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^{32}P-POSTLABELLING: A SENSITIVE TECHNIQUE FOR THE DETECTION OF DNA ADDUCTS

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Content

1. Introduction

Organisms including humans are exposed to many chemical carcinogens by both endogeneous and exogeneous sources[1]. Of over 600 different agents evaluated for their overall carcinogenicity by the International Agency for Research on Cancer (IARC), more than fiftyhave been considered to be carcinogenic in humans[2].

Most of the chemical carcinogens are known to be activated to reactive intermediates that bind to nucleophilic centers in proteins and nucleic acids thereby forming covalent adducts[3,4]. The formation of DNA adducts is regarded as a critical early step in the multistage process of carcinogenesis. Moreover, approximately 90 % of the chemicals considered as carcinogenic for humans form DNA adducts. Although the majority of such DNA damage (DNA adducts) is eliminated by DNA repair processes, some persistent adducts often cause permanent mutations in important growth-controlling genes or loci, resulting in aberrant cellular growth and cancer[5-8]. Therefore, DNA adducts represent a detectable and critical step in chemical carcinogenesis, and thus may serve as an early biomarker for cancer[9]. Thus, the detection of the DNA adducts has become extremely important in risk assessment of chemicals as well as in determinations of exposures to carcinogens for humans. For an assay to be applicable in human exposure settings, it must (i) be sensitive enough to detect low levels of adducts; *(ji)* require only microgram quantities of DNA; *(iii)* give results quantitatively related to exposure; (*iv*) be applicable to unknown adducts that may be formed from complex mixture and (v) be able to resolve, quantitate and identify adducts[10].

Until 1981,the quantitation of DNA adducts has usually required the use of highly radioactive chemical carcinogens (labelled by ^{3}H or ^{14}C)prepared synthetically.Therefore such studies in humans have been impossible. In 1981, the ^{32}P--postlabelling technique[11] was introduced by Randerath and coworkers, and met most of the above mentioned requirements[9-14]. Since then ^{32}P-postlabelling has emerged as

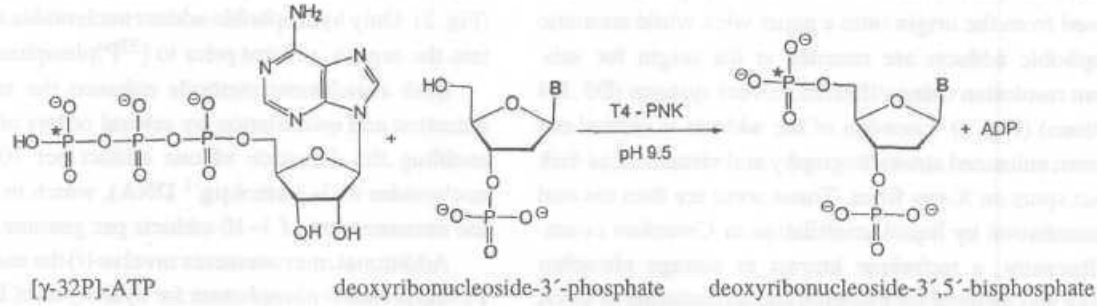

Fig. 1. **Labelling of deoxynucleoside 3'-phosphates by T4 polynucleotide kinase and [γ-^{32}P]ATP**

a major tool for the detection and quantitation of DNA adducts[14a].

The 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 by [γ-^{32}P]ATP and polynucleotide kinase (Fig. 1), and chromatographic separation of carcinogen-nucleotide adducts from non-modified (normal) nucleotides. In this technique, the deoxyribonucleotides are labelled by [^{32}P] after their modification by carcinogens and is, therefore, called ^{32}P-postlabelling.

2. ^{32}P-Postlabelling Methods

2.1. Basic ^{32}P-postlabelling technique

The original "standard" ^{32}P-postlabelling protocol[11,12,15] is shown in Figure 2. 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 ATP and T4-polynucleotide kinase at pH 9.5. This alkaline pH is used in order to minimize the 3'-phosphatase activity of the polynucleotide kinase. The ^{32}P-labelling reaction is sometimes terminated by adding potato apyrase which destroys excess [γ-^{32}P]ATP and carrier ("cold") ATP. ^{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 (Fig. 3). During the first elutions (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) (Fig. 3). 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. Recently, a technique known as storage phosphor imaging was adapted for mapping and quantitation of DNA adducts on chromatograms generated by the ^{32}P-postlabelling assay[16]. It yields an about 10-fold improvement in sensitivity compared to screen-enhanced autoradiography for the detection of ^{32}P (Ref.[17]).

Figure 4 shows examples of autoradiographs of ^{32}P--postlabelling analysis of well-resolved adducts formed by the plant carcinogen aristolochic acid in forestomach DNA of rats (Fig. 4a) 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) (Fig. 4b).

Adduct levels are calculated as relative adduct labelling (RAL) values, which represent the ratio of count rates of adducted nucleotides over count rates of total (adducted and normal) nucleotides[12,14]. Utilizing the "standard" protocol, DNA adducts present at levels of 1 adduct in 10^7 normal nucleotides (0.3 fmol adduct/μg DNA) can be detected.

2.2. Enhancement versions of the method

Several modifications of the standard assay have been employed in order to increase the sensitivity of the method. Two of these are most frequently used and described below.

The first, most current enhancement procedure uses an enzymatic postincubation of DNA digests with nuclease P1 (from *Penicillium citrinum*)[14] (Fig. 2) to enrich adducts. Nuclease P1 dephosphorylates deoxyribonucleoside 3′-monophosphates of normal nucleotides only to deoxyribonucleosides but not of adducted nucleotides. Deoxyribonucleosides do not serve as substrates of T4-polynucleotide kinase for the transfer of [^{32}P]phosphate from [γ-^{32}P]ATP. Adduct enrichment over normal nucleotides thus achieved before labelling, allows the use of larger amounts of DNA (10–20 μg) and of carrier free [γ-^{32}P]ATP.

The second enrichment procedure introduced by Gupta[13] exploits the properties of hydrophobic carcinogen-adducted nucleotides to be extracted into n-butanol in the presence of a phase transfer agent tetrabutylammonium chloride (Fig. 2). Only hydrophobic adduct nucleotides are extracted into the organic solvent prior to [^{32}P]phosphate labelling.

Both enrichment methods enhance the sensitivity of detection and quantitation by several orders of magnitude, enabling the detection of one adduct per $10^{9\text{-}10}$ normal nucleotides (0.3-3 amol.μg^{-1} DNA), 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

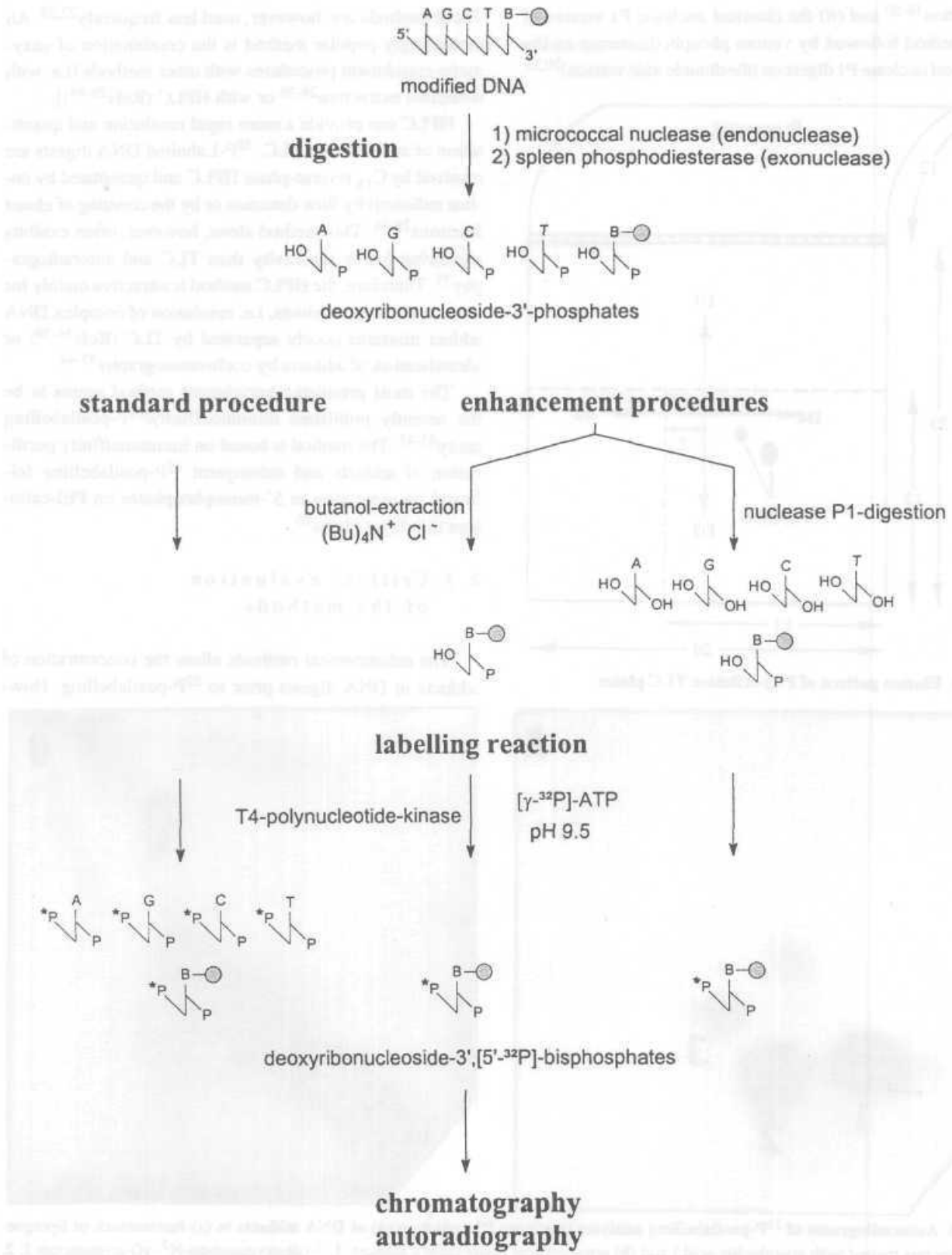

Fig. 2. Scheme of the ^{32}P-postlabelling assay

digestion[18-20] and (*ii*) the classical nuclease P1 version of the method followed by venom phosphodiesterase and/or a second nuclease P1 digestion (the dinucleotide version)[20,21].

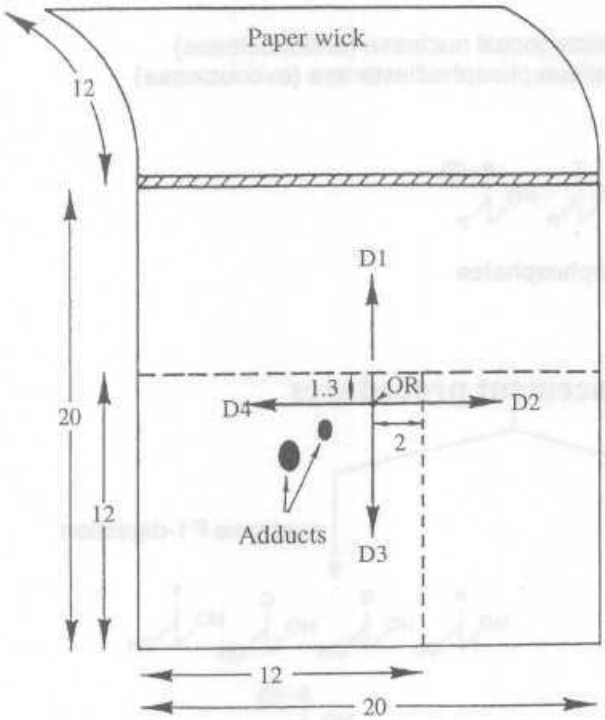

Fig. 3. **Elution pattern of PEI-cellulose TLC plates**

These methods are, however, used less frequently[22,23]. An increasingly popular method is the combination of enzymatic enrichment procedures with other methods [i.e. with n-butanol extraction[24-28] or with HPLC (Refs[29-44])].

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[29-34]. This method alone, however, often exhibits somewhat lower sensitivity than TLC and autoradiography[33]. Therefore, the HPLC method is attractive mainly for some special applications, i.e. resolution of complex DNA adduct mixtures poorly separated by TLC (Refs[34-36]) or identification of adducts by cochromatography[37-44].

The most promising enrichment method seems to be the recently published immunoaffinity/^{32}P-postlabelling assay[45-47]. The 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[46].

2.3. Critical evaluation of the methods

The enhancement methods allow the concentration of adducts in DNA digests prior to ^{32}P-postlabelling. How-

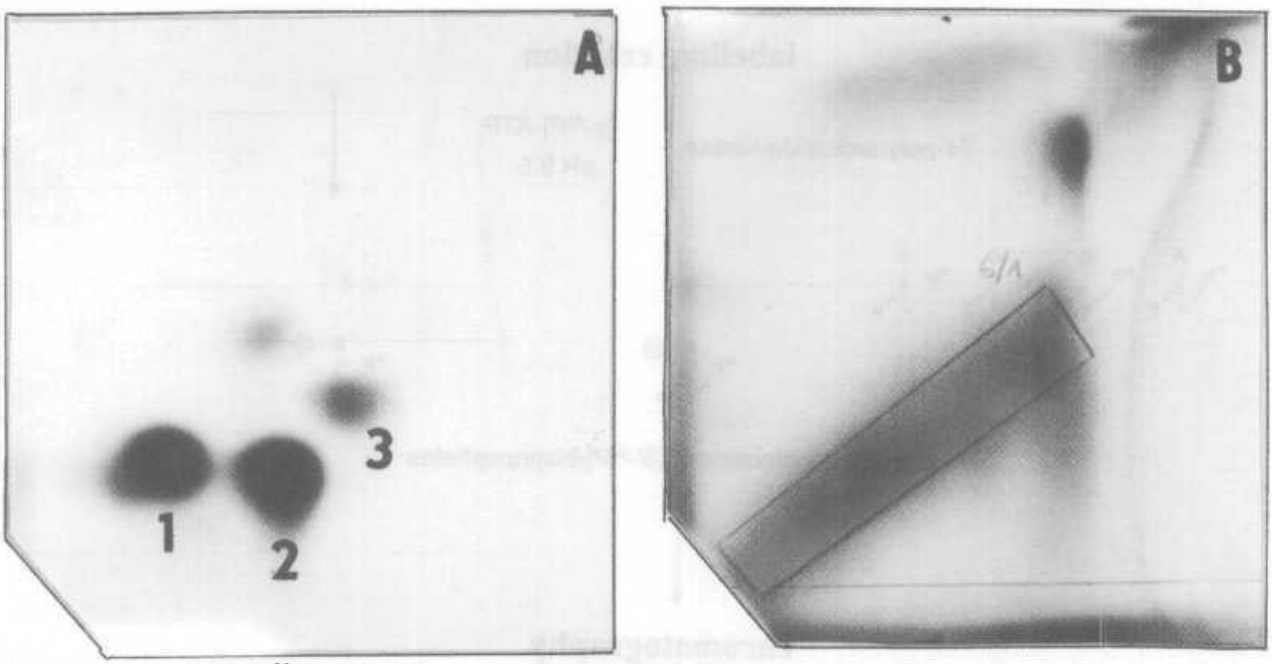

Fig. 4. **Autoradiograms of ^{32}P-postlabelling analyses (nuclease P1-enrichment) of DNA adducts** in (a) forestomach of Sprague-Dawley rats treated with aristolochic acid I and (b) renal cortical tissue from a smoker. 1, 7-(deoxyguanosin-N^2-yl)-aristolactam I; 2, 7-(deoxyadenosin-N^6-yl)-aristolactam I; 3, 7-(deoxyadenosin-N^6-yl)-aristolactam II

ever, 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[11,41,42,46,48-54]. Additionally, the multistep ^{32}P-postlabelling process not only lends itself to potential modifications of several steps but also demands careful control of these many steps in order to obtain reliable qualitative and quantitative results. The incomplete digestion of carcinogen modified DNA, different degrees of resistance of adducted nucleotides to dephosphorylation, incomplete extraction into n-butanol, incomplete ^{32}P-postlabelling, 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[9,55-57]. 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[22,55,58,59] is a limiting factor. The formation of multiple adducts from one carcinogen[55] 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 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 should be used for the exact determination and quantitation of adducts.

Nevertheless, the ^{32}P-postlabelling assay has been shown to be the most appropriate method for the detection of most DNA adducts. Table I shows a brief overview of the most sensitive 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 [immunoassays, fluorescence assay, ^{32}P-postlabelling, mass spectroscopy, electron spray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI)]. Among them, the ^{32}P-postlabelling method is the most sensitive, and, therefore, the most appropriate for human biomonitoring.

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

The increasing popularity of the ^{32}P-postlabelling assay for the determination of modified DNA also evolved from the ability of this method to detect and characterize several DNA lesions[26,56,57] (covalent carcinogen DNA adducts formed from both bulky aromatic chemicals[10] and from small molecules[8,41,53,54,60], oxidative DNA lesions formed by radical oxygen species[22,61], apurinic sites and radiation-induced DNA damage[62,63], cyclic DNA adducts formed from a wide range of bifunctional genotoxic chemicals[64]). Therefore, this method is applicable to identify DNA modifications, which are caused by different multiple effects. Of course, 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

Table I
Methods for the detection of DNA adducts

Method	Quantitation		DNA min. quantity per assay
	Na[a]	amol[b]/μg DNA	
^{3}H or ^{14}C labelled chemical	$1/10^{7}$–10^{9}	3-300	1 mg
HPLC with fluorescence detection	$1/10^{7}$	300	100 μg
Immunoassay (RIA, ELISA)	$1/10^{8}$	30	50 μg
Fluorescence spectroscopy	$1/10^{8}$	30	100 μg
HPLC-MS, GC/MS with ESI or MALDI	$1/10^{9}$	3	100 μg
^{32}P-postlabelling	$1/10^{10}$	0.3	1 μg

[a] Number of adducts per normal nucleotides, [b] amol = 10^{-18} mol

many chemicals but also to confirm their toxic (carcinogenic) effects. 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 (derivatives of aromatic amines[57]) or safrol and related alkenylbenzene derivatives present in plant (vegetable) products[19] or compounds responsible for DNA adduct formation by cola drinking[18]], or food additives and mycotoxins in food (i.e. aflatoxins[56], ochratoxin[65]). On the other hand, some dietary constituents are also analyzed by the ^{32}P-postlabelling assay for their ability to decrease DNA adduct formation or other DNA lesions from carcinogens and might be used for cancer prevention (e.g. chemicals present in green and black tea[57], garlic[66], *Brassicacea* plants[57]).

Another group of chemicals often analyzed are compounds known as environmental pollutants. Single compounds or the natural mixtures are analyzed. Cigarette smoke is the most studied environmental pollutant. A direct correlation of the levels of DNA adducts with smoking habits was shown by ^{32}P-postlabelling[27]. Furthermore, 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[8,9,56,57]. With ^{32}P-postlabelling methods carcinogen DNA adducts, oxidative DNA damage generated by various agents including redox-cycling chemicals, nonmutagenic carcinogens/tumor promoters or chemical mixtures containing or producing radicals are determined[23,56]. In these studies, animal experiments are frequently used, but analysis of DNA from human white blood cells (WBC) is also performed[36,67,68]. DNA adducts of these cells were monitored by ^{32}P-postlabelling in populations living in polluted or unpolluted regions of several countries. The amounts of adducts found in cells correlated with the level of air pollution[28,67]. Therefore, these cells are suggested to be suitable for noninvasive human biomonitoring studies.

^{32}P-Postlabelling is increasingly used to analyse DNA adducts formed from pharmaceutical drugs or their additives in human therapy (e.g. mitomycin, *cis*-platin, cyclophosphamide, cyproterone acetate, tamoxifen)[9,34,40,44,57,69-71]. 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)[20,39,43,52,55,68,72,73].

^{32}P-Postlabelling is also utilized to analyze DNA modifications formed by endogeneous compounds (or endogeneous factors). Randerath and coworkers[74] found DNA adducts even in experimental animals unexposed to any chemicals. These adducts [I-(indigenous) compounds] are found also in humans[56,57] and are derived from endogeneous electrophiles formed in the course of normal metabolism of nutrients and other natural dietary components[56,57,74] and accumulate in an age-dependent manner[56,57,74]. 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. Recent work of Randerath and coworkers shows that both roles are posssible[22,75,76].

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. Therefore, the detection of DNA adducts in tissues of organisms (including humans) exposed to chemicals represents a highly sensitive rapid *in vivo* assay for the genotoxicity 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. This method can be used in prospective studies to assess the risk to humans of their exposure to industrial pollutants, environmental contaminants, food contaminats and drugs. Precautions should be taken when using some individual enhancement procedures of the ^{32}P-postlabelling assay alone. An underestimation or even an overestimation[57] of the number of different adducts present in DNA can occur. The combination of several modifications of the technique leads to more exact determination and quantification of DNA adducts. Moreover, a unification of the ^{32}P-postlabelling assays in individual laboratories over the world should be established[77]. 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 should

help to achieve improvements in cancer epidemiology and in the prevention of cancer.

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M. Stiborová[a], E. Frei[b], C. A. Bieler[b], and H. H. Schmeiser[b] *("Department of Biochemistry, Faculty of Science, Charles University, Prague, [b]Department of Molecular Toxicology, German Cancer Research Centre, Heidelberg, Germany):* **^{32}P-Postlabelling: A Sensitive Technique for the Detection of DNA Adducts**

The ^{32}P-postlabelling assay has emerged as a major method to detect DNA adducts induced by structurally diverse carcinogens and other DNA lesions. The technique comprises enzymatic degradation of DNA to 3'-monodeoxynucleotides, enrichment of adducts, 5'-^{32}P-labelling, adduct separation by TLC (or HPLC), and detection and quantitation of adducts. The review concerns individual ^{32}P-postlabelling techniques (standard procedure, enrichment methods) as well as the applications of the method to different DNA modifications. Recent reports on the application of the ^{32}P-postlabelling technique in human risk assessment using carcinogen-DNA adducts as molecular biomarkers are discussed and a critical evaluation of the assays is presented.