5′-labelling of these nucleotides with an iso-

topically labelled phosphate group, and the resolution, detection and quantitation of the labelled products. Since the development of the original procedure in the early 1980s, many methods have been developed to increase the sensitivity by enrichment of modified nucleotides prior to labelling. The review 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. A review with 179 references.
Keywords: Carcinogens; DNA adducts; Risk assessment; Biomonitoring; ^{32}P-Postlabelling assay; DNA damage; DNA modifications.

1. DNA ADDUCTS AS BIOMARKERS OF CARCINOGEN EXPOSURE

Considerable epidemiological and experimental evidence has indicated that synthetic and naturally occurring chemicals of occupational, environmental, medicinal, and dietary origins play a significant role in the etiology of human cancer[1]. Many chemical carcinogens require activation to reactive intermediates that bind to nucleophilic centres in proteins and nucleic acids thereby forming covalent adducts[2,3]. 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[4]. 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[3–10]. Indeed, associations have been observed between DNA adduct formation, mutagenesis[3,11,12], and tumourigenesis[5,6,12]; while reduction in DNA adduct levels seems to be associated with chemoprevention[13,14]. Therefore, DNA adduct formation in humans was considered to be a promising biomarker utilized in the molecular epidemiology of cancer[9,15–29]. DNA adducts may occur at a number of sites within the DNA molecule; however, adduct formation involves specific electronic and stereochemical factors such that binding, especially with bulky aromatics, is not simply random[14]. Guanine bases in DNA are the predominant sites for attack by chemical carcinogens. The N^7 position of guanine is predominantly modified by alkylation (methylation or ethylation) agents, while aromatic amines and polycyclic aromatic hydrocarbons prefer the C8 and N^2 position, respectively (Fig. 1). Other sites on guanine and sites on other bases may become significantly adducted as well (see arrows in Fig. 1), indicating the multiplicity of adducts formed by a single carcinogen. Although adduct levels are related to exposure, it appears that the extent of DNA

damage occurring at specific sites is more important in terms of carcinogenicity. It was found that some adducts are highly mutagenic and associated with carcinogenesis, while other adducts are not[3,30]. 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[31,32]. 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[33–39], and a relationship between exposure, adduct formation and mutation in the respective tissues has been demonstrated[33–40]. Nevertheless, 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[7]. Of them, methods for the detection and characterization of DNA adducts are the most crucial. Therefore, the development of new sensitive and specific methods for DNA adduct detection is the most challenging task. Development 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.

2. THE "HUMAN SITUATION"

The assessment of human exposure to carcinogenic/mutagenic agents is a complex problem previously limited to epidemiological studies. Animal models have led to the development of the current multistage theories of

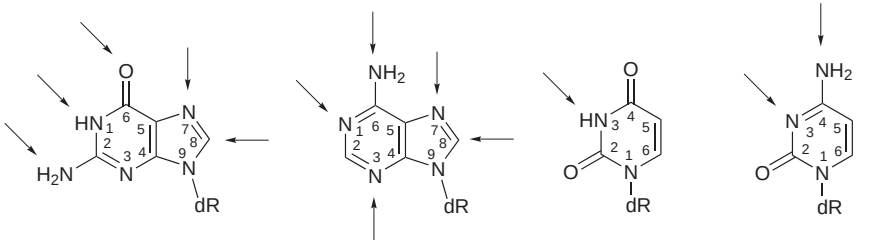

FIG. 1
The preferential binding sites on deoxynucleosides in DNA for electrophiles; dR, 2′-deoxyribose (based on data from[179])

carcinogenesis, and thus have strongly influenced the development of techniques for assessment of carcinogen exposure. The human situation, however, is much more multivariate compared with typical animal studies. There are at least three major factors, inherently unique to human exposure/cancer risk assessment that dictate the applicability of approaches to their detection; (i) large interindividual variation; (ii) exposure to complex mixtures and confounding factors and (iii) inaccessibility of human tissues suitable for analysis[15].

Many chemicals are metabolically activated by oxidative enzymes, such as cytochrome P450 (CYP), to various electrophilic and carcinogenic metabolites. A number of CYP enzymes have been identified in humans with more than 1000-fold interindividual differences in terms of quantity, substrate specificity and tissue distribution[41]. Predisposition to cancer seems to be correlated to genetic polymorphism of these enzymes as well as that of other enzymes including the phase II enzymes of carcinogen biotransformation (e.g. *N*-acetyltransferases, sulfotransferases)[15]. Nevertheless, interindividual variation was only partially explained by genetic polymorphisms of these enzymes and deserves further investigations[42].

DNA repair enzymes, responsible for the removal of adducts, may also play a role in genetic predisposition to cancer. Depressed rates of DNA repair have been observed in patients with *Xeroderma pigmentosum*, a disorder that increases susceptibility to ultraviolet (UV)-induced skin cancer[15,43]. O^6-Alkyl-DNA alkyltransferase, which repairs alkylated O^6-deoxyguanine residues, has also been shown to be decreased in fibroblasts of patients with lung cancer versus non-cancer controls[15,43].

Humans, in addition to their genetic variability, are chronically exposed to low doses of complex chemical mixtures. Contained in these mixtures are many known or potential carcinogens. One may encounter multiple sources of exposure in daily life. In addition to exposure via occupation to environmental/industrial pollution (air, water) and UV radiation, lifestyle is also an important factor when considering the total scheme of exposure events. Cigarette smoking, environmental tobacco smoke, exhaust by-products, foods, etc., may all contribute to a person's cumulative risk of cancer.

Finally, a major difficulty in assessment of human exposure to carcinogens is the inaccessibility of suitable tissues for DNA adducts analysis. Many studies have utilized white blood cells or peripheral blood lymphocytes as surrogate tissues, while others have analyzed samples from autopsy tissue, placental tissues or limited numbers of biopsy samples[44,45]. Further animal

studies are necessary to establish the relationship of surrogate tissue to target tissue and hence their potential for exposure assessment[15].

3. DETECTION TECHNIQUES FOR BIOMONITORING OF DNA ADDUCTS IN HUMANS

The development of appropriate methodologies for detecting and quantifying DNA adducts as biomarkers is dictated by the human scenario described above. 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[46,47].

Until 1980, the detection of DNA adducts has usually required the use of highly radioactive labelled chemical carcinogens (labelled by ^{3}H or ^{14}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[4,7,47–49]. The most frequently used methods for carcinogen DNA adduct detection, which mean 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[50–52], and immunohistochemistry[50,53,54] using adduct-specific antisera, fluorescence and phosphorescence assays[55,56], ^{32}P-postlabelling[1,47,48,57], electrochemical detection[58,59], and mass spectroscopy (i.e. electron spray ionization (ESI)[+], matrix-assisted laser desorption ionization (MALDI) and accelerator mass spectrometry)[60–65]. Recently, another method, capillary electrophoresis and laser-induced fluorescence monitor-

+ *Abbreviations used*: CE, capillary eletrophoresis; CYP, cytochrome P450; dR, 2′-deoxyribose; ESI, electron spray ionization; FAB, fast atom bombardment; HPLC-ESI-MS/MS, liquid chromatography-electrospray ionization tandem mass spectrometry; I-compounds, indigenous compounds; MALDI, matrix-assisted laser desorption ionization; PAGE, polyacrylamide gel electrophoresis; PEI, polyethyleneimine; RAL, relative adduct labelling; SVPD-postlabelling, snake venom phosphodiesterase-based ^{32}P-postlabelling; TLC, thin layer chromatography.

ing, was developed and showed to be promising for DNA adduct measurement[66,67]. Likewise, recent developments in capillary electrophoresis in combination with either immunochemical or mass spectrometric detection techniques may offer new promising approaches with high selectivity[68]. Furthermore, the single cell gel eletrophoresis ("comet") assay might be also 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)[69–74]. 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 usually employs a preliminary derivatization before analysis[61,75]. Major improvements in sensitivity have allowed the measurement of increasingly smaller amounts of adducted species in biological matrices. Although ever improving hardware together with separation techniques may lower detection limits for human biomonitoring, this approach will continue requiring expensive equipment and large quantities of DNA.

Quantitative immunoassays had the disadvantage of requiring usually large amounts of DNA (>100 μg)[7], but this difficulty can be overcome by immunohistochemical adduct localization. While immunohistochemical staining cannot, at present, be regarded as better than semiquantitative, Poirier et al.[7] 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 future due to the availability of small amounts of exfoliated human cells and tissue biopsies[7,76].

Detection of carcinogen–DNA adducts by fluorescence has been used only for 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[55]. The method is sensitive in the range of 1 adduct/10^8 normal nucleotides. Increased sensitivity may ultimately be achived by concentration of large amounts of the fluo-

rescent material for analysis, or the development of more intense fluorophores[55].

The ^{32}P-postlabelling technique was introduced in 1981 by Randerath and coworkers, and meets many of the above-mentioned requirements to be applicable in human exposure settings[15,57,77–79]. Since then ^{32}P-postlabelling has emerged as a major tool for the detection and quantitation of DNA adducts[15,47–49,80–82]. Table I 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 I). Among them, the ^{32}P-postlabelling methods (enhancement versions), utilizing only 5 μg DNA and detecting as little as 1 adduct/10^{10} normal nucleotides are the most sensitive, and, therefore are highly appropriate for human biomonitoring, but do not allow elucidation of structures.

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[4,55,83–93].

3.1. ^{32}P-Postlabelling Techniques

The ^{32}P-postlabelling method is based on the enzymatic hydrolysis of non-radioactive carcinogen-modified DNA to 3′-phosphonucleosides, subsequent [^{32}P]phosphorylation at the free 5′-OH group, and chromatographic separation of carcinogen–nucleotide adducts from non-modified (normal) nucleotides (Fig. 2). In the early 1980s the original, "standard" ^{32}P-postlabelling protocol was developed[57,77,94]. In this technique, carcinogen-modified DNA is digested enzymatically to deoxyribonucleoside 3′-monophosphates with endonuclease (micrococcal nuclease) and exonuclease (spleen phosphodiesterase). Thereafter, DNA hydrolysates (normal and modified deoxyribonucleoside 3′-monophosphates) are converted to 5′-^{32}P-labelled 3′,5′-bisphosphates by incubation with γ-[^{32}P]ATP in the presence of carrier ("cold") ATP and T4-polynucleotide kinase at pH 9.5

TABLE I
Detection methods for human DNA adduct biomonitoring

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

[a] CE, capillary electrophoresis.

(Fig. 2). This alkaline pH is used in order to minimize the 3′-phosphatase activity of the polynucleotide kinase.

The 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

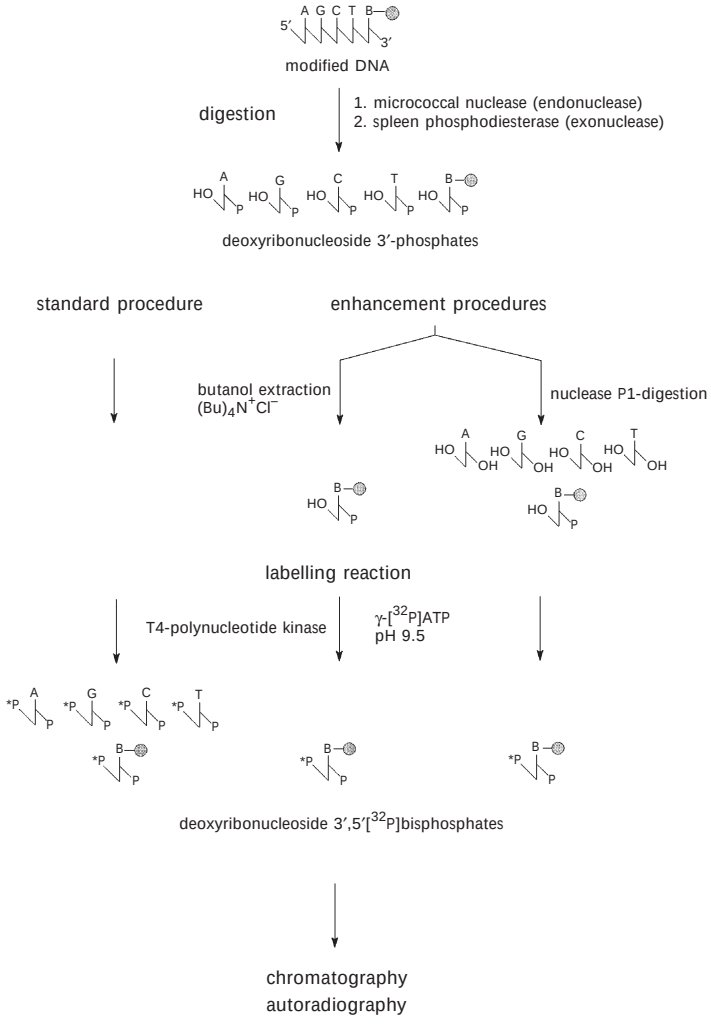

FIG. 2
Scheme of the ^{32}P-postlabelling assay

(PEI) cellulose plates (Fig. 3). During the first elution (D1 and D2 directions) with aqueous electrolyte, labelled unmodified nucleotides and [^{32}P]phosphate are moved from the start onto a paper wick while aromatic hydrophobic adducts are retained at the start for subsequent resolution using different solvent systems (D3, D4 directions) (Fig. 3). Recently, polyacrylamide gel electrophoresis (PAGE) of DNA digests has also proved useful for resolving the ^{32}P-postlabelled species (^{32}P-postlabelling/PAGE analysis)[95]. 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[96]. This technique yields an about ten-fold improvement in sensitivity compared with screen enhanced autoradiography for the detection of ^{32}P [97]. Furthermore, Instant Imager is now also frequently utilized for scanning TLC maps for adducts.

Resolution of adducts depends not only on their physicochemical properties but also on the presence of multiple adducts possessing similar chromatographic mobilities on PEI-cellulose thin layer plates. Figure 4 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[37,98–100] (Fig. 4a) and DNA adducts formed by the complex mixture of carcinogens in to-

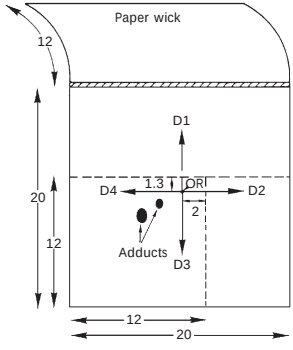

FIG. 3
Elution pattern of PEI-cellulose TLC plates

bacco smoke in human kidney DNA, which migrate poorly resolved along a diagonal radioactive zone[47] (Fig. 4b).

Adduct levels are calculated as relative adduct labelling (RAL) values. RAL values are the ratio of count rates of adducted nucleotides to count rates of total (adducted and normal) nucleotides[77,79]. However, this calculation is based on equal labelling efficiencies of adducts and normal nucleotides[90,101]. The "standard" protocol of ^{32}P-postlabelling method is suitable for most DNA adducts (bulky and non-bulky adducts), but its sensitivity is not sufficient for detection of adducts present at lower concentrations in DNA; utilizing this protocol, DNA adducts present at concentrations 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 γ-[^{32}P]ATP has been shown to enhance the method sensitivity 10- to 100-fold for a number of adducts (intensification procedure)[102,103]. An additional enhancement procedure uses an enzymatic postincubation of DNA digests with nuclease P1 (from *Penicillium citrinum*)[79] (Fig. 2) to enrich adducts. Nuclease P1 preferentially dephosphorylates unmodified deoxyribonucleoside 3′-monophosphates to deoxyribonucleosides and in many cases not the adducted nucleotides. Deoxyribonucleosides do not serve as substrates for T4-polynucleotide kinase for the transfer of [^{32}P]phosphate from γ-[^{32}P]ATP. However, some adducted nucleotides are strongly dephosphorylated by nuclease P1 (e.g., arylamine adducts substituted at C8 of deoxyguanosine), while others are

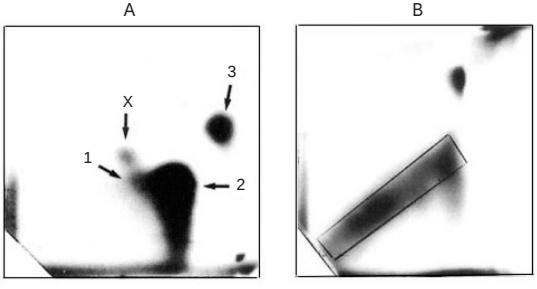

FIG. 4
Autoradiograms of ^{32}P-postlabelling analyses (nuclease P1-enrichment) of DNA adducts in: a renal cortical tissue from patient suffering from Chinese herbs nephropathy generated by aristolochic acid I, b renal cortical tissue from a smoker. Spot 1: dG-AAI, 7-(deoxyguanosin-N^2-yl)aristolactam 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

not (primarily adducts substituted at N^2 of deoxyguanosine). This version of the assay increases its sensitivity significantly (by three orders of magnitude; see Table I). The 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 γ-[^{32}P]ATP.

An additional enrichment procedure introduced by Gupta[78] exploits the properties of hydrophobic carcinogen-adducted nucleotides to be extracted into butan-1-ol in the presence of a phase transfer agent tetrabutylammonium chloride (Fig. 2). 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. More polar adducts containing non-aromatic bulky residues or a small alkyl moiety are, however, hardly extractable into butan-1-ol and cannot be analysed using this version of the ^{32}P-postlabelling technique.

The nuclease P1 and butan-1-ol 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^{10} normal nucleotides (0.3–3 amol/μg DNA) depending on structures of the 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[104,105] and (ii) the classic nuclease P1 version of the method followed by venom phosphodiesterase and/or a second nuclease P1 digestion (dinucleotide version)[105]. These versions are suitable mainly for detection of adducts formed by heterocyclic and aromatic amines, which cause complex modifications of DNA (not only the formation of simple covalent adducts)[104,105]. 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[106]. Because these methods are utilized only for some types of DNA adducts, they are used less frequently[47,81,107,108]. An increasingly popular method is a combination of enzymatic enrichment procedures of the ^{32}P-postlabelling assay with other methods (i.e. with butan-1-ol extraction[1,15,109–115] or with HPLC[47,88,89,98,99,115–122]).

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[116–119,123–125]. This method alone, however, often exhibits somewhat lower sensitivity than TLC and autoradiography[126]. Therefore,

the HPLC method is attractive mainly for some special applications, i.e. resolution of complex DNA adduct mixtures poorly separated by TLC [89,119,127–129] or identification of adducts by cochromatography[47,88,98–100,116–118,121,122].

A promising enrichment method seems to be the immunoaffinity/^{32}P-postlabelling assay[130–132]. 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[131,133,134] .

3.2. Critical Evaluation of ^{32}P-Postlabelling Methods

The enrichment and separation procedures 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 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. Examples of such adducts are those containing a small alkyl moiety or non-aromatic bulky residues or residues with one aromatic ring[47,567,109,112,131,135–140]. 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 causes for underestimation of DNA adducts are as follows: (i) incomplete digestion of carcinogen-modified DNA (e.g. DNA modified by crosslinks), (ii) different degrees of resistance of adducted nucleotides to dephosphorylation (e.g. low resistance of arylamine adducts substituted at C8 of deoxyguanosine), (iii) incomplete extraction into butan-1-ol (e.g. more polar adducts formed by carcinogens containing an alkyl moiety or only one benzene ring in their molecules), (iv) incomplete ^{32}P-postlabelling for several adducts (i.e. series of *N*-(deoxyguanosin-8-yl)arylamine 3′-phosphate adducts, for which substantially higher ATP concentrations are needed than in typical ^{32}P-postlabelling assays[90]), (v) losses of material during manipulations, or (vi) retaining of compounds at the start in the PEI-cellulose TLC [1,15,47,48,107,110,111]. The relatively low efficiency of enzymatic digestion of highly adducted DNA and/or DNA modified by DNA-DNA and/or DNA-protein crosslinking[47,83,84,105,110,111,141,142] is a limiting factor. The formation of multiple adducts from one carcinogen[110,111,141] or the presence of

multiple adducts from different carcinogens (such as environmental pollutants or carcinogens in tobacco smoke, see also Fig. 4) also extremely complicate the evaluation of adducts. Hence, even when digestion of DNA occurs with high efficiency, a combination of several modifications of the ^{32}P-postlabelling method (standard procedure, nuclease P1 or butan-1-ol extraction-enrichment, ^{32}P-HPLC etc.) as well as different chromatographic procedures to separate adducts of different structures[26,81,109,112,143] 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. Lack of adduct standards has, in many cases, limited the interpretation of data to a demonstration of higher adduct levels in exposed groups compared with unexposed groups[48,82]. Nevertheless, exceptions to this disadvantage exist; for example, using specific adduct standards formed by benzo[*a*]pyrene[144,145], heterocyclic amines[93], the anticancer drug tamoxifen[91,92,146,147] or aristolochic acids[27,98–100] (see also Fig. 4a), 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[26,49,148].

Another problem with promoting the ^{32}P-postlabelling technique for biomonitoring is the lack of detection of large amounts of adducts, which are lost by depurination from the DNA. It was described previously that the stable adducts detectable by ^{32}P-postlabelling assay seem to represent only a minor portion of the total DNA adducts formed by several carcinogens (e.g., at least the bulky adducts formed by polycyclic aromatic hydrocarbons or by estrogens such as 17-hydroxyestra-1,5(10)-dien-3,4-dione)[149–151].

3.3. 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 I), and, therefore, it is 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 and radiation-induced DNA damage, cyclic DNA adducts formed from a wide range of bifunc-

tional genotoxic chemicals, and UV-induced photodimers[1,15,26,47–49,78,107–109,135,138,140,152–155]. Therefore, this method is able to detect DNA modifications, caused by different, multiple exposures. 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. Moreover, carcinogen adduct characterization 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 and amines)[1,63,95,105,118,156] or plant (vegetable) products such as safrol and related alkenylbenzene derivatives[104,157], aristolochic acids[77,88,98–100,158], quaternary benzo[*c*]phenanthridine alkaloids sanguinarine and chelerythrine[159], pyrrolizidine alkaloids[160,161] or compounds responsible for DNA adduct formation by cola drinking[1,162]), or food additives and mycotoxins in food (i.e. aflatoxins[1], ochratoxin A [100,158,163]). 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. By this way candidate drugs for cancer prevention (e.g. chemicals present in green and black tea[107], natural or synthetic flavonoids[120,121]) might be found.

Another group of chemicals often analyzed are compounds known as environmental pollutants. Single compounds or natural mixtures are analyzed. Polluted urban air arising from several industrial productions or other sources (chemical or pharmaceutical industries, extensive use of pesticides in agriculture), from heating systems or from incomplete combustion in vehicle engines is analyzed for its potential to form DNA adducts[1,15,32,107,115,122,137,164]. 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[29,113,145,165]. Furthermore, using ^{32}P-postlabelling methods carcinogen DNA adducts and oxidative DNA damage generated by various agents including redox-cycling chemicals, nonmutagenic carcinogens/tumour promoters or chemical mixtures containing or producing radicals are determined[1]. In these studies, animal experiments are frequently performed, but analysis of DNA from human white blood cells is also performed[118,126,133,150,165]. DNA adducts in these cells were monitored by ^{32}P-postlabelling in populations living in polluted or unpolluted regions of several countries[9,26,97,107,114,143,144,164] 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[7]. In addition, urinary excretion of exfoliated bladder cells containing DNA adducts, including alkylation products, oxidized bases and bulky adducts, are excellent markers of exposure[26,167].

^{32}P-Postlabelling is increasingly used to analyze DNA adducts formed from pharmaceutical drugs or additives in human therapy (e.g. mitomycin, *cis*-platin, cyclophosphamide, cyproterone acetate, daunorubicin, tamoxifen, ellipticine)[15,87,91,92,107,119,120,168–170]. 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)[120,121,136,171,172].

DNA modifications formed by endogenous compounds (or endogenous factors) were also analysed by ^{32}P-postlabelling. Randerath and coworkers[108,173,174] found DNA adducts even in experimental animals unexposed to any chemicals. These adducts (I(indigenous)-compounds) are found also in humans; they 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[1,47,48,60,107,173]. It is questionable whether I-compounds are functional modifications that are necessary for normal growth, or are promutagenic lesions, or whether they play both roles. Studies by Randerath and coworkers show that both roles are possible[108,173]. There are two classes of I-compounds, 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[174].

4. CONCLUSIONS

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. 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"[26]. The large interindividual variability in DNA adduct formation, observed in individuals experiencing similar exposures, suggests that genetic differences in carcinogen metabolism, DNA repair and cell-cycle control modulate the individual response to exposure[7,26,33,143,175]. The rela-

tionship between formation of DNA adducts and polymorphisms in some carcinogen-metabolizing genes 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 the interpretation of DNA adduct data currently being collected in epidemiologic studies[54,176–178]. The research activity in this area, promising to resolve this very important factor for cancer development, should therefore be intensified.

The detection of DNA adducts in tissues of organisms (including humans) exposed to chemicals is 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[9,16–29]. Nevertheless, human biomonitoring by ^{32}P-postlabelling has some limitations; for ^{32}P-postlabelling, the adduct recovery, including labelling efficiencies, depends on the type of DNA adduct, thus preventing exact quantitation of unknown adducts. Moreover, the assay can give an estimate of the total adduct burden, but it is only rarely possible to identify specific adducts exactly in human samples. Nevertheless, DNA adducts formed from several carcinogens (e.g. benzo[*a*]pyrene, heterocyclic and aromatic amines, tamoxifen, aristolochic acids) were successfully identified by ^{32}P-postlabelling in human samples. Advances may lie in preparation and use of additional chemical standards and more advanced preparative techniques. Precautions should also be taken when using individual enhancement procedures of the ^{32}P-postlabelling assay alone. An underestimation or even an overestimation[107] of the number of different adducts present in DNA can occur. Combination of several modifications of the technique or of this technique with other methods useful for DNA adduct determination[143] 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 synthetic adduct standards in individual laboratories over the world should be established[49]. 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 achieve improvements in cancer epidemiology and in the prevention of cancer.

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