Methods for Assessing the Effects of Mixtures of Chemicals
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Chemical Dosimetry in Biological Monitoring of Exposure to Toxic Chemicals

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1 INTRODUCTION

Biological monitoring of exposure to chemicals has the objective of defining the so-called 'internal dose' or 'internal exposure level', and has been attempted by a variety of experimental approaches. Two categorical types of measurements can be made for this purpose: (1) measurement of concentrations of chemicals (or their metabolites) in body fluids or excreta; or (2) measurement of toxicological effects (e.g. mutation, sister chromatid exchange, chromosome aberrations, etc.) induced by chemicals in cells and tissues of exposed individuals. As the second approach is the subject of sections 3 and 4, only methodology of the first category will be discussed here.

Quantification of concentrations of individual chemicals or their metabolic derivatives can in principle be carried out on any biological medium that can be obtained for analysis. Thus, measurements could be made in blood, urine, faeces, expired air, adipose tissue, saliva, breast milk, semen, or samples of tissue obtained through biopsy or autopsy. A variety of analytical methodologies have been developed involving direct chemical analysis, immunological analysis, or bioassay depending upon such properties as mutagenicity of chemicals or their metabolites. With the recognition that many compounds require metabolic activation to reactive derivatives to express their toxicological effects, an additional strategy for chemical dosimetry has developed which is based upon the detection and quantitative determination of covalently bound derivatives formed between activated chemicals and cellular macromolecules such as nucleic acids and proteins. The purpose of this review is to provide a summary of currently available methodology for dosimetry applicable to the problem of assessment of human health risk posed by environmental chemicals.

Some general comments are appropriate before specific methodologies are considered. Biological monitoring of exposure to chemicals may have several objectives which may in turn require specific methodological adaptations for different situations. Chemical dosimetry can be used, for example, to insure that current or past exposure does not entail an unacceptable health risk, or to detect potentially excessive exposure before the occurrence of detectable adverse health

effects. The results of a biological monitoring programme can be interpreted on an individual basis, and thus used to estimate for that individual the amount of chemical(s) absorbed during a specific time interval or the amount retained or bound to critical sites. They may also be used to characterize community exposure conditions by analysing results obtained in groups of individuals within the general population. In this respect, biological monitoring is complementary to environmental monitoring, but has certain advantages in evaluating internal dose and estimating health risks (Lauwerys, 1983).

The greatest advantage of biological monitoring is that the data obtained are more directly related to adverse effects than any environmental measurement, and thus provide a better estimate of risk than ambient monitoring. Biological monitoring takes into account absorption by all routes, integrates exposure from all sources, and therefore can be used as a basis for an estimate of total risk from single or multiple chemicals.

Various forms of biological monitoring have long been employed in industrial hygiene. Elkins (1954) was among the first to advocate this approach as an essential element of industrial hygiene programmes, and was a pioneer in establishing correlations between exposure concentrations (i.e. mainly air levels) of industrial chemicals and their concentrations in body fluids. He went on (Elkins, 1967) to propose the establishment of biological threshold limit values for chemicals in biological specimens, comparable to the threshold limit value for substances in air which have been in use for many years. Several monographs and reviews have appeared which deal comprehensively with the general application of biological monitoring, especially in the industrial workplace (Lauwerys, 1983; Linch, 1974; Waritz, 1979).

There are many important considerations governing the kinds of specimens that are useful for biological monitoring. The primary variables include routine availability, metabolic profile of the specific chemical(s) of interest, route of exposure, time of sampling and characteristics of the analytical method to be employed. It is therefore difficult to formulate generalizations, but certain observations can be drawn from experience to date.

Urine is one of the most frequently analysed biological specimens, due to the relative ease of collection and the fact that it contains nearly all exogenous chemicals or their derivatives in amounts that are often proportional to the absorbed dose. Furthermore, quantitation of excretion rates is possible by correction of concentration data to specific gravity or creatinine of spot or timed samples. Faeces are less frequently studied, even though for many chemicals this represents a major excretory route. Collection and preservation are difficult, and few analytical methods have been developed for use on this medium. Furthermore, the capability of the gut microflora to metabolize xenobiotic chemicals introduces a high degree of complexity into the analytical process.

Blood has theoretical advantages for assessing internal dose, since concentrations of chemicals in blood should be highly correlated with exposure,

absorption, activation/inactivation and retention. It is also an optimum source of major proteins such as haemoglobin and albumin, as well as viable cells that can be used as a source of DNA or cultured *in vitro*. There are, however, disadvantages which limit availability and usefulness of blood samples, including the need for invasive techniques for collection and the low concentrations at which most xenobiotics are found in plasma.

Limited use has also been made of other body fluids including breast milk, saliva, and semen for purposes of biological monitoring, but these media have not been extensively exploited. Similarly, as discussed later, analyses of biopsy samples of adipose tissue have been used to assess exposure to certain classes of chlorinated hydrocarbons, but difficulty in obtaining samples of this and other tissues tends to limit their usefulness for large-scale surveys. Tissue samples obtained at autopsy also have limited availability owing to cultural or religious practices, and interpretation of data obtained from them is complicated by the confounding effects of uncontrolled postmortem interval before collection, and instability of chemicals or their metabolites, including macromolecular adducts in the postmortem state. A tissue with great potential usefulness in biological monitoring, but which has received relatively little attention up to now, is the placenta. The use of the placenta as a model for toxicology investigations has been discussed in a recent monograph (Beaconsfield and Birdwood, 1982).

Over the past decade, there has been greatly increased awareness of the large number of carcinogens and mutagens in the environment and concern over the potential health risks posed by their presence. Consequently, many research efforts have been devoted to the development of methodology for establishing the genotoxic properties of chemicals, and for their detection in the environment. Thus, much of the methodology to be discussed in the succeeding sections has to do with monitoring of exposure to genotoxic agents, i.e. carcinogens and mutagens which are thought to exert their effects through mechanisms initiated by covalent binding to DNA. Biological monitoring in the surveillance of exposure to genotoxic agents has been the subject of several recent reviews (e.g. Bridges *et al.*, 1982; Vainio *et al.*, 1981, 1983).

2 DETECTION OF CHEMICAL OR METABOLITE IN BODY FLUIDS OR EXCRETA

2.1 Chemical Analysis

Prevention of excessive exposure to chemicals in industry has been approached traditionally by setting standards for the concentration of compounds in ambient air. Air monitoring has therefore constituted the principal means of assessing exposure. This method obviously takes into account only exposure via the pulmonary route and does not estimate true uptake. It was these shortcomings that have stimulated much research aimed at development of biological

monitoring methods for evaluating individual exposure. Over the past two decades methods have been developed for many substances representing a variety of chemical classes to which people are exposed, principally in the workplace. Lauwerys (1983) has recently summarized these methods from the standpoint of their usefulness in biological monitoring programmes. Table 1 lists those chemicals for which methodology was considered to be sufficiently well developed for application in worker surveillance programmes. Lauwerys (1983) as well as Baselt (1980) and Linch (1974) describe the analytical methodology as well as additional pertinent information including representative values for each of the chemicals in body fluids. These will not be discussed further here.

In addition to their use in monitoring programmes in the workplace, a few of these methods have been applied to population studies involving large numbers

Table 1 Chemicals for which useful biological monitoring methods and hazardous exposure data exist (Lauwerys, 1983)

Inorganic and organometallic substances	
Arsenic (inorganic)	Mercury (inorganic)
Cadmium	Mercury, methyl
Carbon disulphide	Nickel (soluble compounds)
Chromium (soluble compounds)	Selenium
Fluoride	Thallium
Lead	Uranium
Lead, tetraalkyl	
Unsubstituted aliphatic and alicyclic hydrocarbons	
N-Hexane	
2-Methylpentane	
3-Methylpentane	
Cyclohexane	
Unsubstituted aromatic hydrocarbons	
Benzene	Isopropylbenzene (cumene)
Toluene	Styrene
Xylene	Biphenyl
Ethylbenzene	
Halogenated hydrocarbons	
Carbon tetrachloride	Tetrachloroethylene
Chloroform	Vinyl chloride
Dichloromethane	Halothane
1,1,1-Trichloroethane	Polychlorinated biphenyl
Trichloroethylene	
Amino and nitro derivatives	
Ethyleneglycol dinitrate	
Aniline	
Benzidine-derived azo compounds	
Monoacetylbenzidine-derived azo compounds	

Table 1 (*Cont'd*)

Alcohols, glycols and derivatives
- Methanol
- Ethylene glycol
- Diethylene glycol
- Ethylene glycol monomethyl ether (methyl cellosolve)
- Propylene glycol monomethyl ether
- Dioxane

Ketones
- Methylethyl ketone
- Methylbutyl ketone
- Acetone

Aldehydes
- Formaldehyde
- Furfural

Amides
- Dimethylformamide
- Dimethylacetamide

Phenols
- Phenol
- *p-tert*-Butylphenol

Asphyxiants
- Carbon monoxide
- Cyanide
- Acrylonitrile
- Methaemoglobin-forming agents

Pesticides
- Lindane
- DDT
- Endrin
- Organophosphorus insecticides
- Parathion
- Carbaryl
- 2-Isopropyloxyphenyl-*N*-methyl carbonate
- 2,4-D
- 2,4,5-T
- DNOC
- Pentachlorophenol

Hormones
- Diethylstilboestrol

of subjects. For example, chlorinated hydrocarbons have been extensively studied with respect to their storage and accumulation in adipose tissue and other body compartments. Hayes (1975) summarized the very extensive literature concerning levels of DDT and its derivatives in adipose tissue and other body compartments in large numbers of subjects studied over a period of two decades. In a related area, analysis of human milk has been employed to determine

exposure to chlorinated pesticides (Savage *et al.*, 1981) and to polychlorinated biphenyls (Rogan and Gladen, 1983; Rogan *et al.*, 1983).

With reference specifically to environmental carcinogens and mutagens, the field is in a more primitive state of development. The main focus of research has been on the development of analytical methods for detection of carcinogens that can occur as contaminants of food, and consequently most of the existing methods were intended for the purpose of food analysis. Currently available methods are summarized in Table 2, which includes indications of the principal methods of detection together with approximate detection limits. For the most part, the suitability of these methods for analysis of media other than foods has not been evaluated, and in many instances they cannot be used for analysis of metabolites without extensive modifications. They are also characterized by the requirement for relatively sophisticated equipment which limits their utility for surveillance studies requiring analysis of a large number of samples.

A larger number of methods are available for detection of aflatoxins than for any other class of carcinogen. In this case, methods originally developed for analysis of oilseeds and grains, such as those listed in Table 2, have been adapted for analysis of edible tissues and milk of animals in order to minimize human exposure through residues of the parent compounds or metabolites. These methods have also been applied in analysis of tissues of people suspected of having been exposed to aflatoxins. Although many methodological variations have been published, representative current versions can be summarized as follows.

Aflatoxins have been found in organs and tissues of beef, swine, and poultry that have ingested aflatoxin-contaminated feeds. A method for detecting aflatoxin B_1 and its primary metabolite M_1 using two-dimensional thin-layer chromatography (TLC) and fluorimetric densitometry has been reported to have a detection limit of 0.1 ng/g of tissue (Stubblefield and Shotwell, 1981). Another method devised for the same purpose but involving high-performance liquid chromatography (HPLC) with fluorimetric detection had a detection limit of 0.05 ng/g (Gregory and Manley, 1981).

When dairy cattle ingest aflatoxin B_1 via their feed, a significant fraction of the ingested dose appears in milk as aflatoxin M_1, a hydroxylated derivative. Thus, sensitive methods have been developed for analysis of this compound in milk, including those based on TLC separation with fluorescence detection (Gauch *et al.*, 1979; LaFont and Siriwardana, 1981; Patterson *et al.*, 1978; Stubblefield, 1979) which have detection limits in the range of 0.05–0.1 ng/g. HPLC methods of comparable sensitivity have recently been reported (Beebe and Takahashi, 1980; Fremy and Boursier, 1981).

Analyses of human tissues and body fluids have been carried out applying similar methodology in studies attempting to relate aflatoxin exposure to disease in human populations. In evaluating the putative aetiological role of aflatoxins in

Table 2 Detection limits of analytical methods for some carcinogens in environmental media

Carcinogen	Detection method[b]	Detection limit (ng)	References
N-Nitroso compounds			Walker *et al.* (1980)
Volatile nitrosamines	GC/chemi-luminescence	0.5–3	
Non-volatile nitrosamines	None		
Polycyclic aromatic hydrocarbons[a]			Horwitz (1980)
Benzo[*a*]pyrene	HPLC	2–10	
3–6 Ring PAH	HPLC/fluor-escence	20–200	
	GC/FID	2–20	
Diethylstilboestrol[a]	GC/MS	50–100	Horwitz (1980)
Aromatic amines[a]			IARC (1972, 1974, 1982)
Benzidine	GC/EC	1	
	HPLC	180	
AAF	GC/fluorescence	5–25	
Trp-P-1	GC/MS	2.5–25	
2-Naphthylamine	GC/EC	0.5	
Vinyl chloride	GC	1–5	Egan (1978)
Mycotoxins			Horwitz (1980)
Aflatoxin B_1	TLC	0.5–2.0	
	HPLC	2.5	
Aflatoxin B_2	TLC	0.25–1	
Aflatoxin G_1	TLC	0.5–2	
Aflatoxin G_2	TLC	0.25–1	
Aflatoxin M_1	TLC	0.5–2	
Sterigmatocystin	TLC	10–20	

[a] Methods not directly applicable for metabolites of parent substance.

[b] *Abbreviations*: GC = gas chromatography, HPLC = high-performance liquid chromatography, FID = field-ionization detection, MS = mass spectrometry, TLC = thin-layer chromatography.

cases of Reye's syndrome, an acute highly fatal disease of children, aflatoxin B_1 was detected in samples of liver, stool, brain, and kidney at levels in the order of 100 ng/g through application of a TLC method with fluorescence detection (Becroft and Webster, 1972; Shank *et al.*, 1971). These results were extended and substantiated in more recent studies in which HPLC separation with fluorescence detection was used (Nelson *et al.*, 1980; Ryan *et al.*, 1979; Siraj *et al.*, 1981). Aflatoxins have also been detected in human tumours of the liver (Onyemelukwe

et al., 1980; Stora *et al.*, 1981) and lung (Dvorackova *et al.*, 1981) with the TLC methodology listed in Table 2.

Studies in human populations consuming aflatoxin-contaminated peanut butter revealed the presence of aflatoxin M_1 in urine, as detected by TLC analysis (Campbell *et al.*, 1970), and aflatoxins were also found in the urine of Sudanese children suffering from kwashiorkor, examined by HPLC analysis (Hendrickse *et al.*, 1982). Screening methods for detection of aflatoxin and metabolites in human urine (Lovelace *et al.*, 1982) and serum (Lamplugh, 1983) have been reported.

Only a small number of chemical methods for other specific chemical carcinogens have been developed. Matsumoto *et al.* (1981) have developed a method for determination of the carcinogen methylazoxymethanol-*β-D*-glucosiduronic acid in rat bile and urine. Although the method produces good recovery of carcinogen, it is relatively insensitive. Pylypiw and Harrington (1981) reported a method for detecting *N*-nitroso-*N*-methylaniline in urine and serum at levels of 0.01–0.001 ppm.

2.2 Immunological Analysis

The rapidly developing field of immunoassay has up to now principally been applied to the detection of covalent adducts of carcinogens with DNA, as will be discussed subsequently. However, methods based on radioimmunoassays have been reported for two carcinogens.

Johnson *et al.* (1980) developed a radioimmunoassay procedure for 4-acetamidobiphenyl, a metabolite of the carcinogen 4-aminobiphenyl, in urine. Rabbit polyclonal antibodies with high affinity (2.8×10^8 litres/mol) were produced which were capable of detecting the metabolite at levels of about 1 ng (4.8 pmol) in human urine.

Sizaret *et al.* (1982) similarly developed rabbit polyclonal antibodies which not only detected aflatoxin B_1 but also cross-reacted with various aflatoxin metabolites including M_1, the principal urinary excretory product discussed above. The radioimmunoassay developed using these antibodies was capable of following urinary metabolites of aflatoxin administered to rats at doses of 600 pmol or less. The authors thus propose that the assay would be suitable for use in human population studies.

3 DETECTION OF MUTAGENS IN BODY FLUIDS OR EXCRETA

A presumption of genetic hazard can be made if mutagens are found to be circulating in blood or are present in other body fluids. The availability of well-characterized mutation assay systems utilizing microbial cells or mammalian cells in culture makes it feasible to detect and quantify the presence and amounts of mutagenic substances present in body fluids. Further indication of their

character can be gained by determination of the requirement for metabolic activation for mutagenicity.

Although it is not valid to equate mutagenicity of body fluids with actual mutation in the host, it is reasonable to assume that such persons are at elevated risk. A further advantage of this approach is that interpretable data can be obtained in single individuals, making the test especially useful in assessing exposure of small groups.

Potential confounding sources of mutagenic activity in body fluid samples include drugs, food constituents, beverages and life-style factors such as smoking and cosmetics. In some cases, mutagens are excreted in conjugated forms which may require hydrolysis before assay. Certain chemical classes of mutagens (e.g. nitrosamines) may have short half-lives in blood and excreta and therefore be undetectable. Further developmental work will be required to determine which classes of occupational hazards can be detected with mutagenesis assays on body fluids.

Although many mutagenesis bioassay systems are now available utilizing eucaryotic as well as procaryotic cells, practically all of the existing literature concerns detection of bacterial mutagens in urine of exposed persons. The nature of the available findings is illustrated in Table 3.

One of the advantages of the urine-analysis approach is that the samples can be processed before testing. Organic compounds can be extracted and/or concentrated by a variety of techniques, and enzyme treatment will liberate

Table 3 Detection of mutagens in urine by microbial assays[a]

Exposure	Population	Response	Reference
Drugs			
Cytoxan	Patients	+	Siebert and Simon (1973)
Metronidazole	Patients	+	Legator *et al.* (1975)
Niridazole	Patients	+	Legator *et al.* (1975)
Cytostatic drugs	Nurses	+	Falck *et al.* (1979)
Cigarette smoking			
	General	+	Yamasaki and Ames (1977)
	General	+	Putzrath *et al.* (1981)
	Workers	+	Dolara *et al.* (1981)
	Workers	+	Van Doorn *et al.* (1979)
Occupational exposure			
Styrene production	Workers	−	Cerna and Dobias, (1980)
Rubber manufacture	Workers	+	Falck *et al.* (1980)
Coke manufacture	Workers	−	Moller and Dybing (1980)
Epichlorohydrin	Workers	+	Legator *et al.* (1978)
Chemical manufacture	Workers	+	Dolara *et al.* (1981)

[a] Modified from Bloom (1981).

conjugated chemical species. These technical approaches have not yet found wide application, but their feasibility is illustrated by the report of Putzrath *et al.* (1981), who found that if the mutagenic activity of smokers' urine was concentrated by passage through a XAD-2 resin column it could be separated from about 90% of non-mutagenic material by subsequent dichloromethane extraction. Separation of these extractables on HPLC revealed multiple non-polar fractions which were activated to mutagenicity by methylcholanthrene-induced rat liver microsomes. They also remained stable at −20°C for at least three months.

4 DETECTION OF COVALENT ADDUCTS

The rationale underlying the strategy of chemical dosimetry by determining levels of derivatives covalently bound to cellular macromolecules is based on current understanding of the mode of action of genotoxic carcinogens and mutagens, as summarized schematically in Figure 1. Chemicals that are active as carcinogens and mutagens have electrophilic properties, or are metabolically converted into electrophiles. These reactive forms of the compounds undergo attack by nucleophilic centres in nucleic acids and proteins, resulting in the formation of covalent adducts. Particular emphasis has been placed on DNA adducts, since these are postulated to represent initiating events leading to mutation and/or malignant transformation. Indeed, it has been empirically established that the carcinogenic potency of a large number of chemicals bears a proportionality to their ability to bind to DNA—the so-called 'covalent binding index'—when reacted *in vivo* with DNA (Lutz, 1979). Covalent adducts formed in RNA and proteins have no putative mechanistic role in carcinogenesis, but are expected to relate quantitatively to total exposure and activation, and therefore represent dosimeters for both exposure and activating capability.

In the case of DNA and RNA, it is known that covalent adducts have differing levels of stability. Some are removed spontaneously through depurination, for example, whereas others are removed enzymatically in the process of DNA repair. A few are known to remain in DNA for long periods of time. In the few experimental models in which appropriate measurements have been made, adducts removed spontaneously or enzymatically from DNA are excreted in urine in amounts that are reflective of total binding levels. In contrast, those protein adducts that have been examined are stable over the lifespan of the protein, and therefore accumulate over time to give an integrated measure of exposure.

These properties collectively form the basis for several complementary approaches to development of chemical dosimeters, each with its own characteristics, providing different kinds of information. Measurement of DNA adducts *in situ* in the DNA of cells should give the most direct evidence of genotoxic exposure. Measurement of DNA adducts (or products of them) in

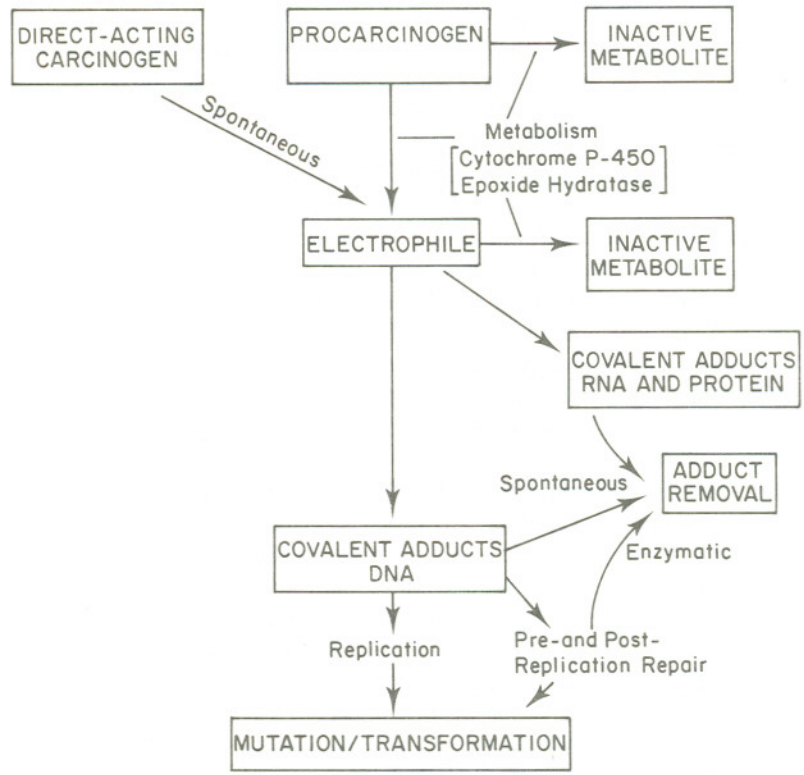

Figure 1 Formation of covalent adducts of carcinogens and mutagens

urine should give an indication of total, recent exposure. Protein adducts, by contrast, should provide an index of total exposure integrated over the lifespan of the target proteins. This rationale is reflected in the experimental approaches summarized in the following sections.

4.1 Protein Adducts

Ehrenberg and Osterman-Golkar (1980) reviewed the rationale and technical requirements for the use of protein alkylation for detecting mutagenic agents. Important among these requirements is that exposure must result in the formation of stable covalent derivatives of amino acids for which assay methods of adequate sensitivity and specificity can be devised. Further, the target protein should be found in easily accessible fluids (e.g. blood), and should be present in concentrations adequate to provide sufficient material for analysis. Among the amino acids likely to be alkylated following exposure are cysteine, histidine, the

N-terminal amino acid of the protein, and lysine. Although any protein could be used for monitoring alkylated derivatives of these amino acids, haemoglobin was suggested by Osterman-Golkar *et al.* (1976) as a suitable dose-monitoring protein, and virtually all of the available literature on this subject concerns studies of haemoglobin alkylation.

Osterman-Golkar *et al.* (1976) established the stability of alkylated residues in haemoglobin modified by ethylene oxide or dimethylnitrosamine, and the equivalence of the half-life of alkylation levels produced by a single dose to the lifespan of haemoglobin in the mouse. Alkylation of haemoglobin in mice treated with vinyl chloride was reported by the same investigators (Osterman-Golkar *et al.*, 1977). Segerback *et al.* (1978) further characterized the experimental model in mice treated acutely and chronically with the direct-acting alkylating agent methylmethane sulphonate in which they demonstrated the validity of the steady-state level of alkyl residues in haemoglobin as a measure of chronic repeated exposure.

Subsequently, Calleman *et al.* (1978) carried out a study of haemoglobin alkylation in people occupationally exposed to ethylene oxide. Blood samples were obtained from persons exposed to doses of ethylene oxide established through continuous air monitoring. Haemoglobin was analysed for the presence of *N*-3-(2-hydroxyethyl)histidine by mass spectrometry and by ion-exchange amino acid analysis. The authors concluded that the haemoglobin alkylation values accurately reflected exposure, and were in good agreement with data derived earlier for ethylene oxide in the mouse.

Farmer *et al.* (1980) have developed a high-resolution GC/MS method for estimating the production of *S*-methylcysteine in haemoglobin following exposure to methylating agents. This method was used to study *in vivo* alkylation of haemoglobin in rats dosed with methylmethane sulphonate (MMS), dimethylnitrosamine (DMN), and the antitumour agent 5-(3,3-dimethyl-1-triazeno)-imidazole-4-carboxamide (Bailey *et al.*, 1981). A linear dose–response curve for MMS was observed over a 100-fold dose range, but the dose–response curve for DMN was non-linear. No alkylation was observed with the antitumour agents, but it may have been overshadowed by a low level of naturally occurring *S*-methylcysteine which was found to be present in haemoglobin of the rat and 13 other animal species. These findings emphasize the importance of careful dose–response studies in animals for each compound for which human exposure data are to be sought by this approach. Farmer *et al.* (1982) have also devised a GC/MS method for the detection of hydroxypropylhistidine in haemoglobin as a measure of exposure to propylene oxide.

Pereira and Chang (1981) surveyed the ability of carcinogens and mutagens representing a broad spectrum of chemical classes to bind covalently to haemoglobin in rats. Animals were dosed with ^{14}C-labelled test compounds at levels of 0.1–10 μmol/kg body weight and blood was collected 24 hours later. Covalent binding was determined by analysis of purified haemoglobin for

Table 4 Covalent binding *in vivo* of ^{14}C-labelled carcinogens/mutagens to rat haemoglobin

Carcinogens	Binding index[a]
Direct-acting carcinogens	
Methylmethane sulphonate	3320
Methylnitrosourea	302
Ethylnitrosourea	217
Methylnitronitrosoguanidine	180
Carcinogens requiring metabolic activation	
Dimethylnitrosamine	697
Benzo[*a*]pyrene	181
3-Methylcholanthrene	165
Diethylnitrosamine	153
Benzene	144
Acetylaminofluorene	108
Dimethylbenzanthracene	99.8
Aflatoxin B_1	39.4
Aniline	24.8
Benzidine	22.5
Chloroform	14.2
Carbon tetrachloride	10.2

[a] Binding index: pmol bound/g haemoglobin/μmol/kg body weight. From Pereira and Chang (1981).

covalently bound radioactivity. Their results are summarized in Table 4, in which compounds are arranged within class in order of decreasing binding capability. All carcinogens/mutagens were found to form covalent adducts with haemoglobin, but the ability to do so varied over a wide range (binding index from 10.2 to 3322). It should be emphasized that the magnitude of this index is *not* reflective of potency as carcinogens for the rat. In those instances (11) in which the compounds were administered at more than one dose, the haemoglobin binding index was also dose related. The authors conclude, therefore, that this approach has potential applicability for dosimetry of some environmental carcinogens at ambient levels of exposure.

4.2 DNA Adducts

The scheme summarized in Figure 1 illustrates the scientific rationale for the measurement of DNA adducts in chemical dosimetry. It is apparent that two experimental avenues are available to obtain information on levels of DNA adducts formed in a given set of circumstances. On the one hand, measurements could be made of the levels of DNA adducts derived from a chemical of interest in

cells of an accessible tissue (e.g. white blood cells, biopsy, or autopsy samples). Providing that the chemical nature and stability of the DNA adducts for the compound of interest had been fully characterized, qualitative as well as quantitative identification of adduct levels could provide for that individual not only an indication of exposure history but also an indication of his capability to activate the carcinogen to DNA-binding forms.

It is well established that all carcinogens thus far studied form a complex spectrum of DNA adducts, involving covalent binding to various nucleophilic sites on all four DNA bases as well as on the phosphate residues of DNA. Thus, from a qualitative viewpoint, detection of all DNA adducts derived from even a single carcinogen can present a very complex analytical challenge. Quantification of adduct levels is complicated even further by the fact that adducts are removed from DNA by chemical or enzymatic processes at different rates, even within the same cell; these rates can also vary substantially from one cell type to another.

Most of the currently available information on DNA adducts in experimental systems has been obtained through the use of physicochemical or radiochemical methods of detection. Usefulness of these methods for detection in human monitoring is limited by their relative insensitivity and inapplicability, respectively. However, immunological techniques are being developed which have promise of utility in detecting DNA adducts in people exposed to environmental carcinogens under ambient conditions. The determination of carcinogen–DNA adducts by immunological procedures has certain advantages over other techniques. The sensitivity is frequently better than that obtainable with radiolabelled carcinogens (which are useful only for experimental purposes in any event). Antibodies are specific for particular three-dimensional structures and can be used to probe the conformation of unknown adducts on DNA. Immunological assays are rapid, highly reproducible and can be used in situations where the cost or availability of radiolabelled carcinogens would be prohibitive. The high sensitivity and capability of detecting non-radioactive adducts would therefore suggest a use in monitoring of human tissues. In addition, immunological techniques can be applied together with morphological procedures (electron microscopy and immunofluorescence) to localize adducts in particular cells, subcellular compartments, or DNA molecules. Current methods for detecting adducts in DNA are summarized below.

A second approach to monitoring DNA adducts is also being explored, taking advantage of the fact that adducts removed from cellular DNA (and also from RNA) are excreted in urine. Their detection and measurement of excretion rates would, in principle, provide information on (recent) exposure history of the subject, and possibly also indications of that individual's capability for DNA repair. Thus, studies of urinary excretion of adducts would provide data complementary to measurement of adduct levels in cellular DNA in the same individual. Experimental progress in this area is summarized below (section 4.2.2).

It is clear that the complexities of this field necessitate extensive further research in method development and careful validation in animal models before interpretable data can be obtained from studies in human populations. However, the potential usefulness of the information to be gained justified the additional research effort, as discussed in several recent reviews (IARC/IPCS Working Group Report, 1982; Perera and Weinstein, 1982; Weinstein, 1983).

4.2.1 Analysis of Cellular DNA

Two methodologies are currently being developed for analysis of DNA for the presence of carcinogen-derived adducts, differentiated by the manner in which the adducts are detected: by immunological techniques on the one hand and by radiochemical labelling on the other.

For immunological detection, antisera have been raised in rabbits against the RNA and DNA adducts of aromatic amines, polycyclic aromatic hydrocarbons, aflatoxins, and methylating and ethylating carcinogens. High-affinity antisera have been elicited with either nucleoside adducts covalently bound to a protein carrier, or modified DNA electrostatically coupled to a protein carrier. The properties of these antisera have been reviewed by Poirier (1981). Monoclonal antibodies have also been produced that bind carcinogen–DNA adducts with high affinity. These are reviewed by Muller and Rajewsky (1981).

Characteristics of currently available antibodies against carcinogen-modified DNA are summarized in Table 5. Two very preliminary studies are in progress which utilize these antibodies in attempts to determine the occurrence of benzo[*a*]pyrene adducts in white blood cells and tissues of individuals who might have received substantial exposure to this compound, such as roofers, shale oil workers, and lung tumour patients. Experience to date indicates that the methodology has potential value for epidemiological studies, but that significant problems remain to be resolved. These problems and some suggestions for potential areas for field studies are discussed in the IARC/IPCS Working Group Report (1982).

Postlabelling methods to detect and characterize carcinogen–DNA adducts have been described by Gupta *et al.* (1983) and Haseltine *et al.* (1983). The experimental strategy and procedures involved in the method are summarized in Figure 2. Carcinogen-adducted DNA is subjected to enzymatic analysis under conditions which, when carried to completion, produce a mixture of normal and adducted nucleotides, with the phosphate localized on the 3′-position of deoxyribose. These nucleotides are then subjected to phosphorylation through the action of polynucleotide kinase, using γ-^{32}P-ATP as the source of ^{32}P. This substrate can be obtained with extremely high specific activity, so that nucleotides are radiolabelled in the 5′-deoxyribose position also at a high specific activity. Unmodified nucleotides are removed by TLC or HPLC, and the mixture of adducted nucleotides is resolved on two-dimensional TLC and subjected to

Table 5 Antibodies available against DNA modifications induced by carcinogens[a]

Carcinogen	DNA adduct	Affinity constant (l/mol)	Sensitivity	Assay
N-Nitroso- and alkylating agents	Me–O^6–Gua	2.7×10^{10}	60 fmol	RIA[d]
	Et–O^6–Gua	2.0×10^{10}	40 fmol	RIA
	Bu–O^6–Gua	2.7×10^{10}	10 fmol	ELISA[e]
	Et–O^4–Thy	1.3×10^{9}	40 fmol	RIA
	Bu–O^4–Thy	8.8×10^{8}	240 fmol	RIA
	Bu–O^2–Thy	1.1×10^{10}	450 fmol	RIA
	Me–N^7–CMP	1.0×10^{8}	70 fmol	RIA
Aromatic amines	AAF–C^8–Gua	6.0×10^{9}	100 fmol	RIA
			5 fmol	ELISA
PAH	Benzo[*a*]pyrene–DNA	1.1×10^{8}	5 pmol	RIA
			5 pmol	ELISA
Mycotoxins	AFB_1–DNA		50 fmol	ELISA
	AFB_1–N^7–Gua DNA[b]		100 fmol	ELISA
	AFB_1–FAPY–DNA[b]		100 fmol	ELISA
	AFB_1–N^7–Gua[b]		100 fmol	ELISA
	AFB_1–FAPY[c]		100 fmol	ELISA
Ultraviolet light	Thymine dimer–DNA		80 fmol	RIA

[a] Modified from IARC/IPCS Working Group Report (1982).

[b] Groopman *et al.* (1982).

[c] Hertzog *et al.* (1982).

[d] RIA (radioimmunoassay): 50% tracer-antibody binding by adduct.

[e] ELISA (enzyme-linked immunosorbent assay): sensitivity is that giving 50% inhibition in competitive assays.

autoradiography. The detection of adducts and quantitative estimation of their levels are achieved by densitometry of the autoradiograms.

Gupta *et al.* (1982) have applied this method to studies of DNA modified by bulky aromatic carcinogen adducts formed *in vivo* with derivatives of 2-aminofluorene, acetylaminofluorene, and benzo[*a*]pyrene. In its most sensitive form, the method showed great sensitivity, with the ability to detect adducts at a frequency of 1 in 10^7–10^8 DNA bases. This approach therefore has great potential as a method for application to human population studies. However, much further development is required. For example, in its present form, the method is only applicable to DNA modified by bulky, aromatic carcinogens, and new hydrolysis and separation techniques will be required to enable its application to alkylated DNA. Furthermore, identification of individual adducts will be impossible until reference standards for each adduct are made available and their properties determined. Since, as discussed earlier, each carcinogen

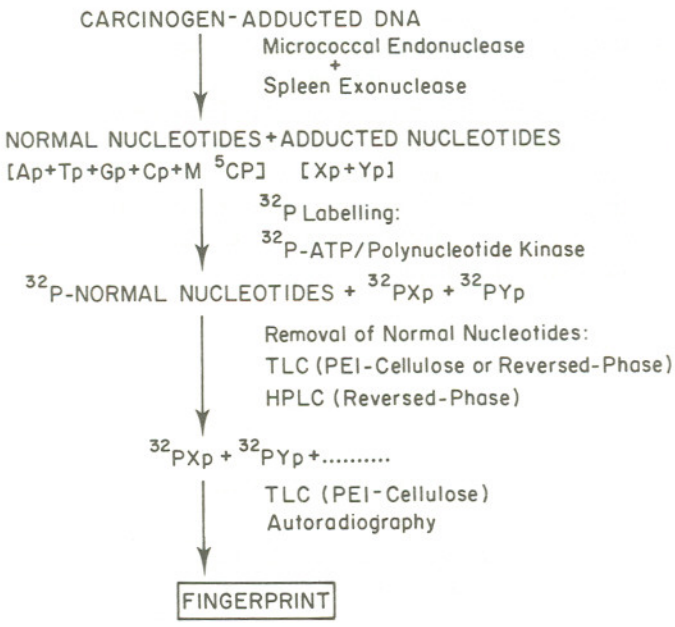

Figure 2 Detection of carcinogen–DNA adducts by ^{32}P post-labelling analysis. From Gupta *et al.* (1982)

forms a complex mixture of DNA adducts, this development will require much additional research effort.

4.2.2 Urinary Excretion of Adducts

Exploitation of the detection of DNA adducts in urine for dosimetry has been undertaken in two experimental models.

Bennett *et al.* (1981) found that rats dosed with aflatoxin B_1 excreted in their urine a large fraction (about 35%) of the major N^7-guanine adduct of the carcinogen over the 48 hours after a single injection. The adduct was isolated from urine by the combined use of preparative and analytical HPLC and was quantified by absorbance at 365 nm. The method allowed reproducible quantitative measurement of adduct in urine from rats treated with doses as low as 0.125 mg/kg body weight. Further, application of the method in rats treated with different doses of carcinogen showed that the amount of adduct excreted bore a constant relationship to peak adduct levels in the livers of animals treated with the same doses. Thus, one condition of adequacy as a dosimeter was met, namely, quantitative reflection of adduct levels in the target tissue.

However, the limit of sensitivity of the method was still inadequate to detect ambient exposure levels of aflatoxins known to occur in human populations, and further methodological development was required. Donahue *et al.* (1982) report improvements that attain that objective. These improvements consist of modifications in the chromatography, but most importantly improved sensitivity by substituting for the absorbance measurement of the earlier method radiolabelling of the adduct with ^{3}H-dimethylsulphate and ultimately determination of the radioactive product, ^{3}H-9-methylguanine. The essential features of this method are summarized in Figure 3.

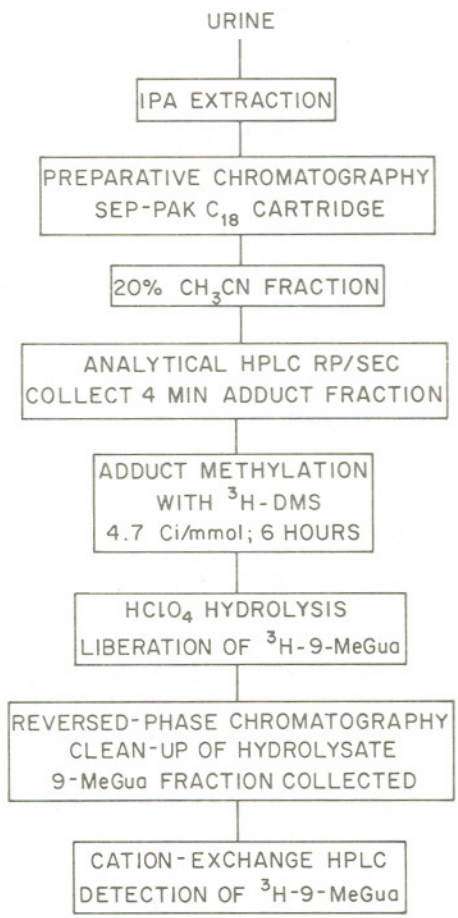

Figure 3 Analysis of urine for AFB_1–N^7-guanine. From Donahue *et al.* (1982)

Experiments with similar objectives were carried out with the carcinogen dimethylnitrosamine by Hemminki and Vainio (1982) and Hemminki (1982). Rats were injected with ^{14}C-dimethylnitrosamine and urine was collected over the succeeding five days. Radioactivity was extracted and separated by Sephadex G-10 chromatography, and the main DMN-derived adducts were tentatively identified. These included *N*-acetyl-*S*-methylcysteine, 1-methylhistamine, *S*-methylcysteine, and methionine, allantoin and 7-methylguanine. Although dose–response experiments were not carried out, these results illustrate the potential applicability of the approach to human monitoring if suitable detection methods for the adducts can be devised. They also illustrate the complexity of the adduct mixtures formed by alkylating agents such as DMN.

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