

Review

³²P-Postlabelling and mass spectrometric methods for analysis of bulky, polyaromatic carcinogen-DNA adducts in humans

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ABSTRACT

There has been significant recent progress toward the development of human carcinogen-DNA adduct biomonitoring methods. ³²P-Postlabelling is a technique which has found wide application in human studies. ³²P-Postlabelling involves enzymatic preparation and labelling of DNA samples, followed by chromatographic separation of carcinogen-nucleotide adducts from unadducted nucleotides. Thin-layer ion-exchange and high-performance liquid chromatography (HPLC) have been utilized. This paper critically reviews ³²P-postlabelling methods for analysis of bulky, polyaromatic carcinogen-DNA adducts and details a strategy to optimize this technique for monitoring human samples. Development of a human carcinogen biomonitoring method requires that the biomarker meet certain criteria: that the biomarker be responsive to exposures known to increase human cancer risk, to reductions in those exposures, and to the influence of metabolic differences. In addition, reliable samples must be available by non-invasive means. The ability of ³²P-postlabelling to meet these criteria is traced in the literature and discussed. Identification of specific carcinogen-DNA adducts is a difficult task due to the low (femtomole) levels in human target tissues. Because co-chromatography in thin-layer chromatography (TLC) is generally not considered to be proof of chemical identity, both synchronous fluorescence and HPLC in conjunction with ³²P-postlabelling and TLC are used to confirm the identity of specific carcinogen-DNA adducts in human samples. Mass spectrometry is a highly specific method, the sensitivity of which has been improved to the point which may allow its use to confirm the identity of carcinogen-DNA adducts isolated by ³²P-postlabelling and other methods. The literature relating to the use of mass spectral techniques for carcinogen-DNA adduct analysis is reviewed.

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LIST OF ABBREVIATIONS

CAD	Collisionally activated dissociation	ECNI	Electron-capture negative ionization
CI	Chemical ionization	EI	Electron impact
D1	Acronyms used to describe the multi-dimensional mobile phase chromatographic developments used during 32 P-postlabelling	FAB	Fast atom bombardment
D5		GC	Gas chromatography
D8C	Dibenzofur, 4-glycidyl-2-phosphates	HPB	4-Hydroxy-1,3-pyridyl-1-butanol
dpN	Deoxynucleotide-3'-phosphates	HPLC	High-performance liquid chromatography
		ICR	Ion cyclotron resonance
		LD-TOF	Laser desorption time-of-flight
		MOCA	Methylene bis-2-chloroaniline

MS	Mass spectrometry
MS-MS	Tandem mass spectrometry
MSTFA	N-Methyl-N-trimethylsilyltrifluoroacetamide
NICI	Negative-ion chemical ionization
PEI	Polyethylenimine
RAL	Relative adduct labelling
SIM	Selective ion monitoring
TLC	Thin-layer chromatography
TOF	Time-of-flight

1. INTRODUCTION

1.1. Biology of carcinogen-DNA adducts

Carcinogen-DNA adducts are the covalent reaction products of electrophiles and nucleophilic sites on DNA. They are the initial damage to DNA induced by many, but not all, carcinogens. For example, to potent carcinogens, 2,3,7,8-tetrachloro-*p*-dioxin and methapyrene, apparently do not form DNA adducts [1,2]. Nonetheless, the carcinogenicity of several chemical series of compounds can be predicted by their ability to bind DNA [3,4]. Carcinogen-DNA adducts can cause errors during cell and DNA replication. DNA polymerase may insert an incorrect base on the newly synthesized strand opposite the carcinogen-DNA adduct because of altered hydrogen bonds [5,6]. Or, the carcinogen-DNA adduct may distort the DNA helix allowing insertion or deletion of bases or breaks in the DNA backbone [7-9]. *In vivo* mutations and chromosomal damage *in vivo* are correlated with carcinogen-DNA adduct levels [10-14]. Mutations and chromosome translocations caused by some carcinogens are those responsible for oncogene activation [15-19].

Organisms have evolved systems to lessen the genetic impact of lesions like carcinogen-DNA adducts. Fig. 1 illustrates schematically events which might take place following carcinogen-DNA adduct formation. Many DNA adducts are repaired prior to cell replication, restoring the original DNA sequence [20-23]. The rate of repair depends upon the structure of the particular DNA adduct [21,23]. For example, hydroxyla-

tion at the C-8 position of deoxyguanosine can occur at the rate of 50 000 times per cell per day [24,25]. However, this lesion is rapidly repaired, usually before a cell can divide [24]. DNA adducts of bulky carcinogens, like the polycyclic aromatic hydrocarbons and aromatic amines which will be discussed here, occur less frequently, but are also repaired more slowly. For example, 7,12-dimethylbenzanthracene-DNA adducts persist for longer than 42 weeks in experimental animals given a single dose [26]. Agents which increase cell proliferation and "promote" growth alter the balance between repair and replication, in some cases allowing the damage to become fixed before repair can take place [27]. In addition, some promoting agents apparently select for the initiated cells [27].

DNA replication fixes permanently the damage caused by the carcinogen-DNA adducts [28]. However, the vast majority of mutations do not induce tumors because they do not occur in critical areas of the genome. The size and organization of eucaryotic DNA is in itself protective, insuring that critical mutations are rare events. The number of genes regulating critical cellular functions, including growth, differentiation, and communication, is probably small. Only about 100 genes, of the roughly 30 000 potential proteins, are recognized as having oncogenic potential [29]. In many cases, very specific targets in these genes must be mutated for oncogenic activation.

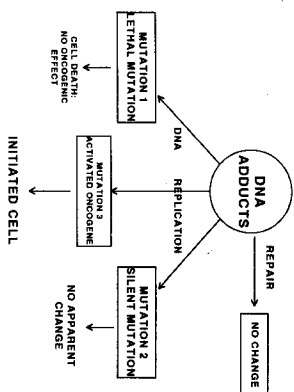


Fig. 1. Several possible biological outcomes resulting from carcinogen-DNA adduct formation.

For the *ras* gene family, only mutations in 12 specific base pairs out of a total of about 450 in the gene will produce an active oncogene [18]. In the total of about $6 \cdot 10^9$ base pairs in the human genome, only 10^3 to 10^4 may represent critical targets. Further, more than one oncogene must be activated in order for a cell to be transformed [29]. The number, and the temporal sequence, of these activations is also tissue-specific [29]. These factors combine to reduce the likelihood that any given DNA adduct will result in a tumor.

Formation of carcinogen-DNA adducts requires exposure, absorption, a specific metabolism which produces activated electrophiles, and non-repair of the lesion once formed. Measuring carcinogen-DNA adducts integrates for individual differences in these parameters. Monitoring carcinogen-DNA adducts may also be useful in disease prevention. Because there is a low probability that any given carcinogen-DNA adduct will initiate tumorigenesis, there is a corresponding high probability that, if the exposure is terminated, cancer can be prevented. The potential impact on cancer incidence of using tools like carcinogen-DNA adduct analysis to reduce the number of mutagenic hits is illustrated by the hypothetical situation presented in Fig. 2. We propose that the pattern of cancer incidence with increasing age may be a function of the accumulation of critical mutagenic "hits" within actively dividing

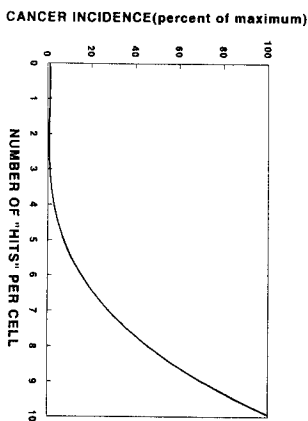


Fig. 2. Hypothetical relationship between the number of critical mutations ("hits") per cell and cancer incidence (data based on the age-related cancer rates).

stem cells. The main implications of this hypothesis are: (1) therapeutic measures to cure cancer may ultimately fail because there is a high probability that cells in a target organ may have accumulated a number of mutations; certain therapies may, therefore, contribute to increasing the mutagenic load most inopportunistly; and (2) decreasing the number of hits sustained by a cell might have a dramatic effect upon cancer incidence. Monitoring carcinogen-DNA adducts will be useful to decrease the mutagenic load if the exposures which cause them are terminated. The impact of intervention will be particularly great if it is done early in the individual's exposure history.

1.2. Chromatographic methods for adduct analysis

Synthesis of chemical carcinogens incorporating a radioactive carbon or hydrogen advanced tremendously the understanding of how these agents affect DNA [4]. Brooks and Lawley [30] demonstrated that DNA binding was a better predictor of carcinogenicity for a series of polycyclic aromatic hydrocarbons than was RNA or protein binding. Chromatographic methods, including liquid chromatography (largely with Sephadex LH-20 columns) and reversed-phase high-performance liquid chromatography (HPLC), were then employed to isolate specific carcinogen-DNA adducts [31,32]. However, radioisotopic methods cannot, obviously, be used in human exposure settings. Therefore, radio-mimetic methods have been supplanted for human monitoring by techniques which do not require radiolabelled carcinogen. Nonetheless, radiolabelled carcinogen-DNA adduct standards remain invaluable as positive controls and to define detection conditions for other methods, including ^{32}P -postlabelling. Immunoassays, electrochemical detection, synchronous fluorometric HPLC procedures, ^{32}P -postlabelling and, most recently, mass spectrometric (MS) methods have been developed and proven to be sufficiently sensitive to be used in human studies. These qualities have permitted for the first time monitoring of those human populations with potential carcinogen exposure. This paper will focus on the practical aspects of ^{32}P -postlabelling applications and new developments in MS toward monitoring of bulky, polycyclic aromatic carcinogen-DNA adducts in humans. Excellent work has been done using the other methods mentioned above [24,25,33,34].

2. ^{32}P -POSTLABELLING: PREPARATION AND ENZY-MATIC PROCEDURES

^{32}P -Postlabelling has been used extensively in determinations of the carcinogen-DNA adduct load in animals and humans. The technique was developed by Gupta and co-workers [35-41] as a progression from their earlier interest in assays of modified tRNA bases. The method involves incorporation of a ^{32}P label into DNA deoxynucleotides *after* the DNA has been isolated from the tissue of interest (hence the name). This label is then followed during subsequent chromatographic steps designed to resolve carcinogen-DNA adducts from unadducted deoxynucleotides and from each other. ^{32}P -Postlabelling has several advantages over other methods for carcinogen-DNA adduct analysis. The technique can be very sensitive when [^{32}P]ATP of high specific activity is used. The working range of the assay includes one adduct per 10^7 - 10^{10} unadducted nucleotides, corresponding to attomoles of carcinogen-DNA adducts per μg of DNA and the range of adducts expected in human samples [42,43]. This level of sensitivity can be achieved with DNA samples in the microgram or nanogram range [42-45]. In addition, ^{32}P -postlabelling is, within certain limits of molecular size and polarity (discussed below), a general method sensitive to damage produced by a variety of chemical carcinogens.

On the other hand, ^{32}P -postlabelling is a complex assay involving reagents (enzymes) which have varying Michaelis-Menten constants for the substrates (nucleotides and carcinogen-DNA adducts). There is also significant variability in the physical and chemical behavior of adducts formed from different carcinogens, which has a marked effect upon their behavior in the chro-

matographic systems. When the specific exposure is not known, it is also difficult to assign chemical identity to any of the carcinogen-DNA adduct "spots" resolved in the thin layers, without independent confirmation. Carcinogen-DNA adduct standards are invaluable to determine labelling efficacy and chromatographic behavior. Persons wishing to use ^{32}P -postlabelling to analyze human tissues should interpret their results with caution until they have gained sufficient experience with the technique to appreciate these foibles. For example, the shortcomings listed above make it inappropriate to assign absolute values to carcinogen-DNA adducts in any human study unless adduct identity and recovery have been established with reliable carcinogen-DNA adduct standards.

A schematic for preparation of material for ^{32}P -postlabelling chromatography is given in Fig. 3. DNA thought to contain carcinogen-DNA adducts is isolated from the tissue of interest, then hydrolyzed to 3'-phosphodeoxynucleotides using micrococcal endonuclease and spleen phosphodiesterase. The 3'-phosphodeoxynucleotides are 5'-labelled using polynucleotide kinase and [^{32}P]ATP. The 3',5'-bisphosphodeoxynucleotides are then chromatographed in two stages. Adducted 3',5'-bisphosphodeoxynucleotides are separated initially from the majority of the vast excess of unadducted nucleotides. Then,

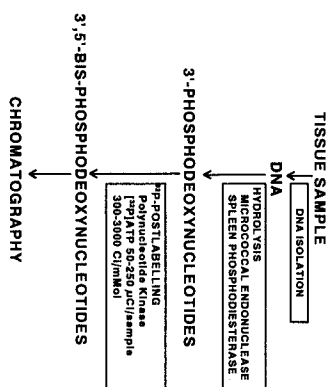


Fig. 3. Schematic of ^{32}P -postlabelling methods.

the various carcinogen-DNA adducts are resolved from each other. Anion-exchange thin-layer chromatography (TLC) using a polyethylencitrimine (PEI) stationary phase on flexible plastic sheets has been the most common method for ^{32}P -postlabelling analyses. Carcinogen-DNA adducts on the chromatograms are visualized by autoradiography. Individual carcinogen-DNA adduct spots may then be excised from the chromatogram, using the autoradiogram as a template. The carcinogen-DNA adduct spots and appropriate background areas are then counted in a liquid scintillation counter using either Cerenkov counting or a fluor-containing fluid [42]. The levels of carcinogen-DNA adducts are estimated using the relative adduct labelling (RAL) which is defined as:

$$\text{RAL} = \frac{\text{cpm}_{\text{adducts}}}{\text{cpm}_{\text{unadducted nucleotides}}} \times 10^3$$

Unadducted nucleotides may be determined empirically in an aliquot of the sample, or estimated from the specific activity of the [^{32}P]ATP multiplied times the amount of DNA in the sample, assuming complete labelling and that 1 μg DNA equals 3240 pmol of deoxynucleotide phosphate [42].

The use of reversed-phase HPLC ^{32}P -postlabelling separations is increasing, a trend which is expected to continue. These methods will be discussed in some detail below.

^{32}P -Postlabelling involves the use of large quantities of ^{32}P -labelled materials. This radioactive emits energetic β -particles which can also produce X-rays. Exposure protection, safe handling precautions, and constant monitoring are absolutely required for this technique. Specific precautions and descriptions of shielding devices can be found in the references [39,46].

2.1. [^{32}P]ATP synthesis

[^{32}P]ATP used for labelling is generally synthesized in the laboratory from $\text{H}_2^{32}\text{PO}_4$ using the methods of Johnson and Walseth [47]. [^{32}P]ATP may also be purchased from vendors,

however, this is often not cost-effective due to the large quantities of material required. [^{32}P]ATP synthesis utilizes several of the enzymes involved in glycolysis to first label D-glyceraldehyde-3-phosphate with [^{32}P]orthophosphate followed by phosphoglycerate kinase-catalyzed transfer of the ^{32}P to ADP. Synthetic efficiencies of 98% and greater and specific activities of approximately 3000 Ci/mmol are possible. Periodically, problems with synthesis are encountered which seem to be traceable to the orthophosphate supplied from the vendor. Typically, when problems occur, synthetic yields are on the order of 50%. We have found that the addition of fresh labelling cocktail, increasing the incubation time and/or temperature (to 37°C) can often significantly increase the yield of a bulky synthesis mix. In this regard, our results are similar to those of Johnson and Walseth [47] which suggested that an inhibitor of ATP synthesis which is occasionally present in some batches of [^{32}P]orthophosphate can be diluted out by the addition of buffer or cocktail. To reduce the liquid volume used in the assay, we require that the isotope be supplied at concentrations greater than 300 mCi/ml.

Poor ATP synthesis can pose problems for ^{32}P -postlabelling if no adjustment is made to account for the reduced activity. In particular, carrier-free labelling (see below) can be affected since the degree of intensification is dependent upon the ratio of [^{32}P]ATP to 3'-phosphodeoxynucleotides [26]. In addition, care must be taken to assure there is sufficient [^{32}P]ATP to label all adducts and residual normal nucleotides when using HPLC, nuclease P_1 or *n*-butanol extraction enhancement procedures.

2.2. DNA isolation

Any DNA isolation technique can be used for ^{32}P -postlabelling provided it is borne in mind that polynucleotide kinase uses ribonucleotides as substrates as readily as deoxyribonucleotides and that the enzyme has a near-absolute requirement for 3'-phosphonucleotides [48]. Therefore, it is important that RNA be removed as completely as possible. In the methods we currently

use, 0.1 g of homogenized tissue is digested with 0.24 mg of RNase A and 40 U of RNase T₁. This treatment digests RNA into the corresponding ribonucleotides which remain in solution when the DNA is precipitated by the addition of alcohol. We find that this treatment is sufficient for most tissue sources, yielding A_{230}/A_{260} ratios generally about 0.45 which do not change when additional RNase is added. However, we do find that blood lymphocytes or splenocytes require a second incubation with additional RNases before A_{230}/A_{260} ratios stabilize.

2.3. DNA hydrolysis

Hydrolysis of DNA specifically to 3'-phosphodeoxynucleotides by micrococcal endonuclease and spleen phosphodiesterase is a critical step in ^{32}P -postlabelling. The concentration of hydrolytic enzymes, pH, and the length of incubation are factors which must be considered. Unadducted DNA is the normal substrate for these enzymes, and we begin hydrolysis optimization by ascertaining which concentration of the enzyme mix will hydrolyze unadducted nucleotides most ef-

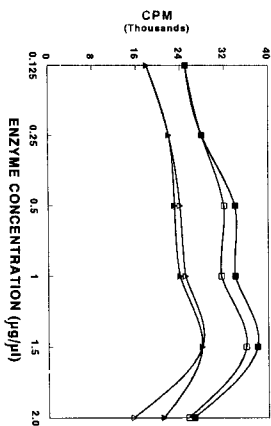


Fig. 4. Effect of concentration of micrococcal endonuclease and spleen phosphodiesterase on the labelling of the resulting unadducted 3'-phosphodeoxynucleotides. DNA (1 μg) was incubated with a cocktail containing the indicated amount of each enzyme for 3 h at 37°C and pH 6. The remainder of the experiment was conducted as described in ref. 38 except that the specific activity of [^{32}P]ATP used was 12 Ci/mmol. (▲) Deoxyguanosine-3',5'-bisphosphate; (△) deoxythymidine-3',5'-bisphosphate; (□) deoxyadenosine-3',5'-bisphosphate.

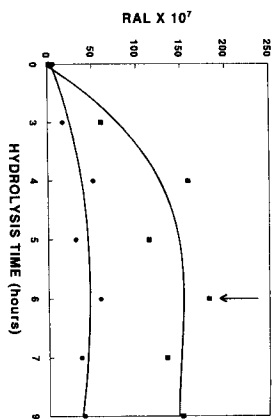


Fig. 5. Effect of the length of hydrolysis time on reported DNA adduct levels. A mouse was treated with 100 μmol of dibenzol[*a*]carbazole (DBC) topically. Liver DNA was isolated, hydrolyzed, and ^{32}P -postlabelled as described [14,26] except that hydrolysis incubation time was varied. RAL is defined in the text. Indicated values are the means of two separate analyses. (●) DBC adduct 6; (○) DBC adduct 3 (adduct numbering is as described in the references [53]). The arrow indicates the hydrolysis time adopted for routine analysis of these samples.

fectively while holding hydrolysis time constant at 3 h. This procedure has been described in detail by Reddy *et al.* [38]. It is important to determine whether the yield of nucleotides and the purine-to-pyrimidine ratio is as expected for the amount of DNA added [38]. Fig. 4 shows a typical hydrolysis profile for various dilutions of a hydrolysis mix. Note that an optimal yield of unadducted nucleotides is reached when the concentration of the hydrolysis mix is 1.5 $\mu\text{g}/\mu\text{l}$ of each enzyme. Whenever we begin analysis of a new type of carcinogen-DNA adduct, we extend our analysis of hydrolysis conditions by varying incubation time, while holding enzyme concentration constant. In this phase, the relative yields of the specific carcinogen-DNA adducts are used as the criterion. Fig. 5 shows the time course curves for RAL of dibenzol[*a*]carbazole-DNA adducts. Note that optimal RAL is reached when the DNA is labelled following 4-6 h incubation with the hydrolysis enzyme mix at 37°C. At the same time, we determined that there is no loss of the unadducted nucleotides under these conditions. Fig. 6 shows time course curves for benzo[*a*]pyrene DNA adducts hydrolyzed at either pH 6 or pH 7. Also note that there is an approxi-

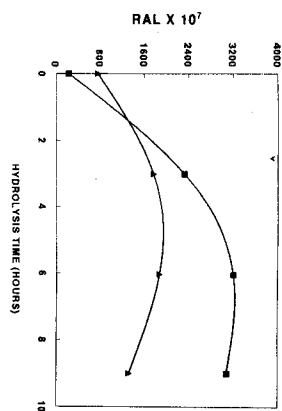


Fig. 6. Effects of hydrolysis time and hydrolysis pH on labelling of benzo[a]pyrene DNA adducts. Calf thymus DNA was reacted *in vitro* with benzo[a]pyrene-7,8-diol-9,10-epoxide. DNA was solvent-extracted, then precipitated, hydrolyzed and ³²P-labelled as described [14,26] except that hydrolysis incubation time and pH was varied. Indicated values are the means of two separate analyses. (■) pH 7; (▲) pH 6.

mate doubling of RAL values at pH 7 at 6-9 h of incubation.

We have investigated hydrolysis conditions in this way for several compounds (including polycyclic aromatic hydrocarbons, aromatic amines, and N-heterocycles) and have not as yet been able to define a relationship between structure and the need for extensive hydrolysis. N-(Deoxyguanosin-8-yl)-4-aminobiphenyl, the major 4-aminobiphenyl-DNA adduct, is effectively hydrolyzed using a 3-h incubation at pH 6, for example. On the other hand, some 6-nitrochrysene-DNA adducts require altered hydrolysis conditions. There are two distinct metabolic routes which lead to the formation of DNA-binding species for the compound 6-nitrochrysene [49]. One of these routes proceeds from the formation of 6-nitrochrysene-1,2-dihydrodiol, presumably via p-4501a1-mediated aromatic epoxidation [50]. Alternatively, oxidation can occur at the nitrogen which results in the formation of adducts at the C-8 position of deoxyguanosine, a site preferred by many aromatic amines [50]. We found that altering the pH of hydrolysis from 6 to 7 increased RAL values for the N-hydroxy-6-aminochrysene adducts 7-fold. However, no effect of pH was seen with the adducts resulting from exposure to 6-nitrochrysene-1,2-dihydrodiol [49].

Gorelick and co-workers [51,52] also reported that fluoranthene-DNA adducts require individualized hydrolysis conditions. Based on the different pH values optimal for micrococcal endonuclease (pH 9.2) and spleen phosphodiesterase (pH 6.6), these workers recommend that fluoranthene adducts be hydrolyzed first for 2 h with micrococcal endonuclease at pH 8.5, followed by reduction of the pH to 7.3 (with the addition of ethylenediaminetetraacetate to chelate Ca²⁺) and an additional 2-h incubation with added spleen phosphodiesterase [51]. This procedure may prove useful for other adducts as well.

2.4. ³²P-Labeling with polynucleotide kinase

Polynucleotide kinase is ideally suited for an application such as ³²P-postlabelling. This enzyme has a substrate specificity which includes all the known ribo- and deoxyribonucleotides [35,36,38]. Therefore, it was not surprising that carcinogen-adducted 3'-phosphodeoxynucleotides are labelled by this enzyme. Voticka and Hemminki [53] found that, under the recommended labelling conditions, a wide structural variety of carcinogen-DNA adducts were effectively labelled. What was somewhat surprising, however, is that a wide variety of carcinogen-DNA adducts are labelled preferentially to unadducted nucleotides (see below).

With standard or cold-carrier labelling conditions, there is an excess of ATP having a specific activity of approximately 300 Ci/mmol, and 3'-phosphodeoxynucleotides are rate-limiting. Nucleotides are labelled quantitatively, and the reported RAL is an accurate estimate of the level of carcinogen-DNA adducts in the sample [39]. If, however, conditions are adjusted so that there is an excess of 3'-phosphodeoxynucleotides and a limiting amount of [³²P]ATP, 3'-phosphodeoxynucleotides will be ³²P-labelled according to the affinity of the enzyme for the individual substrate. It was found that, under these conditions, the apparent levels of certain carcinogen-DNA adducts were intensified [54]. The degree of intensification is widely variable depending on the carcinogen-DNA adducts and the relative con-

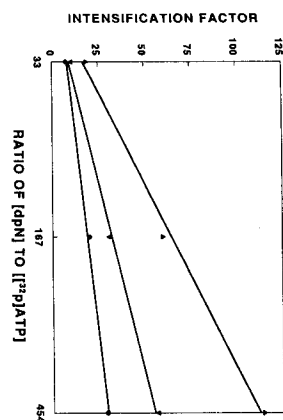


Fig. 7. Effect of the relative concentrations of nucleotide-3'-phosphates (dpm) to [³²P]ATP on the intensification of dimethylbenzanthracene-DNA adducts by ³²P-postlabelling using carrier-free conditions (plotted from data in ref. 56).

centrations of [³²P]ATP and 3'-phosphodeoxynucleotides [26]. For example, Schut *et al.* [54] saw that specific 7,12-dimethylbenz[*a*]anthracene adducts were intensified from 20- to 60-fold. Schut *et al.* [55] reported that intensification of dibenz[*a,h*]acridine-DNA adducts ranged from 10 to 25 times. We have seen that N-deoxyguanosin-8-yl-N'-acetylbenzidine is intensified 3.6-fold, while the corresponding N'-acetylbenzidine adduct is intensified approximately 18-fold [14]. Randerath *et al.* [26] found that altering the relative concentrations of 3'-phosphodeoxynucleotides and [³²P]ATP changed the intensification of the particular carcinogen-DNA adduct. As is shown in Fig. 7, while the change in intensification is linear for each specific carcinogen-DNA adduct over a 10-fold range, slopes for different 7,12-dimethylbenzanthracene-DNA adducts differ significantly.

Although in carrier-free conditions polynucleotide kinase labels some adducts in preference to unadducted nucleotides, the adducts in a sample are not quantitatively labelled. There is such a vast excess of unadducted deoxynucleotides in most samples that not all carcinogen-DNA adducts are found by the enzymes. Thus, the sensitivity of carrier-free methods is limited, practically, to about 5 adducts per 10⁸ unadducted nucleotides. Prolonged autoradiographic exposure of the chromatograms (three to seven days) can in-

crease the visual sensitivity of the method, but it is difficult to discern the radioactive signal as different from background [56].

Nonetheless, carrier-free labelling is an excellent method for analyzing samples from experimentally exposed animals. Also, the first reports that carcinogen-DNA adducts were elevated in certain human populations utilized carrier-free ³²P-postlabelling [56,57].

2.5. Enhancement strategies

In order to radioactively label all the carcinogen-DNA adducts in a sample containing 2 µg DNA, about 37 mCi of [³²P]ATP with a specific activity of 3000 mCi/mmol (8.1 · 10¹⁰ dpm) would be required [42]. The expense and safety hazards associated with handling such prodigious amounts of ³²P have deterred most investigators from this approach. Since the detection limit of carrier-free labelling is on the order of 5 carcinogen-DNA adducts per 10⁸ unadducted deoxynucleotides, and the levels of bulky carcinogen-DNA adducts expected in human samples may range from 1 adduct per 10¹⁰ to 10⁷ unadducted nucleotides, alternative strategies to improve or "enhance" the sensitivity of ³²P-postlabelling have been developed. These procedures are shown schematically in Fig. 8. The basis for each enhancement procedure is removal of the majority of the hydrolyzed, unadducted 3'-phosphodeoxynucleotides prior to ³²P-postlabelling. Because relative enrichment of carcinogen-DNA adducts of several orders of magnitude can be attained in some cases, there is the added benefit that the amount of radioactive material which must be handled can be reduced. The major enhancement methods are nuclease P₁ digestion of the deoxynucleotide-3'-phosphates, *n*-butanol extraction of the carcinogen-DNA adducts, and pre-separation of the carcinogen-DNA adducts from unadducted deoxynucleotide-3'-phosphates using a chromatographic system (HPLC). Each enhancement method is based on the differential behavior of unadducted nucleotides from carcinogen-DNA adducts. However, it cannot be assumed *a priori* that any particular adduct class

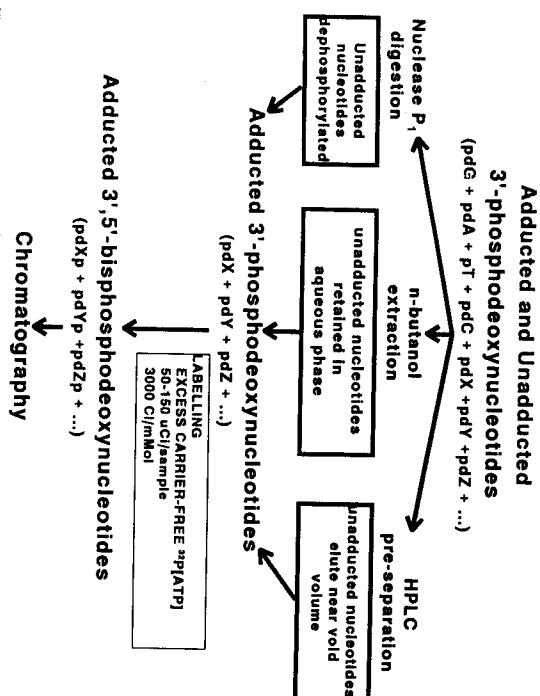


Fig. 8. Procedures to enhance ^{32}P -postlabelling of carcinogen-DNA adducts. Unadducted 3'-phosphodeoxynucleotides are removed prior to ^{32}P -postlabelling by enzymatic dephosphorylation (nuclease P_1 procedure), organic extraction (*n*-butanol extraction procedure), or chromatographic separation of the nucleotides (HPLC procedure). Carcinogen-DNA adducts are then quantitatively labelled with an excess of [^{32}P]ATP. Chromatography proceeds as described in ref. 60.

will behave sufficiently different from unadducted deoxynucleotide-3-phosphates in order to be completely recovered by any enhancement system. Indeed, significant in the advances of the last several years have been experiments which have documented the limitations of the enhancement procedures.

2.5.1. Nuclease P_1

Reddy and Randerath [42] discovered that nuclease P_1 , which 3'-dephosphorylates unadducted nucleotides, did not hydrolyze the phosphate from certain carcinogen-DNA adducts. Polynucleotide kinase has a very specific requirement for nucleotide-3-phosphates. Nuclease P_1 -hydrolyzed unadducted nucleotides will not be appreciably labelled, while all the carcinogen-DNA adducts in a sample may be labelled. If the particular carcinogen-DNA adduct is completely resis-

tant to nuclease P_1 , there will be a dramatic increase in the number of labelled deoxynucleotides. For those compounds which are highly resistant, treatment with nuclease P_1 can bring about a 2-3 order of magnitude increase in sensitivity [42]. The nuclease P_1 procedure is very straightforward and has become the most commonly used ^{32}P -postlabelling technique in human monitoring (see below). Hydrolyzed samples are simply incubated for 30-40 min at 37°C with nuclease P_1 prior to labelling [42].

2.5.2. *n*-Butanol extraction

Reddy and Randerath [42], in describing the nuclease P_1 procedure noted that all carcinogen-DNA adducts tested were not enhanced to the same degree. This was followed by detailed studies by Gupta and Earley [58] and Gallagher *et al.*, [59] who noted that there were classes of com-

pounds only partially resistant to nuclease P_1 , which were slightly enhanced or underreported when this method was used. Notable in this group were the aromatic amines. Gupta [43], extending the work of Kadlubar, found that an *n*-butanol extraction could enhance the detection of several series of carcinogen-DNA adducts, including those of aromatic amines. In the presence of a phase transfer agent, usually tetrabutylammonium, many adducted 3'-phosphodeoxynucleotides are extracted from the aqueous phase into *n*-butanol, while unadducted deoxynucleotides-3-phosphates remain in the aqueous phase. The relative extractability of any carcinogen-DNA adduct will depend upon its polarity and lipophilicity. These differ between particular carcinogen-DNA adducts, because of different chemical structure, and the position of DNA binding. We view *n*-butanol extraction and nuclease P_1 digestion as complementary procedures which yield information on populations of carcinogen-DNA adducts which are only somewhat overlapping. For example, in the studies detailed below, we found that, while there were similarities between chromatograms from nuclease P_1 - and *n*-butanol-extracted samples of the same tobacco smokers (notably, the presence of a diagonal zone of adducts), the chromatographic behavior of the discrete adducts imbedded within and around the zones were subtly different [60]. Simply stated, nuclease P_1 -resistant adducts were not superimposable upon the major *n*-butanol-extracted adducts. Because these methods complement each other, we propose that both be used to analyze human samples.

2.5.3. HPLC pre-separation

Chromatographic separation of unadducted nucleotides prior to ^{32}P -labelling has become a focus of interest. Dunn and San [61] have developed methods to pre-purify adducted 3'-phosphodeoxynucleotides following DNA hydrolysis. Unadducted 3'-phosphodeoxynucleotides in the samples are injected into a reversed-phase HPLC system and eluted with methanol-1 M ammonium phosphate (5:95), then adducts are eluted by increasing methanol concentration to 70%. Frac-

tions are collected, dried, then ^{32}P -postlabelled and spotted on individual PEI chromatographic plates and analyzed as described above. These methods have been used extensively to determine the levels of carcinogen-DNA adducts in fish [62]. This method of adduct enhancement is very promising. Sample throughput is reasonable at about 30 min (or about 8 h to prepare sixteen samples). Sensitivity is similar to that of other adduct enhancement methods. On the other hand, the requirement for high-molarity mobile phases is a definite drawback from the point of view of equipment maintenance and associated costs. We have attempted to decrease buffer molarity through the use of ion-pairing reagents, with mixed results, as the salts from the latter accumulate during concentration and interfere with subsequent chromatography. There have been no reports indicating the sample recovers for any series of carcinogen-DNA adduct; therefore, it is difficult to determine whether some adducts elute in the void volume, are retained on the column, or are degraded during the drying process. Dunn (personal communication) reports that the spectrum of carcinogen-DNA adducts enriched by this method is similar to that of nuclease P_1 to the first approximation. If this is the case, time may be better used in the nuclease P_1 method where all the samples in a run can be handled in batch fashion. Nonetheless, different chromatographic systems may make this method quite useful, in particular for low-molecular-mass carcinogen-DNA adducts. Using these techniques, Dunn *et al.* [63] reported that carcinogen-DNA adduct levels were elevated in lung biopsy samples from human smokers.

Unadducted 3'-phosphodeoxynucleotides are eluted first in reversed-phase HPLC. We have seen that peak tailing of the unadducted 3'-phosphodeoxynucleotides can pose a problem even when several minutes separate the respective peaks, due to the great excess of unadducted deoxynucleotides. This factor makes it more difficult to establish reproducible conditions of [^{32}P]ATP excess.

Kadlubar *et al.* [64] used reversed-phase HPLC as both a separation and an analytical system to

close to the origin. However, when we analyzed an *in vitro* synthesized 4-aminobiphenyl-DNA adduct standard, we found that we could recover only about 0.3% of the adducts by ^{32}P -postlabelling. However, we did notice that, on the D1 autoradiogram, there was a large radioactive spot which migrated above the area where we usually cut the tab for contact transfer (as much as 4 cm above the origin). When this activity was removed and transferred to a new D3 plate (Fig. 10), the adduct pattern characteristic of 4-aminobiphenyl was obtained and the recovery increased over 100-fold. We found that adducts were retained much closer to the origin and recoveries increased when 0.8 M sodium phosphate buffer (pH 6.8) or 0.65 M sodium phosphate (pH 6.0) was used as in lanes C and D [60]. We later found that carcinogen-DNA adducts resulting from *in vitro* microsomal activation of 6-aminochrysene-1,2-dihydrodiol also migrated 4-5 cm from the origin. In contrast, carcinogen-DNA adducts from DNA modified *in vitro* with N-hydroxy-6-aminochrysene were retained at the origin. To deal with this potential problem, we employed an experiment originally performed to demonstrate that benidine-DNA adducts were not lost by preparing the transfer tabs in the usual way (lanes A + B) [70]. The length of the tab is determined by the size of the magnet used during transfer (2.5 cm, see below) (lanes E and F). Keeping tab size constant, tabs were prepared which included a progressively larger area above the origin (lanes G-L). We found that our best recovery of both 4-aminobiphenyl- and 6-aminochrysene-1,2-dihydrodiol-DNA adducts came when tabs were removed as in lanes K and L. Fortunately, cutting off the tab at this point did not significantly increase background radioactivity transferred to the plates. We subsequently adopted these methods for our human studies [60].

The above work is also significant to the single-sheet chromatographic technique because adducts may migrate off the area of the plate retained following D1 and D2, and be similarly discarded. These findings stress the importance of *in vitro* adduct standards in developing methods.

These recovery problems could not have been solved without the use of these materials.

3.1.2. Resolution of carcinogen-DNA adducts

Magnet-mediated, contact transfer of the tabs cut out from the D1 chromatogram is performed essentially as described by Lu *et al.* [67]. Fig. 10 shows how the chromatogram is developed and the appearance of the resulting carcinogen-DNA adducts. The transfer complex (magnet + cutout + new PEI sheet + magnet) is developed in the D3 direction (as noted above, D2 is omitted using the contact transfer technique) using the mobile phase listed in Table 1. Plates are then rinsed with water to remove the D3 solvent, dried under cool air, rotated 90° counterclockwise, pre-developed in water, then developed using one of the D4 solvents listed in Table 1. The D5 is in the same direction as D4 and a 2.5-cm Whatman No. 1 wick is attached before development and removed afterward.

These methods have been used to detect a wide variety of carcinogen-DNA adducts [39,42,43,58,67]. There are many occasions when carcinogen-DNA adducts are not sufficiently resolved using a given mobile phase. Changing the concentration of the mobile phase by adding water can sometimes bring about adequate resolution [55]. If this fails, the mobile phase may be changed with good results. Several mobile phase systems are given in Table 1.

3.2. HPLC analysis of ^{32}P -postlabelled DNA

It should also be possible to use HPLC to resolve ^{32}P -postlabelled carcinogen-DNA adducts. Levy and Weber [71] used HPLC separation techniques in their analysis of 2-aminofluorene-exposed mice. Samples were *n*-butanol-extracted as described above, labelled, then injected into a reversed-phase HPLC system as 3'-5'-bisphosphodeoxynucleotides. A gradient from 10 to 50% acetonitrile in 30 mM potassium phosphate, pH 6 was used. Adducts were collected in timed fractions and their levels determined by Cerenkov counting. Gorelick and co-workers [51,52] have also used HPLC with some success with flu-

oranthrene in animal studies. To decrease the background radioactivity due to unadducted nucleotides, Gorelick and co-workers [51,52] developed an extensive post-hydrolysis clean-up involving reversed-phase Sep-Pak enrichment and brief nuclease P₁ digestion. The remaining 3'-phosphodeoxynucleotides were then ^{32}P -postlabelled, and redigested extensively with nuclease P₁ to remove 3'-phosphates from all deoxynucleotides. This digest was injected into a reversed-phase HPLC system and chromatographed using a methanol-0.1 M ammonium acetate, 1 mM ammonium dihydrogenphosphate, pH 5.7 program which runs for 85 min [52].

Good sensitivity was reported in these animal studies. However, resolution of individual carcinogen-DNA adducts and the level of background activity could pose problems in human studies with the techniques used by Levy and Weber [71]. Gorelick and co-workers [51,52] were able to decrease background significantly with the extensive sample clean-up employed. However, there have been no published reports to date involving these techniques in human exposure studies.

4. GENERAL ISSUES IN THE ANALYSIS OF HUMAN TISSUES

The potential applications of ^{32}P -postlabelling to studying carcinogen exposure in humans are exciting and may prove to be extremely useful in reducing carcinogenic exposure and risk. No other technique is presently as sensitive, or requires such small amounts of tissue or DNA for analysis. These characteristics make ^{32}P -postlabelling most amenable to non-invasive sampling. Nonetheless, there are several analytical issues which should be addressed. First is the issue of carcinogen-DNA adduct identification. The number of potentially carcinogenic compounds in the human environment must surely number in the hundreds. For example, one authority lists 43 organic carcinogens of occupational importance and several of these are mixtures which contain multiple components [72]. The potential exists, therefore, for many carcinogen-DNA adducts to

have similar chromatographic behavior in any given system. In fact, a diagonal zone is observed in autoradiograms from many human samples which likely consists of radioactivity from overlapping carcinogen-DNA adducts [73]. This interpretation is reinforced by the data which shows that, when animals are treated with carcinogenic complex mixtures (cigarette smoke condensate, or coke oven, coal soot, or diesel exhaust particulate extracts), a similar diagonal zone is observed following ^{32}P -postlabelling [73]. Intensity of the diagonal zone was also related to the carcinogenicity of the mixture. Characterization of the minor components of these zones will likely be difficult. However, we have seen that several adducts are outstanding in the diagonal zone, or resolve to areas on the periphery of the diagonal zones. These adducts may be identified. The issue of carcinogen-DNA adduct identification can be addressed successfully through the use of standards and corroborating techniques. The identity of several carcinogen-DNA adducts has recently been established in human and animal studies by co-chromatography with synthetic adduct standards in multiple thin-layer and HPLC systems [60,74]. Mass spectral and nuclear magnetic resonance data are preferred methods of chemical identification. As the sensitivity of these methods increases and their tissue requirements decrease, they will be used to augment techniques such as ^{32}P -postlabelling. Several approaches are reviewed below.

From the preceding discussion, it is likely that specific adducts, and even entire classes of adducts, may be considerably underestimated or completely lost when doing ^{32}P -postlabelling, depending upon the analytical plan. The obvious recourse would be to analyze each sample under a variety of different enhancement and hydrolytic protocols. Since humans are often exposed to complex mixtures of carcinogens, time constraints make this possible. We have proposed that both *n*-butanol extraction and nuclease P₁ methods be used on each human sample because of the different adduct populations they enhance [60]. A reasonable alternative would be to optimize hydrolysis, labelling and chromatographic

conditions for carcinogen-DNA adducts thought to result from the particular exposure. If that specific carcinogen-DNA adduct is present, it can then be quantitated accurately. In addition, if other adducts on the chromatographic maps can be identified using standards or other methods, and their recoveries under those conditions calculated, they can also be quantitated separately.

Absolute accuracy of carcinogen-DNA adduct quantitation in human samples is not possible presently with ^{32}P -postlabelling due to the losses which can occur at various points in the analysis. However, the effect of variable adduct recovery can be minimized simply by adopting consistent methods of hydrolysis and chromatography before the study begins and by maintaining these strictly during the complete study term. This done, analytical variation will be minimized, and results will be internally consistent and completely valid — even if the quantitative data are not directly comparable to other studies.

Analytical techniques capable of detecting carcinogen-DNA adducts in human samples require validation on several levels to predict human risk reliably. Early studies which showed that carcinogen-DNA adducts were related to cancer, mutation and other genotoxic endpoints demonstrated biological plausibility, i.e. carcinogen-DNA adducts are an important link in the causal chain in the activity of many carcinogens [10-14]. ^{32}P -Postlabelling played a prominent role in ascertaining these genetic interactions [10,14].

4.1. Studies in groups with increased cancer risk

The first issue for human samples is whether the biomarkers will be increased in the target organs of groups known to be at increased cancer risk. Cigarette smoking is the obvious starting point for these studies because this habit causes a significant fraction of all human tumors. In particular, from 50-85% of tumors in the lung and urinary bladder are caused by tobacco smoking [75]. Early studies were concerned with the relationship between cigarette smoking and easily obtainable tissues. Everson *et al.* [56] used carri-

er-free techniques to demonstrate that carcinogen-DNA adducts were increased significantly (about 20-fold) in the placentas of current smokers. (The absolute value of this difference is problematic because all samples were assayed near the limit of detection of this variant of the assay.) The immediate implication of this work, that the offspring of smokers may be exposed to carcinogens via the maternal blood supply, has had considerable public health impact. Manchester *et al.* [33] also examined placental DNA by both ^{32}P -postlabelling and synchronous fluorescence. They found that the placentas of smokers (and in some non-smokers) contained carcinogen-DNA adducts consistent with exposure to polycyclic aromatic hydrocarbons, specifically benzo[a]pyrene. Adducts with similar chromatographic behavior as the benzo[a]pyrene-DNA adduct standard were visualized after either nuclease P_1 or n -butanol extraction. Unfortunately, this study did not report the number of individuals tested, nor were summary statistics given to determine whether there was an association between the presence of carcinogen-DNA adducts and a smoking habit. All the samples from smokers positive for carcinogen-DNA adducts by synchronous fluorescence were also positive by ^{32}P -postlabelling. However, at least one sample from a non-smoker exhibited apparent carcinogen-DNA adducts by ^{32}P -postlabelling but not by synchronous fluorescence.

4.1.1. Tobacco smoking and the lung

The lung is the major target for tobacco smoke carcinogenicity [75]. Depending upon the study, smokers are thought to be from 5 to 12 times more likely to develop lung tumors than non-smokers [75]. Phillips *et al.* [76] were the first to study the levels of carcinogen-DNA adducts in human lung surgical samples. Using total nuclease P_1 adducts as the criteria, they saw an average of 11.3 adducts per 10^8 unadducted nucleotides in eighteen smokers, 2.4 per 10^8 in five non-smokers (*t*-test, $p \leq 0.05$) and 5.2 adducts per 10^8 in four ex-smokers. In addition, they saw a significant correlation between the number of cigarettes smoked per day and the level of carcinogen-DNA adducts (correlation coefficient 0.724, $p \leq 0.001$). Van Schooten *et al.* [77] saw that carcinogen-DNA adduct levels were increased in the normal tissue, but not the tumor tissue of smokers, consistent with what is known about the biology of the tumors (specifically their rate of cell turnover and metabolism [78]). These workers noted that while the reported levels of carcinogen-DNA adducts were 5-10 times higher by an enzyme-linked immunosorbent assay than by ^{32}P -postlabelling, the data from the two methods were well correlated (correlation coefficient 0.97, $p \leq 0.001$) [77].

We have similarly found that there are significantly increased levels of carcinogen-DNA adducts in the lungs of tobacco smokers. Samples were analyzed by both n -butanol extraction and nuclease P_1 . Fig. 10 shows the mean carcinogen-DNA adduct levels for several smoking-related carcinogen-DNA adducts. The relative increase in smoking-related carcinogen-DNA adducts in the lung is approximately proportional to the increased risk of lung cancer seen in cigarette smokers. For example, adducts P3 and P4 and

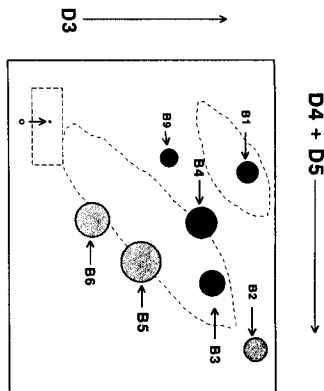


Fig. 10. Chromatogram used to resolve carcinogen-DNA adducts (D3 chromatogram). Tabs from D1 (Fig. 9) are placed as indicated by the rectangular dotted lines at the origin. The directions of solvent development are given by the arrows. Two carcinogen-DNA adducts (B5 and B6) were found not to be related to smoking exposure. The other carcinogen-DNA adducts indicated in the figure (in this case, n -butanol-extracted adducts) were smoking-related. Dotted lines indicated where diagonal zones of adducts are often seen.

total nuclease P_1 -enhanced adducts (PZTOT) are from 5 to 7 times higher in the smokers than in the non-smokers. It is interesting to note that nuclease P_1 -enhanced adducts seem to be the more sensitive marker of the exposure. Although there is considerable overlap in sensitivities, nuclease P_1 is thought to enhance carcinogen-DNA adducts related to exposure to polycyclic aromatic hydrocarbons, while n -butanol extraction enhances carcinogen-DNA adducts resulting from exposure to aromatic amines. The lung has a high capacity to metabolize polycyclic aromatic hydrocarbons to carcinogens and very little activity of the specific P-450 (1a2) responsible to the activation of aromatic amines to carcinogenic forms [79] (F. Kadlubar, personal communication). In addition, P-450 1a2 has been shown to be inhibited by cigarette smoke condensate [80].

In this regard, Geneste *et al.* [81] have very recently shown that nuclease P_1 -enhanced DNA adducts were elevated in current smokers and, in this group, there was a significant positive correlation between aryl hydrocarbon hydroxylase activity and total carcinogen-DNA adducts.

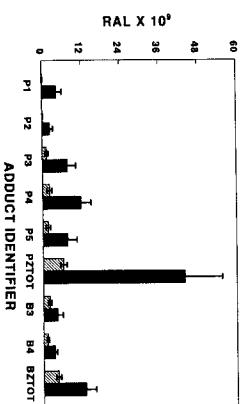


Fig. 11. Mean levels of smoking-related carcinogen-DNA adducts in surgical samples of human lung from smokers and non-smokers. Adduct identifiers are given on the abscissa, where P indicates a nuclease P_1 -enhanced adduct and B and n -butanol-extracted adduct. PZTOT and BZTOT refer to the total adducts within the diagonal zones. Bars indicate the standard error of the mean. All comparisons for carcinogen-DNA adducts between smokers and non-smokers shown are statistically significant at the 0.05 level.

4.1.2. Tobacco smoking and the urinary bladder

The urinary bladder is the other target for tobacco smoke carcinogenicity. It has been estimated variously that smokers have from 2-9 times greater risk for the development of urinary bladder cancer than non-smokers [75]. We have stud-

ied the levels of carcinogen-DNA adducts in conal biopsies of men in an attempt to determine whether tobacco smoking is related to increased carcinogen-DNA adduct levels [60]. Chromatograms from an ex-smoker and a current smoker are compared in Fig. 11. Note the appearance of



Fig. 12. Autoradiograms of ^{32}P -postlabeled DNA samples from an ex-smoker (A and B) who quit twenty years prior to sampling, and a current smoker (C and D). *n*-Butanol-extracted samples are given in A and D, while the nuclease P_1 enhanced samples are given in B and C [60].

diffuse zones of activity in the autoradiograms for the current smokers, and the distinct adduct spots near and within these zones. Mean *n*-butanol-extracted carcinogen-DNA adduct levels for smokers and non-smokers are given in Fig. 12. In the urinary bladder, as opposed to the lung, the levels of these *n*-butanol-extracted carcinogen-DNA adducts seem a more sensitive marker. Thus, it seems, from our studies of lung and bladder, that the polycyclic aromatic hydrocarbons in tobacco smoke act locally at a site of rapid metabolism in the lung, while the aromatic amines in tobacco smoke are absorbed systemically. These compounds are metabolized by the liver, then re-enter the bloodstream as the activated N-hydroxylated forms which are capable of binding to urothelia following renal filtration. We also saw that the increase in mean carcinogen-DNA adduct levels in the bladder approximately mirrored in the increased cancer risk for tobacco smoking in that organ. Smoking-related carcinogen-DNA adducts were increased 1.6- to 9-fold in this study. We found that adduct B4 shared chromatographic identity in several thin-layer ion-exchange systems with the major adduct from DNA that was reacted with N-hydroxy-4-aminobiphenyl *in vitro*. Modifying the methods of Schneider *et al.* [74] by the addition of citric acid to the elution buffer, several adducts (including adduct B4) were eluted from the thin layers and injected as bisphosphates on a reversed-

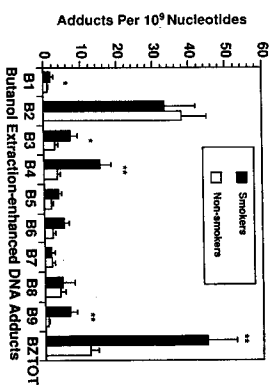


Fig. 13. Mean levels (and standard errors of the means) of *n*-butanol-extracted carcinogen-DNA adducts in the urinary bladder of smokers and non-smokers. Statistically significant differences are indicated by * ($p \leq 0.05$) or ** ($p \leq 0.001$) [60].

phase HPLC system [60]. It was found that adduct B4, but not others, co-chromatographed synthetic N-deoxyguanosin-8- γ -[4-aminobiphenyl] standard (Fig. 13). These data strongly implicate 4-aminobiphenyl as a major cause of carcinogen-DNA adducts in the human urinary bladder.

4.1.3. Tobacco smoking and the oral cavity

The oral cavity is thought to be another target tissue for tobacco smoke carcinogenicity [82]. Smokers are estimated to be at 6-fold increased risk. There is also strong evidence for a synergy between smoking and alcohol consumption in oral cancer, as the combination increases risk to about 17-fold [83]. There is cytogenetic evidence indicating that the oral mucosa cells of smokers are exposed to clastogens, as an increase in micronuclei was observed [84]. However, the evidence suggests that carcinogen-DNA adducts detectable by ^{32}P -postlabeling are not strongly associated with exposure in this tissue. Neither Dunn *et al.* [63], nor Chacko and Gupta [85] were able to detect any smoking-related carcinogen-DNA adduct in sampled oral mucosa. Foiles *et al.* [86] did report a significant elevation of carcinogen-DNA adduct levels in the oral mucosa of current cigarette smokers. Persons who reported regular alcohol consumption had a significantly lower level of DNA adducts than non-drinkers. Unfortunately, the sample size was small, not allowing tests of the interaction between the two exposures. To date, there have been no studies determining the levels of nuclease P_1 -enhanced adducts in this tissue.

The carcinogen-DNA adduct levels in a variety of tissue of tobacco smokers was determined by Randerath *et al.* [87] who saw the highest levels in heart muscle and lung, as well as kidney, aorta and bladder.

The skin is a target tissue for exposure to several mixtures known to contain polycyclic aromatic hydrocarbons [88]. Two recent case studies have shown that psoriasis patients treated with coal or juniper tar had increased levels of carcinogen-DNA adducts in skin biopsies [89,90].

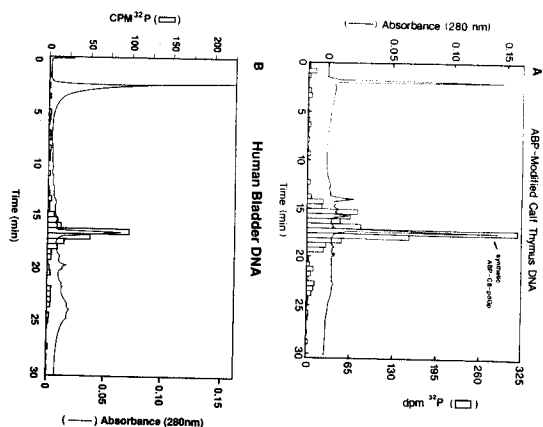


Fig. 14. (A) HPLC analysis of an *in vitro* modified 4-aminophenyl DNA that was ^{32}P -postlabelled and eluted from the chromatogram. A tritiated synthetic marker was added prior to injection and the absorbance at 280 nm follows this marker. (B) HPLC of human urinary bladder DNA treated in a similar fashion. The absorbance at 280 nm of the tritiated marker is given along with the ^{32}P from the eluted sample. Tritium data are not shown for the sake of clarity; however, this activity eluted in the same fraction in both cases [60].

4.1.4. Metabolism as a source of inter-individual variability

Highly correlated dose-response curves are the rule in animal experimental studies [14]. Several factors can confound similar findings in human studies. Often, there are problems with self-reports or exposure monitors which do not integrate exposure of the same period as the biomarker. On the other hand, the human population is highly variable in response. A 50-fold variation in the ability of human lungs to metabolize benzo[a]pyrene was recently reported [79,91]. Several investigators have seen significant correlation between reported current smoking and carcinogen DNA adducts [60,76]. However,

there is often a significant spread in the data. Fig. 14 shows the dose-response relationship in our recent study of the urinary bladder. Note the variability in individual response. In addition, none of the other smoking-related adducts were correlated significantly with dose. This suggests to us that individual factors are at play which strongly modify the effect of dose. In an important study, Geneste *et al.* [81] found that total carcinogen-DNA adducts in the lung were positively correlated with aryl hydrocarbon hydroxylase (P-450) activity, showing that drug metabolism is a potent effect modifier. In a preliminary report, we have seen that there is an inverse correlation between glutathione S-transferase μ activity and total nucleic acid adducts in the human lung [92]. In both cases there were extreme effects, suggesting the possibility for highly sensitive and resistant populations.

4.1.5. Passive smoking

Passive smoking has been reported recently to be associated with increased cancer risk [93,94]. Randerath *et al.* [95] noted that passive smoking was involved in two of three non-smokers who were positive for a smoking-related adduct in the lung. Similarly, in the bladder, we have noted that those non-smokers who reported passive smoking exposure had the highest levels of smoking-related DNA adducts [60]. While these reports are tantalizing, they are based on very small samples. An experimental study by Holz *et al.* [96] saw no effect in passive smokers. However, this study was flawed by limited exposure and analysis of a non-target tissue (see below). Further studies on this issue should be conducted to determine if these preliminary findings are valid.

4.1.6. Exposure intervention

Another issue in evaluating ^{32}P -postlabelling as a method for carcinogen-DNA adduct analysis in humans is that of intervention and its effects on marker levels. The risk of tobacco-induced lung and bladder cancer decreases with time when an individual quits the habit [75]. However, most epidemiological studies show that the risk does not return to that of never-smokers. For ex-

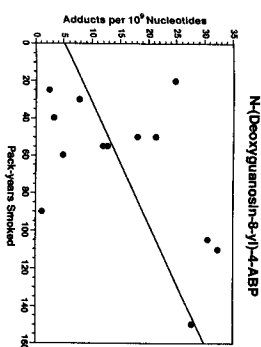


Fig. 15. Relationship between self-reported tobacco smoke exposure and levels of N-(deoxyguanosin-8-yl)-4-aminophenyl in urinary bladder samples of tobacco smokers.

ample, in the urinary bladder, one study showed that ex-smokers were at an almost 2-fold increased risk, while smokers were about 5 times more likely to develop cancer [97].

Several of the studies cited above have examined the effects of smoking cessation. Phillips *et al.* [76] reported an intermediate level of DNA adducts in a small group of ex-smokers. In the same tissue, Randerath *et al.* [98] saw that ex-smokers had carcinogen-DNA adduct levels very similar to that of the non-smokers. Again, however, the sample was very small. Fig. 16 compares the levels of *n*-butanol-extracted carcinogen-DNA adducts in urinary bladder between twenty ex-smokers and nine never-smokers [60]. There were no statistically significant differences between these groups. Since carcinogen-DNA adducts are diluted by cell replication, adduct levels will decrease as the cells normally divide; however, mutations induced will persist. We believe that mutations formed during the smoking tenure add to the total number of "hits" a cell receives and are responsible for the residual excess risk of urinary bladder cancer seen in ex-smokers. As noted in the initial report, this finding may somewhat limit the utility of carcinogen-DNA adduct analysis in risk estimates of individuals who are no longer exposed, because their risk will likely be underestimated [60]. The importance of doing evaluations and interventions early in the exposure history is manifest.

These data also address another issue, namely,

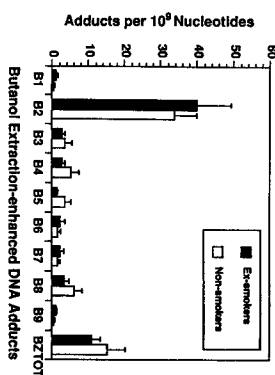


Fig. 16. Levels of *n*-butanol-extracted DNA adducts in the urinary bladders of ex- and never-smokers [60].

the suggestion that carcinogens (or their metabolites) interfere with the results of ^{32}P -postlabelling. It has been shown that, at high concentrations and in the absence of DNA, certain carcinogen metabolites are substrates for the polynucleotide kinase-catalyzed labelling reactions and are detectable by the chromatographic system. Therefore, these metabolites might be responsible for some of the "DNA adducts" seen with ^{32}P -postlabelling, particularly, the diagonal zone of activity [33]. However, recent data have shown that the residence time of carcinogens bound to particles in the lung is very short [99,100]. It is also well known that parent compounds and metabolites disappear rapidly from the body—in a matter of days—when exposure is terminated [101]. Adduct levels are, however, persistent. Therefore, it would seem very unlikely that metabolite labelling is a factor in human studies.

Carcinogen-DNA adducts, as determined by ^{32}P -postlabelling, are related to tobacco smoking and are significantly increased in target organs (lung and urinary bladder). The degree to which the reported range of adduct level increases reflects the range of excess cancer risks provided by epidemiological studies for those tissues is remarkable. In these target tissues there is often a dose-response evident, although individual differences in drug metabolism and detoxication are strong effect modifiers [81,92]. These data indicate that carcinogen-DNA adduct analysis by ^{32}P -postlabelling can integrate for individual re-

sponse factors and have value in human risk assessment. When persons quit smoking, their carcinogen DNA adduct levels decrease. There have also been two anecdotal reports suggesting that, at least in the target organs, ^{32}P -postlabelling may be sensitive enough to discern an effect of passive smoking exposure.

4.2. Non-invasive biomonitoring approaches

The above studies provide strong evidence that the magnitude of biomarker (carcinogen-DNA adduct) response is concordant with the risk associated with various exposures to tobacco smoke and other agents. However, to be useful in human risk assessment and biological monitoring, it must be possible to obtain samples by relatively non-invasive means. Clearly, direct, non-invasive target organ analysis is not possible from internal organs such as lung, liver, or urinary bladder. The focus of research in this regard has been to explore the validity of surrogate tissues sources. There have been two general approaches to the problem. One approach has been to explore the use of blood lymphocytes as a surrogate tissue for all target organ effects. This approach is based on the ease of obtaining blood samples and the reasonable amount of cells and DNA available in these samples. Blood is also the central compartment, and any blood-borne metabolites could potentially interact with lymphocytes and their DNA. Because some leukocytes are usually non-dividing, they should also integrate the exposure over their lifespan.

4.2.1. Blood leukocytes

Studies investigating the response of blood leukocytes to the carcinogens in tobacco smoke have been disappointing, generally. Phillips and co-workers [102,103] saw that the mean level of nucleic acid-enhanced carcinogen-DNA adducts in a small group of smokers and non-smokers was identical, although there was more variation in the smokers. Jahnke *et al.* [104] reported very similar results with a larger population. Sveta and Hemminki [105], recognizing these data and the fact that the granulocytic fraction of leuko-

cytes (40-75% of the total) have a lifespan of about one day, while T-lymphocytes may live for several years, took a different approach and analyzed these cellular fractions separately. The overall leukocyte carcinogen-DNA adduct level was twice as high in this group of smokers over non-smokers; however, this difference was not statistically significant. When analyzing the cellular fractions, these authors found that the carcinogen-DNA adduct levels in lymphocytes of smokers were 2.4 times higher ($p \leq 0.05$) than the levels in their non-smoking counterparts, while the levels in the granulocytes was essentially identical for both groups. On the same note, Holz *et al.* [96] examined monocytes ($t_{1/2} = 8$ h) of experimentally exposed smokers before and after exposure and found that there was a qualitative difference in the autoradiograms with several smoking-related carcinogen-DNA adducts appearing after exposure. Notably, the smoking-related adduct pattern was often different for different individuals. Passive smoke exposure was said to have no effect, as non-smokers in the same room as the smokers reportedly did not show changes in their carcinogen-DNA adduct patterns. Tobacco smoke, although it is responsible for as much as 30% of all cancers, is not a potent carcinogen on a per dose basis. Years of exposure are often required to elicit tumors in humans and animals. It may not be surprising that brief exposures to passive smoke do not result in overt increases of carcinogen-DNA adducts, particularly in non-target cell type. These studies together indicate that only specific populations of leukocytes are responsive to the effects of tobacco smoke, making cell separation a virtual necessity when doing studies of this type.

Reddy and Randerath [106] investigated the relationship between carcinogen-DNA adduct levels in leukocytes and a number of internal target organs for several carcinogens and tobacco smoke condensate in an animal model. They found that the qualitative adduct pattern was similar in leukocytes and internal target organs for most single carcinogens. However, the levels in lymphocytes reflected target organ levels only for benzo[a]pyrene. For two other carcinogens,

dibenz[*a,h*]carbazole and safrole, the leukocyte levels were 22 and 51 times lower than in the target organ. In addition, no adducts were detected in leukocytes of the animals treated with tobacco smoke condensate, while the total ^{32}P -postlabelling nucleic acid adducts in the lungs were 1 per 10^7 unadducted nucleotides. We have also studied the relative DNA adduct levels in dogs treated with 4-aminobiphenyl and found that the carcinogen-DNA adduct in the leukocytes were from 50 to 80 times lower in these cells than in the bladder urothelium, and that there was no correlation between these levels for individual dogs (unpublished data).

4.2.1.1. Blood leukocytes and occupational studies. Because of the ease of obtaining samples, several very interesting occupational studies have been done using blood leukocytes. Leukocytes from Finnish foundry workers were analyzed by ^{32}P -postlabelling [107-109]. The carcinogen-DNA adduct levels in these cells were increased in those groups of workers who were exposed to the highest levels of polycyclic aromatic hydrocarbons. These were significantly different from controls, whereas the levels in workers with low exposure were not. In addition, workers returning from vacation had adduct levels one half as high as when they were working steadily [107]. Finally, while the animal experiments cited above suggested that the levels of benzo[a]pyrene-DNA adducts in leukocytes were reflective of the levels in the target organs, no adducts which shared chromatographic behavior with *in vitro* standards were seen conclusively in this work [107-109]. This suggests that the main exposures in this occupation are to agents other than benzo[a]pyrene.

Occupational exposures to coke oven emissions are associated with lung cancer [110]. Hemminki and co-workers [111-113] studied a group of these workers, local controls, and rural controls in Poland. They found that the levels of nucleic acid-enhanced carcinogen-DNA adducts were related to exposure and significantly elevated in the coke oven workers over the rural controls. Further, the workers employed in coke oven batteries displayed the highest levels, significantly greater than that of those who did no bat-

tery work. Astonishingly, the levels in the local controls (people in Silesia not working in coke ovens) were not statistically different from all coke oven workers, albeit somewhat lower. The levels in these "non-exposed" controls were equivalent to that reported in the Finnish foundries, above. Considering that these persons are exposed to lower environmental levels continuously, it may not be surprising that carcinogen-DNA adducts accumulate in people when the "ambient" levels are so high (reported to range to $0.05 \mu\text{g}/\text{m}^3$). These data also suggest that ^{32}P -postlabelling may be also useful in determining general environmental exposures, at least of the magnitude seen in this area. There was also no effect of tobacco smoking on adduct levels in this work.

Coal tar pitch is used in the production of the anodes in aluminum reduction. Significant exposure to coal tar pitch volatiles — known to be potent lung carcinogens — can occur when the electrodes are heated during electrolysis. Anodes can be pre-baked, or formed during the reductive process [114]. The former results in potential exposure to workers in the pre-bake plant, the latter in exposure to the aluminum workers. Schoket *et al.* [115] investigated the level of carcinogen-DNA adducts in lymphocytes of workers in two such plants. They found that adduct levels were increased in workers employed at one plant where hygienic conditions were poor, while levels were not increased in the second plant, where exposures were thought to be lower.

Roofers may also be exposed to significant levels of polycyclic aromatic hydrocarbons [116]. A blood leukocyte study was conducted to determine if this exposure resulted in increased covalent DNA modifications [117]. The levels of carcinogen-DNA adducts were elevated in roofers, as opposed to their controls. In the exposed group, smoking was not associated with the reported levels of adducts. However, in the controls, only the smokers showed detectable levels. One advantage of carcinogen-DNA adduct analysis, in common with all biological monitoring, is that exposure by all routes is considered. In this study,

only the amounts of polycyclic aromatic hydrocarbon and benzo[a]pyrene in skin wipes were correlated significantly with carcinogen-DNA adduct levels, while the levels of total polycyclic aromatic hydrocarbons and benzo[a]pyrene in air were not. This data reinforces the value of biological monitoring where the exposure route may be other than by air.

4.2.2. Exfoliated urothelial cells

We have attempted to develop an alternative approach to non-invasive monitoring for carcinogen-DNA adducts. Since many carcinogens are organotropic in their effects, generally due to specific distribution or metabolic parameters, we have concentrated on developing methods to monitor genetic changes in the target organs by monitoring the cells exfoliated from the organs and collected in body fluids. In particular, we have developed modifications of ^{32}P -postlabelling, so that carcinogen-DNA adduct levels in exfoliated urothelial cells can be measured [45]. These cells have long been used to detect the presence of urinary bladder malignancies. The number of cells exfoliated daily is fairly small (10^5 – 10^6); therefore, we reasoned that there would be no advantage to using adduct intensification procedures and risk the loss of particular adducts. Instead, we increased the amount of [^{32}P]ATP to the point that all available nucleotides would be labelled quantitatively, making this analysis, effectively, a variant of the standard ^{32}P -postlabelling conditions. We originally showed that these techniques would be useful in an animal model. We treated dogs 5 times per week with 5 mg/kg 4-aminobiphenyl orally and measured the adduct levels in the exfoliated urothelial cells [45]. We found that the levels increased with increasing dose and, after six weeks exposure, reached steady state at levels seen in the urinary bladder when the animal was sacrificed. This study was followed up by an investigation of the carcinogen-DNA adduct levels in the exfoliated urothelial cells of tobacco smokers [44]. It was found that the carcinogen-DNA adduct levels were higher in tobacco smokers than in non-smokers, but that the differences were not statistically significant.

nificant due, we think, to high levels of background activity in some non-smoking samples that were misinterpreted as carcinogen-DNA adducts. However, we found that there were statistically significant correlations in the tobacco smokers between certain smoking-related adducts and reported tobacco smoking, urinary mutagenicity and 4-aminobiphenyl-hemoglobin adducts. An adduct which co-chromatographed with the major adduct in a synthetic 4-aminobiphenyl-DNA standard was the marker best correlated with 4-aminobiphenyl-hemoglobin adducts in the same individual ($r = 0.601$, $p \leq 0.01$). This study demonstrates that the measurement of target organ carcinogen-DNA adducts will be possible for persons exposed to bladder carcinogens. We are currently working on reducing the amount of background radioactivity seen in some samples by reducing interfering contaminants in the cell pellets. We are looking forward to using these techniques in human occupational studies. Such non-invasive methods should be very useful in monitoring the kinetics of DNA damage in humans as well as in intervention studies. As a note in proof, Kaderlik *et al.* [118] have very recently reported the results of a case study involving a worker who was overexposed accidentally to methylene bis-2-chloroaniline (MOCA). Specifically, MOCA-DNA adducts were observed in the exfoliated urothelial cells following the exposure.

4.3. Assay and individual variation

4.3.1. Inter-laboratory variation

Quantitation is a major concern with ^{32}P -postlabelling. Adduct recoveries are variable. Relatively minor changes in protocols and techniques may introduce large differences in reported values. In addition, each laboratory has adopted its own ^{32}P -postlabelling "style". These factors tend to increase reservations about the absolute value of reported results. As mentioned above, these criticisms are legitimate. However, the results within any given study should be consistent and valid, provided methods were not changed during the course of the work.

Three laboratories analyzed the same leukocytes from iron foundry workers [119]. While there was a 3-fold range of the reported values for the mean values of study group and controls, the levels reported by each lab for individual persons were correlated. These correlations varied, ranging from 0.45 to 0.62, but all were significant at the 0.01 level. Similar results were seen in the study of Polish coke oven workers [111]. Again, there was a 2-fold difference in the reported adduct levels, but the values were correlated significantly ($r = 0.66$, $p \leq 0.01$).

^{32}P -Postlabelling results have also shown to be correlated with the results of immunoassay in human studies, although generally the reported levels are 2 to 10 times lower [77,111].

4.3.2. Repeated samples: sampling, run-to-run, and inter-individual variability

An issue in human monitoring that has not been adequately addressed is that of measurement accuracy, repeatability, and consistency. Since we are dealing with rare events using assays that are often pushed to near their limit of detection, the potential exists for misclassification, particularly when judgement is based on data from a single sample. The work of Savelle and Hemminki [105] is very important in this regard. These authors sampled smokers and non-smokers on two separate occasions three weeks apart and analyzed each sample on four separate occasions. Measuring levels in T-lymphocytes, they report that there were differences between the four independent analyses of the same sample, but these were not statistically significant ($p \leq 0.289$). However, the intra-individual and between-sample differences and their interaction were statistically significant ($p \leq 0.028$, $p \leq 0.002$ and $p \leq 0.009$, respectively). Very similar data were obtained from analysis of granulocytes, as well. These data are interesting and suggest, but do not prove, that there can be significant temporal differences in response even in cells which turn over very slowly.

^{32}P -Postlabelling has proven to be a valuable, versatile tool in ascertaining the effects of human exposure to carcinogens. The method has limita-

tions, which should be understood so as to minimize negative effects. One of the limitations of ^{32}P -postlabelling is the lack of a conclusive identification system. There have been rapid strides in increasing the sensitivity of MS which may allow it to be used in tandem with ^{32}P -postlabelling to provide positive identification of the carcinogen-DNA adducts in human samples.

5. ROLE OF MASS SPECTROMETRY IN CARCINOGEN-DNA ADDUCT ANALYSIS

5.1. General issues

As indicated from the ^{32}P -postlabelling discussion, analysis of carcinogen-DNA adducts from biological matrices is a challenging task which requires a method to have high sensitivity and specificity. MS is a highly specific method, and recent developments have increased sensitivity to the point where analysis of human DNA samples by this method may now yield fruitful results.

The common goal in DNA adduct analysis by MS is to measure the particular carcinogen adducted to DNA from biological matrices. These carcinogen-DNA adducts can be detected as the parent chemical compound released from DNA or as the adducted bases, nucleosides, or nucleotides. A more difficult task is to determine the type and position of carcinogen bonding to the DNA, for example, bonding through a specific nitrogen or oxygen on the base. As indicated, a method which could survey and measure different types of DNA adducts would be beneficial in determining the full impact of a carcinogenic insult. At this time, the goal of measuring a single carcinogen or DNA adduct from biological matrices is sufficiently challenging; however, as MS methods continue to advance, a survey method may be developed that could attain the goal of complete multi-analysis of different types of DNA adducts in biological matrices.

5.2. Analysis of human DNA adducts by mass spectrometry

Gas chromatography (GC) combined with MS

has been in existence for three decades. The typical ionization modes, electron impact (EI) and chemical ionization (CI), in the majority of cases do not have sufficient sensitivity for DNA adduct analysis. To enhance these limits and to allow for GC derivatization of the carcinogen and/or adduct was necessary. Mass spectral data was compared to fluorescence methods for the analysis of benzo[a]pyrene anti-diol-epoxide-DNA adducts in humans [120]. A selective ion monitoring (SIM) mode was utilized and the derivatization was performed with a mixture of trimethylchlorosilane, hexamethyldisilazane, and pyridine. Although the epoxide-DNA adduct was not quantitated by the GC-MS method, a synthetic sample at 100 pg could be detected easily. Nitroamine-DNA adducts in smokers and non-smokers were assayed by GC-MS with negative-ion chemical ionization (NICI) [121]. Acid hydrolysis of DNA generated 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB). The HPB detected concentration levels by GC-MS were approximately 1 fmol/mg DNA. Methylated bases were analyzed by GC-MS from urine using the SIM mode after derivatization with *N*-(tert-butyl dimethyl silyl)-*N*-methyltrifluoroacetamide [122]. Several excellent reviews have recently been published that discuss GC-MS methods for the quantitative analysis of DNA adducts [123], and analytical approaches for protein-carcinogen adducts via MS [124]. Many of the citations referenced in these review articles will not be repeated here; however, modern and novel MS techniques will be considered for their potential in carcinogen-DNA adduct analysis.

Fast atom bombardment (FAB) has been employed to measure 7-methylguanine in human urine via tandem mass spectrometry (MS-MS) with collisionally activated dissociation (CAD) [125]. Identification of alkylated DNA bases in urine was accomplished using MS-MS [126]. FAB ionization was utilized with deuterium exchange experiments. These experiments were capable of resolving ethylated guanine from *N*2-dimethylguanine.

5.3. Analysis of non-human and synthesized DNA adducts by mass spectrometry

Before proceeding with difficult and possibly expensive analysis of human subjects for DNA adducts, experiments can be conducted utilizing animal DNA or synthesized materials. Numerous studies have been performed to evaluate carcinogenic behavior of a compound in animals, and to evaluate the effectiveness of techniques for quantitation and characterization of carcinogen-DNA adducts.

DNA from calf thymus was subjected to γ -rays to investigate free radical-induced damage via analysis with GC-MS [127]. Trimethylsilylated derivatives were characterized by GC-MS with EI. SIM was also demonstrated for quantitative purposes, but no detection limits were reported. Electrophoretic labelling of nucleosides can enhance detection limits when GC-MS is utilized in an electron-capture negative ionization (ECNI) mode. Trainor *et al.* [128] have successfully used pentafluorobenzyl and similar derivatizing reagents for generating electrophoretic derivatives. Structural elucidation of deoxynucleosides was achieved with this technique using EI probe, and methane CI in the positive-ion mode. A comparison of ECNI with GC-MS and moving belt HPLC-MS was made [129]. A quantity of 100 pmol was examined by GC-MS for various derivatizing reagents with 5-hydroxymethyluracil, and the relative molar responses reported. Although the method has great potential for analysis, the electrophoretic derivatives were separated via HPLC. Hydrolysis can occur: it was noted that the hydrolysis half-lives of these derivatives ranged from one to twelve days. Chemical transformation of DNA adducts with GC-MS and ECNI-MS can also enhance the detection limits [130]. Reactions of potassium superoxide with DNA were discussed. Alkaline hydrolysis of 4-aminobiphenyl-DNA adducts followed by negative-ion GC-MS gave 56% recovery with observed adduct levels of 1 adduct in 10^8 in some instances [131].

The utility of FAB-MS to characterize DNA adducts has been demonstrated for 6-nitrochry-

sene and 6-aminochrysene after treating rat hepatocytes [50]. Oligonucleotides modified by carcinogens were examined by FAB-MS-MS [132]. Analyses were performed in negative-ion mode after modification with (\pm) *r*-7,8-dihydroxy-7,9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene.

Procedural steps have been set to identify DNA adducts by their CAD fragments after FAB ionization [133]. Picogram levels of an unknown polycyclic aromatic hydrocarbon nucleoside adduct were detected using FAB on a triple quadrupole MS-MS system [134]. As seen in Fig. 16, the constant neutral loss mass spectrum of an *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) derivative produced ions at m/z 484, 633, and 705 indicative of silyl losses, and 500 pg were detected. PAH-DNA adducts at levels of 200 pg were observed by FAB using a high-resolution four-sector MS-MS system and a 15-cm photodiode array detector [135].

HPLC with moving-belt interface using electrophoretic derivatization and NICI gave observed levels at 9.9 pg and 180 fg of respective nucleosides in an ECNI mode [136]. Thermospray MS and thermospray MS-MS were employed to examine the deoxyguanosine carcinogen adduct of 2-acetylaminofluorene [137]. The thermospray mass spectrum in Fig. 17 shows the intact nucleoside adduct with ions detected for the protonated and sodiated parent as well as for the aglycon. Because of the soft ionization mode of thermospray, the DNA adduct can be observed as an

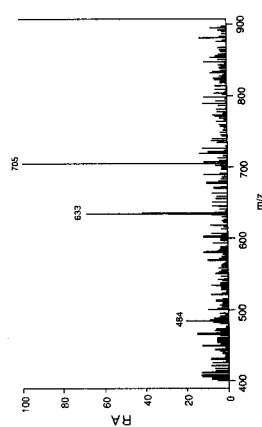


Fig. 17. Fast atom bombardment via constant neutral loss of MSTFA-derivatized *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene; molecular mass = 488; quantity observed is 500 pg.

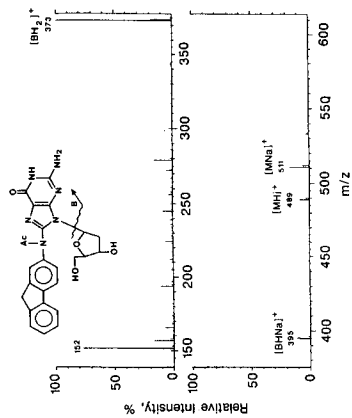


Fig. 18. Thermospray mass spectrum of *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene; molecular mass = 488.

intact species. Thermospray combined with HPLC was utilized to examine DNA adducts formed with mitomycin C, porfomycin, and thiotepta [138]. Thermospray ionization was combined with electrochemical oxidation to detect the 4-aminobiphenyl adduct of deoxyguanosine [139]. Increased ion intensities were noted via direct injection of the DNA adduct after electrochemical cleavage into the sugar, purine, and carcinogen components. Dynamic FAB with capillary electrophoresis was utilized to measure DNA adducts at levels of 75 pg [140]. The measurement was performed by low-energy MS-MS (triple quadrupole) and high-energy (magnetic sector) MS-MS.

Ion cyclotron resonance (ICR) mass spectrometers have been utilized to investigate modified and unmodified bases and nucleosides [141]. Ionization was performed with a self-chemical ionization mode. The site and extent of alkylation of DNA by carcinogens was studied by MS-MS with desorption chemical ionization [142]. Use of the MS-MS method indicated an enhancement in specificity and sensitivity for analysis purposes. Laser desorption time-of-flight (LD-TOF) MS was reported to be capable of detecting 20 fmol of material [143]. Differentiation of bases and carcinogens could be made with the LD-TOF measurement. Carcinogenic polycyclic aro-

matic hydrocarbons adducted to DNA were analyzed by laser desorption and liquid metal ion-secondary ionization via TOF-MS and compared [144]. Using cinnamic acid derivatives in the matrix, levels of 1.5 pmol were observed.

The analysis of DNA adducts at low levels in biological matrices involves tedious preparation and sensitive analytical procedures. New MS ionization modes and instrumental designs can push back the frontiers of carcinogen-DNA adduct analysis. Other ionization modes such as electrospray need to be evaluated for DNA adduct measurements. Furthermore, these newer methods may actually achieve routine analysis of adducted oligonucleotides. The future looks promising, but challenging, for this area of biomarker risk assessment. It is believed that chromatography will continue to play an important role in developing these methods, and novel MS ionization modes, mass analyzer designs, and detection schemes will definitely enhance the capability of detecting and identifying DNA adducts.

6. CONCLUSIONS

The analysis of carcinogen-DNA adducts by ^{32}P -postlabelling has tremendously increased our knowledge concerning the biologic effects of these lesions. These methods have proven to be sensitive to carcinogenic exposure and interventions, and have been useful in the development of non-invasive biological monitoring methods. The methods have also shown themselves to be responsive to individual variability in metabolism. While further validations are needed for individual risk assessment, it would seem that groups with higher levels of carcinogen-DNA adducts demonstrated by ^{32}P -postlabelling may indeed be at increased risk, strongly suggesting that intervention be enacted. The utility of ^{32}P -postlabelling to cancer prevention is promising.

The major weakness of ^{32}P -postlabelling is lack of specific qualitative information regarding identity of the carcinogen-DNA adducts. Presently, ^{32}P -postlabelling has been combined with HPLC and synchronous fluorescence to characterize specific carcinogen-DNA adducts. MS al-

so has significant potential to augment the information obtained by other techniques. The sensitivity of MS has increased greatly in the past few years and, if these trends continue, these methods will greatly increase in importance in the analysis of carcinogen-DNA adducts in humans.

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