REVIEW

DNA modification in carcinogen risk assessment in relation to diet: recent advances and some perspectives from a MAFF workshop

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Food is one of the ultimate complex mixtures to which man is exposed and which cannot easily be dispensed with. Apart from certain well-studied microcomponents (for example, food pyrolysates, Sugimura (1990)) human exposure to genotoxic agents arising from macrocomponents has been relatively little studied from the standpoint of DNA damage. The results of epidemiological studies into the relationship between diet and cancer have left many researchers with the impression that it is an intrinsically intractable problem which is perhaps best left well alone. However, given the popular conception that the normal human diet is 'safe' and that such risks as there may be are due to contamination by pesticide and other 'chemical' residues, there is clearly a need to evaluate the possible avenues open to investigators and which are likely to yield meaningful results which would enable scientifically-based advice to be given to the public as to the best dietary habits. This overview of the current state of methodology for measurement of DNA damage in relation to diet as well as a summary of current MAFF-supported work and future prospects in this area arose out of a workshop entitled 'DNA modification in carcinogen risk assessment' held in London on November 18, 1996†. The object of this report is to summarize the results presented at the workshop and also to indicate the significance of the MAFF-funded programme within the broader context of recently published studies from the international scientific community. Hence, a comprehensive review of all aspects of diet-related DNA damage is beyond the scope of this article.

The workshop was organized as part of the MAFF Risk Assessment Research Programme and contributes to an interdepartmental initiative, the Government/Research Councils Initiative on Risk Assessment and Toxicology (RATSC) which aims to bring together regulators and toxicologists to discuss their respective perspectives on current problems in the risk assessment of chemicals. Further aims of RATSC are to identify subjects for future detailed workshops on specific issues and to identify priorities for research into toxic chemical risk assessment. The membership of RATSC is drawn from a wide range of Government Departments and Research Councils and is chaired by Dr David Shannon (MAFF Chief Scientist).

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Introduction

The incidence of some of the major cancers (such as gastrointestinal tract e.g. oesophagus, stomach and colon/rectum] and breast), as well as other chronic diseases such as heart disease, varies widely over the world (IARC 1990). The sometimes extreme extent of the variation (for example, of oesophageal cancer within China where there is a 20-fold difference between the highest and lowest rates) has lead to the conclusion that environmental and/or lifestyle factors have a major role in their aetiology (Doll and Peto 1981, Tomatis *et al.* 1990). Striking evidence for this conclusion has come from studies of migrant populations, such as Japanese living in Hawaii or on the west coast of the United States, where their cancer rates begin to reflect those of the local population within one generation. Thus, immigrant Japanese men living in Hawaii have a rate of gastric cancer (46 per 100 000) which is lower than their contemporaries living in Japan (79 per 100 000), and which approaches the level of the Hawaiian population of US origin (16 per 100 000) (Parkin 1993). There is good evidence that changes in consumption of specific dietary components are linked to this effect (Kolonel *et al.* 1981). This situation clearly demonstrates that environmental or lifestyle factors can both increase and decrease the risk of chronic diseases. The underlying aetiology of the major cancers noted above has not been unambiguously elucidated but many epidemiological studies have highlighted various dietary risk, and protective, factors which account for some of the variability, such as high consumption of fat and red meat as risk factors for colorectal cancers and a lack of fresh fruits and vegetables increasing risk of stomach cancer.

Cancer is a genetic disease inasmuch as the phenotype of the cancer cell is typified by genetic instability and a high level of mutations in certain key genes, such as tumour suppresser and DNA repair genes. Recent major advances in the techniques of molecular biology and genetics of cancer have lead to the discovery of inherited cancers, such as hereditary non-polyposis colon cancer (HNPCC), for which the causal factor appears to be the presence of germline mutations in genes for DNA repair enzymes (Modrich 1994). However, the inherited

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cancers appear to account for only 5–10% of the total incidence at any site. Nonetheless, the significance of these studies for the bulk of non-inherited (spontaneous) cancers is that they indicate that the acquisition of one or more somatic mutations in key genes may lead to the disease. Exposure to genotoxic (i.e. DNA-damaging) agents which give rise to these somatic mutations may lead to an increased risk of cancer. However, the magnitude of the risk is clearly linked to statistical considerations of the probability that a certain dose of agent at the target cell population will be sufficient to result in enough mutagenic damage in one or more cells to give rise to a tumour.

What, then, is the evidence that exposure to DNA-damaging agents results in increased risks of cancer? Table 1 shows a list of the exposures for which there is good evidence of carcinogenicity to human (IARC Group 1). It is worth emphasizing the point that, in most cases (the exceptions being medicinal drugs), the cancer risk was detected in human populations where the exposures were not particularly high when compared with high dose animal bioassays. In almost every case the agents were found to damage DNA, either by formation of characteristic DNA adducts (e.g. from aflatoxin B_1 or 4-aminobiphenyl) or by increasing 'generic' damage such as oxidation. Furthermore, in almost all the cases clear dose–response relationships were seen which suggest that exposure to the agent was a key determinant in the risk (as opposed to host susceptibility being the major risk factor). It is interesting to note that there are only a few exposures on the list which are related to diet – aflatoxins, alcoholic beverages and Chinese-style salted fish. This may appear paradoxical given the large contribution that diet appears to make to cancer risk (Doll and Peto 1981), however it is worth noting that one of the criteria that IARC uses to select exposures for consideration is that there is a body of relevant data on which to base an evaluation. For diet there is often simply not enough information available on exposure to foodborne genotoxic components or on the interactions of dietary constituents with host factors (such as chronic infectious agents) which give rise to endogenous exposures.

Most of the chemicals which are known to be carcinogenic to humans and experimental animals are genotoxic, that is, they cause damage to genomic DNA. Moreover, much of this DNA damage is known to lead to mutations at DNA replication unless some sort of repair takes place. This was elegantly and unambiguously demonstrated by Loechler and his colleagues (Green *et al.* 1984) when they incorporated a single O^6-methylguanine residue into a plasmid and showed that, on replication, it resulted in a mutation consistent with the mispairing predicted by Loveless (1969). If the modified plasmid was exposed to the repair enzyme O^6-alkylguanine alkyltransferase prior to replication then no mutation was detected. Similar results have been obtained with incorporation of single DNA adducts representing some of the major classes of genotoxic carcinogens, including such small modifications as the oxidized DNA bases (reviewed by Grollman and Shibutani 1994). So, within a given cell population there is an interplay between the formation of DNA adducts and their repair.

Aflatoxins (naturally occurring mixtures of)	Erionite	(Oestrogens steroidal)
		— oestrogen replacement therapy
Alcoholic beverages	Ethylene oxide	Oestrogen–progestin combinations
Aluminium production	Haematite mining + radon	— sequential oral contraceptives
Aminobiphenyl	*Helicobacter pylori* (infection with)	— combined oral contraceptives
Arsenic and arsenic compounds	Hepatitis B virus (chronic infection with)	*Opisthorcis viverrini* (infection with)
Asbestos	Hepatitis C virus (chronic infection with)	Phenacetin-containing analgesics
Auramine manufacture	Iron and steel founding	Radon and its decay products
Azathioprine	Isopropyl alcohol manufacture (strong acid)	Rubber industry
Benzene		Salted fish, Chinese style
Benzidine	Magenta manufacture	*Schistosoma haemotobium* (infection with)
Betel quid with tobacco	Melphalan	Shale oils
Boot and shoe manufacture and repair		
Chlornaphthazine	8-Methoxypsoralen + UV	Solar radiation
Bis(chloromethyl)ether	Methyl-CCNU	Soots
Chorambucil	Mineral oils	Sulphuric acid mists
Chromium (VI)	MOPP therapy	Talc-containing asbestiform fibres
Coal gasification	Mustard gas	Thiotepa
Coal-tar pitches	Myleran	Tobacco products
		— smokeless
Coal tars	2-Naphthylamine	Tobacco smoke
Coke production	Nickel and nickel compounds	Treosulphan
Cyclophosphamide	Oestrogens (nonsteroidal)	Vinyl chloride
	— diethylstibestrol	
		Wood dust

Table 1. A list of exposures which have been found to be carcinogenic to humans (compiled from IARC Monographs Supplement 7

It would appear that, of the large number of cells which contain damaged DNA and/or mutations following exposure of an organism to a genotoxic agent, only a minute fraction proceed to the stage of acquisition of the full tumour phenotype at which point rapid proliferation occurs and the frank tumour appears. The latter stages of this process (that is, from the initiated cells through to the carcinoma) have been characterized in some detail for colorectal cancers (Fearon and Vogelstein 1990, Figure 1).

In many respects, therefore, the link between DNA damage and tumorigenesis would appear to be made (at least for colorectal cancer, which is one of the best characterized human cancers). However, it is worthwhile quoting from a consensus statement which appeared in a recent volume on '*Mechanisms of Carcinogenesis in Risk Identification*' (IARC 1992). 'DNA replication is an essential component of cell proliferation and entails a small risk of mutation in the absence of any abnormal exposure. Given these circumstances, it is reasonable to assume that all the necessary stages in the process of carcinogenesis occur with a non-zero probability in all individuals, whether as the result of their ubiquitous exposure to the very wide variety of agents in typical daily life or spontaneously. This perspective has two important scientific and practical consequences: first, a change in exposure that affects the rate of any of the stages in the carcinogenic process will affect the subsequent incidence of cancer: second, it is unrealistic to assume that each case of cancer has an identifiable cause'. The latter conclusion is particularly significant in view of the widespread belief in both the scientific community and among the general public that the new molecular techniques in cancer research will lead to the identification of the individual's risk of cancer (see for example, Marx 1991). However, the former conclusion indicates that changes in the levels of DNA damage (among other things) will be accompanied by changes in outcome (cancer risk) at the population level.

There is increasing evidence that human DNA is subjected to continuous assault by highly reactive chemical agents. For the damage caused by such agents to have biological consequences (such as mutation and ultimately cancer) one or more of several possibilities have to occur: (a) the damage has to be in DNA at the time of replication, (b) there has to be some deficiency or inefficiency in DNA repair which means that the damage is not removed prior or subsequent to replication, and (c) that the damage has to occur at a site in a gene which will result in altered function of the gene product. It is known, however, that DNA is efficiently repaired. Moreover, if certain kinds of damage are present at the replication fork, cell division will be arrested until the damage can be repaired. If it cannot be repaired the cell will not normally be allowed to divide and will be diverted into a pathway leading to cell death (apoptosis).

Thus it can be seen that only a fraction of all DNA damage will lead to a permanent change in DNA (mutation) and that in all likelihood several such changes will be required in a cell for the full cancer genotype to be acquired and for the phenotype to be manifested.

This situation is summarized in a very diagrammatic form in Figure 2. Cells at each stage will have accumulated a number of genetic changes. However, the accumulated changes need not necessarily have occurred in the same order in different cells or cell populations. At the last stage there may be only one cell which acquires the full cancer phenotype and becomes the pregenitor cell of a monoclonal colony of tumour cells.

From the point of view of detecting and measuring the role of food-related DNA damage in cancer risk we need to consider what information is potentially available at any of the stages shown in Figure 2.

The extent and identity of DNA damage at the early stages of carcinogenesis (in the upper part of the diagram) is related to exposures to genotoxic agents and the interactions of these exposures with the host and is probably a better marker of exposure than of risk. The closer that one gets to the tumour then measurements of biological changes (mutations in key tumour suppressor genes or oncogenes) are more likely to be indicative of risk. Several practical problems are apparent in the use of markers for pre-tumourous cells. The first is that the size of the population of cells which have accumulated these changes is likely to be very small and the problem would then be to select these cells for analysis from the vast majority of normal cells. It is nonetheless obvious that advances in molecular biology may lead to the development of techniques to overcome these methodological difficulties. A second problem is that the pattern of molecular changes which would be detectable in these cells would not necessarily be very informative about the exposure or exposures. The idea of using 'mutational spectra' to identity the source of certain patterns of

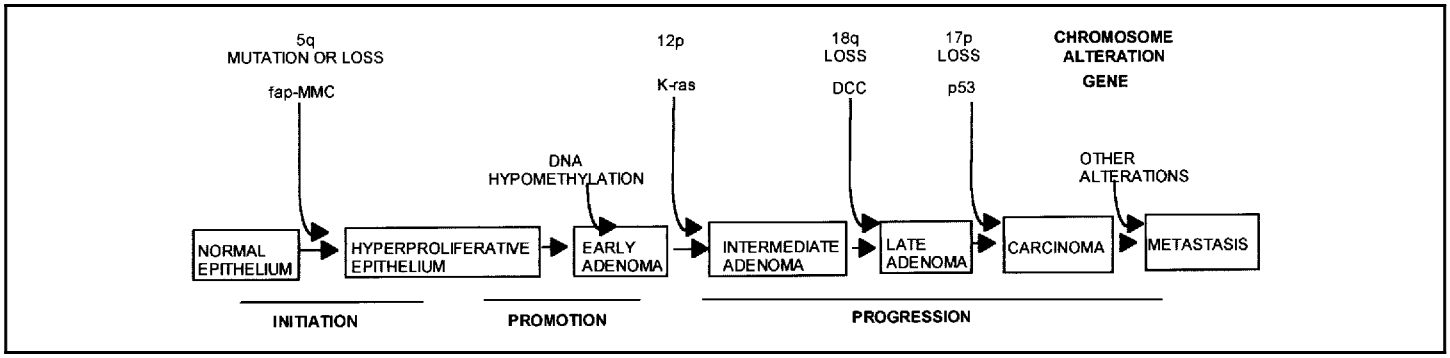

Figure 1. Multistep model of carcinogenesis (Fearon and Vogelstein 1990)

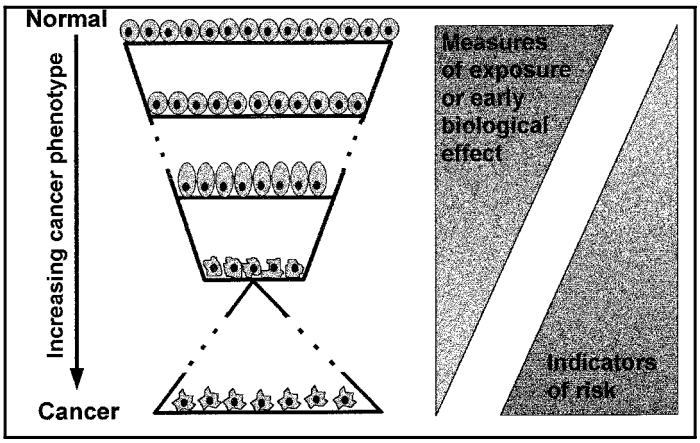

Figure 2. Diagrammatic representation of the progression from normal cells to tumour cells. The wedges on the right illustrate the relative value of biomarkers of exposure and risk at each stage.

genotoxic damage has been around a long time but despite experimental promise the prospects of the technique being useful in the near future in human studies are not particularly bright (Kat and Thilly 1994). A final comment with regard to the use of molecular genetic markers of carcinogen exposure is that the measurements may only be made when essentially irreversible changes have taken place (such as the presence of a mutated *p53* gene). Thus the prospects of any kind of intervention to reduce the risk of cancer in the population under study is somewhat limited.

Overall, present knowledge of the processes involved in chemical carcinogenesis and the availability of sensitive analytical techniques to measure DNA damage are such that the time is ripe for detailed studies of the relationships between diet and cancer. For future applications markers of diet-related DNA damage or constituents could be used as surrogate markers of outcome, albeit somewhat crude in view of the difficulty in extrapolating risk of outcome from early molecular events. This would lead to a dramatically reduced time required to study the risk or benefit for any particular food constituent with respect to DNA damage.

Techniques for measurement of diet-related DNA damage

For the most part the available analytical methods have been developed to such an extent that they are now capable of detecting certain types of DNA damage in nominally 'unexposed' humans. The issue is therefore primarily one of relevance and interpretation.

The complex nature of the diet probably results in the formation of many different types of DNA damage. One technique which has the potential for detecting a broad range of modified DNA bases is ^{32}P-postlabelling. ^{32}P-Postlabelling was originally developed by Randerath and Randerath (1993) to measure naturally occurring modified bases in RNA but was subsequently adapted for use to determine DNA adducts. In the original procedure, DNA was enzymatically digested to 3′-nucleotides which were then phosphorylated in the 5′-position using ^{32}P-ATP and T4 polynucleotide kinase. The adducted nucleoside-3′, [^{32}P]5′-bisphosphates were separated from unmodified nucleotides by two-dimensional TLC and the radioactive adduct spots were detected by autoradiography. It has been the subsequent introduction of adduct enrichment steps, primarily, butanol extraction, nuclease P1 treatment, chromatographic separation, and immunoaffinity clean-up, leading to increases in specificity and sensitivity which has made the method so useful to human biomonitoring studies (Figure 3). Butanol extraction is a solvent–solvent partitioning procedure in which bulky aromatic and/or lipophilic nucleotide adducts are extracted preferentially into a butanol phase from an aqueous acidic phase in the presence of a phase transfer reagent, tetrabutylammonium chloride (Gupta 1993). Nuclease P1 dephosphorylates normal nucleotides to nucleosides, but does not attack most aromatic or bulky nucleotide adducts. Since nucleosides are not substrates for kinase, only adducted nucleotides are ^{32}P-labelled in the subsequent steps (Reddy 1993). HPLC separation with a post column radioactivity monitor is being increasingly used as a

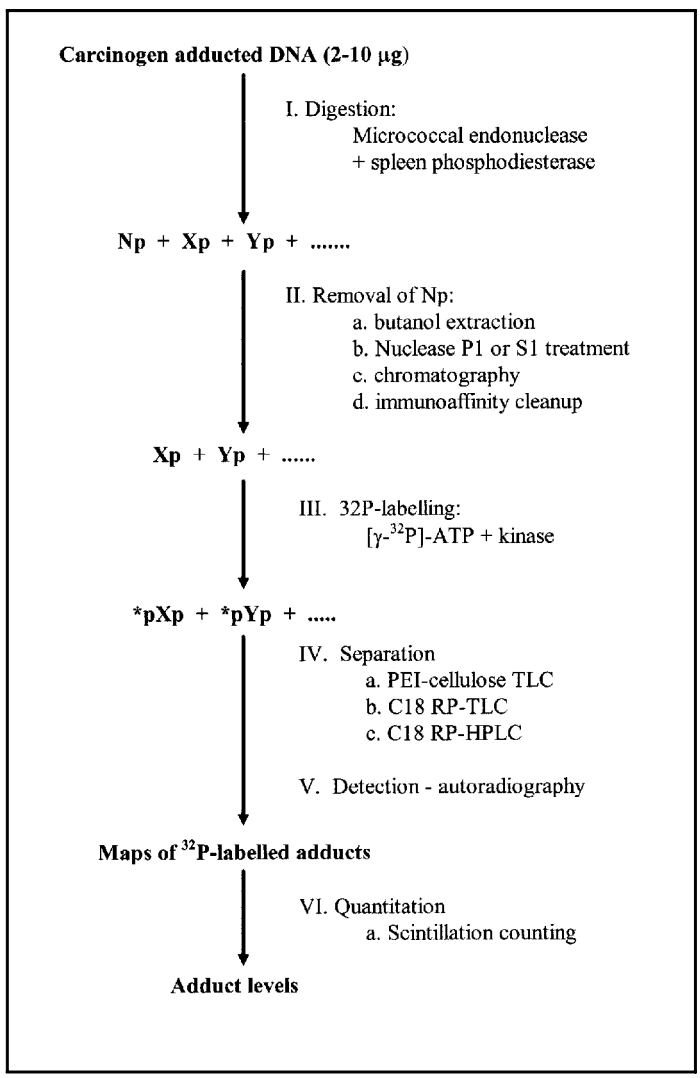

Figure 3. Experimental strategy employed in the analysis of carcinogen–DNA adducts by the bisphosphate version of the ^{32}P-postlabelling assay (redrawn and modified from Reddy (1993).

means of separating and quantifying ^{32}P-labelled nucleoside-3′,5′-bisphosphates modified by bulky carcinogens (Pfau *et al.* 1993). Alternatively, HPLC or column chromatography can be used to enrich adducts prior to ^{32}P-postlabelling, especially in the case of alkyl adducts (Wilson *et al.* 1988, Mustonen *et al.* 1991, Mustonen and Hemminki, 1992, Kato *et al.* 1993), etheno adducts (Misra *et al.* 1994) and malondialdehyde adducts (Vaca *et al.* 1992). Immunoaffinity purification of O^6-alkyl- and *N7*-alkyl-deoxyguanosine adducts prior to ^{32}P-postlabelling has been successfully used to measure DNA damage in human tissues (Kang *et al.* 1992, 1995, Haque *et al.* 1994).

Other techniques are being incorporated into human studies because they offer either exquisite sensitivity (e.g. accelerator mass spectrometry [AMS]) or the possibility of using small amounts of DNA (e.g. immunoslotblot assay) are described below.

AMS is a technique for the direct quantitation, with great sensitivity, of radioisotopes such as ^{14}C. An AMS instrument is a tandem mass spectrometer in which the two mass spectrometers are separated by an electrostatic (Van der Graaf) accelerator (Vogel and Turteltaub 1994). The important thing to note is that AMS measures isotopes only and provides no molecular or structural information, in contrast to the more conventional mass spectrometric techniques. Measurement of DNA adducts using ^{14}C-labelled carcinogens involves isolation of the exposed DNA and conversion of the sample into a form of carbon compatible with the ion source of the instrument. For ^{14}C, graphite has been found to be ideal. Carbon from purified DNA is isolated by oxidizing the sample directly to CO_2, N_2 and H_2O. The CO_2 is cryogenically separated and reduced to filamentous graphite for introduction into the ion source. Any molecular information about the adducts must be obtained before the AMS analysis using techniques such as HPLC or TLC (Turteltaub *et al.* 1993a, b, c).

A number of studies in experimental animals have demonstrated the utility of AMS for measuring DNA adducts after administration of doses of carcinogen that are similar to the human exposure from foods. For example, ^{14}C-MeIQx (a heterocyclic amine found in many cooked meats [Eisenbrand and Tang 1993]) was administered to rats for 7 days at doses from 5 pg kg^{-1} to 5 mg kg^{-1} intragastrically and the level of ^{14}C bound to DNA was determined by AMS. A clear dose–response was seen at levels of MeIQx of 1 ng kg^{-1} day^{-1} up to 100 ng kg^{-1} day^{-1} which corresponds to typical levels of human exposure through the diet (Turteltaub *et al.* 1993b). Similarly, formation of AFB_1–DNA adducts in rat liver, kidney, lung, colon and spleen was linear with dose over a range of 0.6 ng kg^{-1} to 13 μg kg^{-1} (Cupid and Garner, unpublished data).

The doses of ^{14}C-labelled carcinogens that would be required for human studies are small but there are clearly ethical considerations involved in administration of radioactive carcinogen to human subjects at any dose. This is a limitation of the current AMS methodology in human studies. Nevertheless, some studies have been possible, using strictly controlled conditions wherein patients were administered trace amounts of radiolabel and a dose of carcinogen equivalent to levels commonly found in a normal western diet, approximately 6 h before undergoing colon surgery. Colon samples taken at surgery following administration of AFB_1 contained DNA adducts in the order of 1 per 10^{12} nucleotides, which was approximately 100 times lower than observed in rat colon after administration of the same dose (15 μg kg^{-1} body weight) (Cupid and Garner, unpublished data). In contrast, the level of MeIQx–DNA adducts detected in human colon was higher than in rats or mice treated with an equivalent dose of MeIQx (Turteltaub *et al.* 1997). The sensitivity of AMS has also been used to confirm results obtained by ^{32}P-postlabelling, something that is almost impossible to do by any other technique. This approach has been used for 7-ethyldeoxyguanosine (Kato *et al.* 1993) and tamoxifen (Martin *et al.* 1995).

The immunoslotblot (ISB) technique (Figure 4) was originally developed by Nehls *et al.* (1984) for the detection of O^6-ethyldeoxyguanosine in intact DNA. With a suitable antibody the technique can be used for other adducts and has recently been used in several studies on diet-related DNA damage. The major advantage of ISB over many other methods of measuring DNA damage is that it only requires a small amount of DNA (typically 1 μg). During the Workshop three examples of the use of ISB were presented and these are briefly described below.

An antibody against O^6-carboxymethyldeoxyguanosine (O^6-CMdG) was found to recognize the adduct in intact DNA and an ISB method was set up (Harrison *et al.* 1997). Using 1 μg of DNA per well the limit of detection was 1 fmol and this was sufficiently sensitive to detect O^6-CMdG in DNA extracted from human gastric biopsy samples (Harrison *et al.*, unpublished results).

An ISB method has also been used to detect DNA adducts in rats treated with low levels of aflatoxin B_1 (10 μg kg^{-1} day^{-1}). The sensitivity of the aflatoxin B_1–DNA assay was 1 fmol per well and it was shown that up to 25 μg of DNA could be loaded to each well (Phillips *et al.*, unpublished results).

In the case of food flavourings, high affinity antibodies have been prepared against protein conjugates of methyleugenol (Gardner *et al.* 1996). The antibodies were found to recognize DNA adducts and a resulting sensitive ISB method (*ca* 1 adduct per 10^9 bases) has the potential for use in biomonitoring (Caldwell *et al.*, unpublished results).

Applications of DNA adduct measurements in human and experimental studies

There have been a number of studies in humans where biomarkers of DNA damage have been used in connection with the influence of diet and these will be summarized in the following sections.

Aflatoxin B_1 and hepatocellular carcinoma

A study of the role of aflatoxin B_1 and hepatocellular carcinoma is the only one to date in which biomarkers of DNA damage have been used in a prospective epidemiological study with cancer as the outcome. Althoug

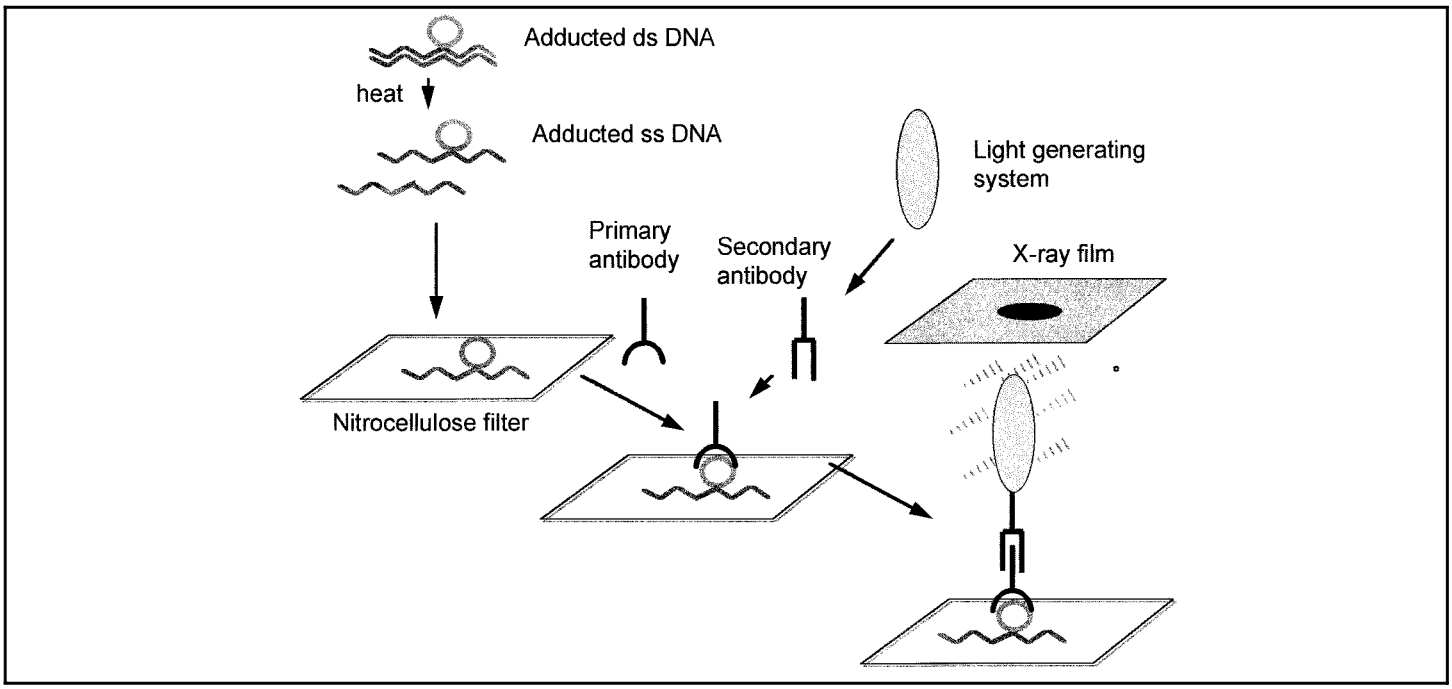

Figure 4. Diagram of the principle of the immunoslotblot assay.

of little, if any, relevance to the risk of hepatocellular carcinoma in the UK, the Shanghai study reported below can be considered as a paradigm for future opportunities which are certain to arise as large ongoing prospective studies, with biological sample banks, in the UK and Europe reach maturity.

Primary liver cancer, in particular, hepatocellular carcinoma (HCC), is one of the leading causes of cancer mortality in Asia and Africa. The geographical distribution of HCC is similar to that of hepatitis B viral (HBV) infection and to areas where foodstuffs are commonly contaminated with AFB_1 (IARC 1990). Although it has been difficult to separate unambiguously the contributions of AFB_1 and HBV to the aetiology of HCC, there is ample experimental evidence that AFB_1 alone is a hepatocarcinogen (Busby and Wogan 1984). AFB_1 is metabolized to its 8,9-epoxide and forms characteristic adducts with DNA and proteins which form the basis of a number of biomonitoring approaches (Groopman *et al.* 1991, Weaver and Groopman 1994). The measurement of the major guanine–AFB_1 adduct has been used extensively to monitor human exposure to AFB_1, and the rationale for this approach will be reviewed here.

The major AFB_1–DNA adduct *in vitro* and *in vivo* which can be released from DNA by depurination has been identified as 8,9-dihydro-8-(*N* 7-guanyl)-9-hydroxy-AFB_1 (AFB_1–Gua, Essigman *et al.* 1977, Croy *et al.* 1978). AFB_1–DNA undergoes ring-opening to give 8,9-dihydro-8-(*N*5-formyl-2,5,6-triamino-4-oxyopyrimidin *N*5-yl)-9-hydroxy-AFB_1 (Hertzog *et al.* 1982), which is relatively stable in DNA. These two products account for >95% of the AFB_1 residues bound to DNA (Groopman *et al.* 1991). AFB_1 binds to rat liver DNA in a linear dose-dependent manner, even at low doses (Appleton *et al.* 1982), and good correlations have been observed between urinary excretion of AFB_1–Gua and both administered doses of AFB_1 and AFB_1–DNA in rat liver DNA (Bennett *et al.* 1981).

Autrup *et al.* (1983) detected AFB_1–Gua in urine samples collected from subjects in an area of Kenya where food samples were contaminated with AFB_1 and where the incidence of liver cancer is high. The identity of urinary AFB_1–Gua was confirmed by fluorescence spectrophotometry and comparison with an authentic sample. In a subsequent study a regional variation in urinary AFB_1–Gua was observed, but only moderate correlations with liver cancer risk were obtained (Groopman *et al.* 1985). The analysis of the large numbers of urine samples which are required in order to carry out molecular epidemiological studies on the role of AFB_1 in liver cancer has been greatly expedited by the development of monoclonal antibody-based immunoaffinity columns which allow rapid purification of AFB_1–Gua prior to quantitative analysis (Groopman *et al.* 1985, Autrup *et al.* 1987).

Several recent studies of human populations with exposure to dietary AFB_1 and elevated liver cancer risk illustrate the value of urinary AFB_1–Gua as an informative biomarker. In samples collected from subjects living in Guangxi province, in the People's Republic of China, a good correlation was found between dietary AFB_1 and AFB_1–Gua over a 1-week period. A somewhat poorer correlation was observed when daily intake and the following day's excretion were used. Both of these correlations were superior to those obtained when urinary excretion of AFB_1 metabolites, either as a total or individually, was used (Groopman *et al.* 1992a). A reasonably good correlation was found between dietary AFB_1 intake and total urinary AFB_1 metabolites in subjects from the Gambia, but a better correlation was obtained using AFB_1–Gua. Interestingly, no difference was detected in AFB_1–Gua excretion between hepatitis surface antigen positive and negative carriers for the same dietary intake of AFB_1 (Groopman *et al.* 1992b, Harris 1994).

The culmination of much of this w

hepatocellular carcinoma was a recent study carried out in China. A cohort of 18244 mostly middle-aged (45–64 years) men residing in Shanghai was accrued between 1986 and 1989. In addition to an in-person interview regarding dietary and other past exposures, each subject provided a single void urine sample. After close to 70000 person-years of follow-up, 55 cases of incident HCC were identified. A nested case-control analysis showed highly significant associations between the presence of urinary aflatoxins (including AFB_1–Gua), serum hepatitis B surface antigen positivity and HCC risk. Risk was especially elevated in individuals who were positive for both of these biomarkers (RR = 59.4) (Ross *et al.* 1992, Qian *et al.* 1994). Interestingly, a cohort analysis using the 55 cases of HCC revealed no strong or statistically significant association between HCC risk and dietary aflatoxin consumption as determined from the interview combined with a survey of market foods in the study region. In the authors' words – 'our results underline the importance of biomarker measurements in assessing the aflatoxin–HCC association in epidemiological studies' (Qian *et al.* 1994).

The formation of DNA adducts by AFB_1 has been associated with high incidences of a specific mutation in codon 249 of the *p53* tumour suppresser gene from hepatocellular carcinomas from regions of the world where aflatoxin contamination of foods is common (reviewed by Greenblatt *et al.* 1994). A lower incidence of this mutation was observed in Thailand where aflatoxin contamination is lower than in China or other parts of South-East Asia (Hollstein *et al.* 1993) and it was absent in HCC in Alaskan natives where an inherited genetic disposition to the disease is suspected as the major risk factor (De Benedetti *et al.* 1995).

Food-derived heterocyclic amines

The food-derived heterocyclic amines are the reaction products of proteins that are formed during the heating (cooking, broiling, frying or grilling) of protein-containing foods. The chemistry, formation, occurrence and biological activity of this now quite extensive group of compounds has been comprehensively reviewed by Eisenbrand and Tang (1993). In general the heterocyclic amines are metabolized to biologically active intermediates in a similar manner to the aromatic amines and give rise mainly to C-8 guanine adducts. This pathway has been well characterized for PhIP (see below) (Figure 5). There have been a number of estimates as to the level of human exposure to food-derived heterocyclic amines which arrive at a similar figure of 0.3–5 mg per person per year. However, there are going to be large interindividual variations because of the different ways in which food is prepared and it is important to have reliable and informative biomarkers of exposure to these genotoxic compounds.

Amongst the many heterocyclic amines that have been identified in cooked meat, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) has attracted particular attention because it tends to be the most abundant of them. PhIP induces colon and mammary tumours in rats and lymphomas and hepatic tumours in mice. Interestingly, the colon tumours that are produced in rats by PhIP have a high frequency of microsatellite instability which is similar to that seen in inherited and sporadic colorectal cancers (Canzian *et al.* 1994). The major DNA adduct formed by PhIP has been characterized as a C-8 derivative of 2′ -deoxyguanosine (Lin *et al.* 1992) and has been detected in target tissues in experimental animals by ^{32}P-postlabelling (Friesen *et al.* 1994). PhIP–DNA adducts have recently been detected in human colon samples at levels of 3 adducts per 10^8 normal nucleotides and it is of particular interest to note that the positive samples detected by ^{32}P-postlabelling were also analysed by NICI GC–MS of the derivatized PhIP obtained by alkaline hydrolysis of DNA (Figure 6) and gave clearly positive and quantifiable results by this unambiguous and sensitive procedure (Friesen *et al.* 1994).

Recent developments have focused on the detection of PhIP

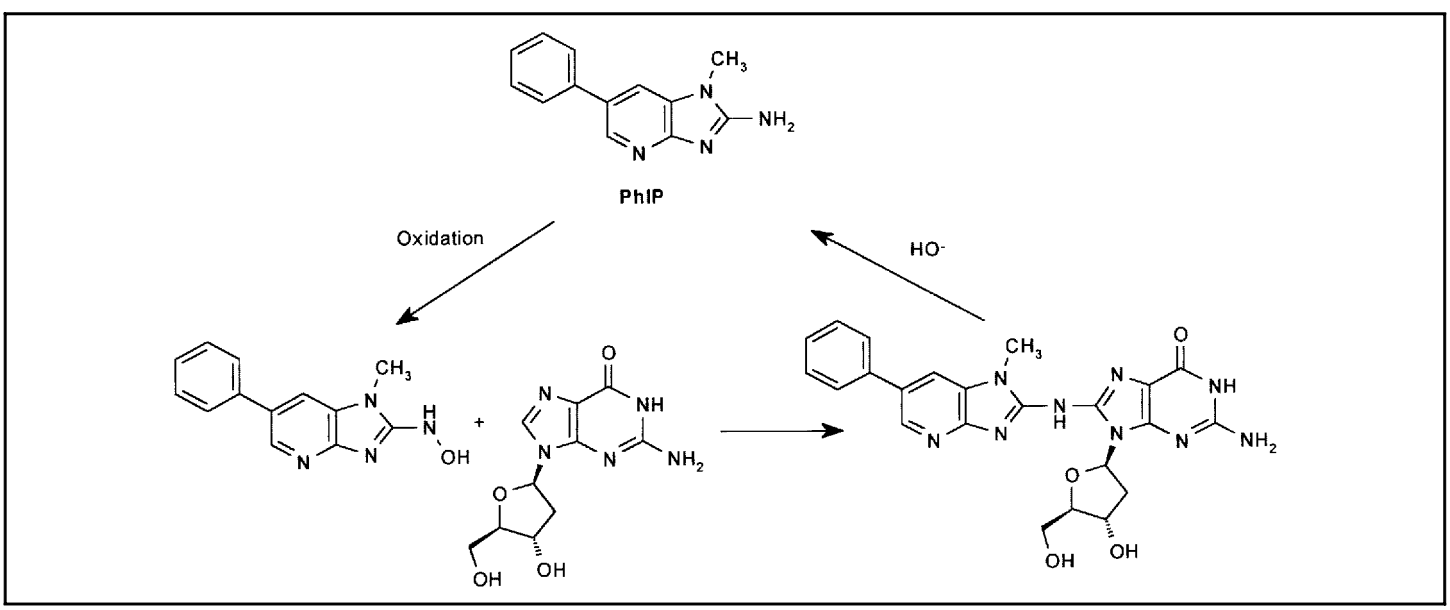

Figure 5. Formation and alkaline hydrolysis of C8–PhIP–dG adducts.

adducts in defined DNA sequences. Using a selectable marker gene, *hprt*, as a target in Chinese hamster V79 cells genetically engineered to express CYP1A2, it has been demonstrated that PhIP preferentially causes mutations at 5′ GGA3′ (Boobis *et al.*, unpublished observations).

Protein adducts of food-derived heterocyclic amines have not been exploited as biomarkers of exposure in the way that haemoglobin adducts have been used very successfully for aromatic amines from cigarette smoke (Bryant *et al.* 1988) and occupational exposures (Schütze *et al.* 1995). However, recent results suggest that the activated metabolites of food-derived heterocyclic amines do not form significant amounts of protein adducts. In the presence of human albumin, human liver microsomal fraction metabolizes radioactive MeIQx and PhIP extensively to their N-hydroxy derivatives and concomitantly radioactivity becomes bound to the albumin. However, the level of this binding is extremely low. Indeed, the level of MeIQx bound to serum albumin obtained from human volunteers was below the limits of detection of the currently available assay (29 atmol MeIQx mg^{-1} albumin) (Boobis *et al.*, unpublished observations).

DNA damage derived from natural food flavourings

The allylbenzenes are anutrient food flavour chemicals which are present in the human diet and are ingested daily at doses of the order of mg kg^{-1} day^{-1}. These compounds, which include safrole, oestragole and methyleugenol, have been shown to cause hepatotoxicity and hepatocarcinogenicity when administered chronically to rodent test species, at doses of many mg kg^{-1} day^{-1} (Miller 1982). Their carcinogenicity is believed to involve metabolic activation by cytochrome P_{450}-mediated 1′-hydroxylation of the side chain and sulphation, yielding carbonium ions which react with DNA and thereby cause genotoxic damage. The following strategy has been used to explore whether these compounds pose a threat to human health (Gardner *et al.* 1996).

Development of methods for quantitation of urinary DNA adducts derived from the allylbenzenes has been undertaken. Protein conjugates were synthesized by coupling 1′-acetoxyallylbenzene derivatives of both methyleugenol and oestragole (models for the reactive sulphate conjugates), or the corresponding cinnamic acids, to rabbit antiserum albumin. These were used as immunogens to produce rabbit antisera. The anti-(methyleugenol adduct) antisera were shown by ELISA to specifically recognize the dimethoxybenzene ring of the compound, indicating that they can be used for specific immunodetection of methyleugenol-derived adducts. The anti-(oestragole adduct) antisera recognized a variety of chemical derivatives bearing aliphatic or allyl side-chain modified benzene rings, and could be of value for detection of adducts derived from many different allylbenzenes. The antisera were further shown by ELISA to recognize calf thymus DNA reacted *in vitro* with the corresponding 1-acetoxyallyl-benzenes, but not calf thymus DNA alone. DNA adducts have been detected in the livers of rats given methyleugenol or oestragole using [^{32}P]-postlabelling, and also ELISA and slot-blotting using the specific polyclonal antisera. The [^{32}P]-postlabelling assay has revealed the presence of multiple DNA adducts, while the ELISA and slot-blot assays demonstrated dose-related formation of allylbenzene–DNA adducts in rat liver after chronic treatment. Detection of urinary allylbenzene–DNA adducts should be feasible by immunoaffinity chromatography, then analytical HPLC with immunodetection.

The enzymology of 1′-hydroxylation and sulphation of methyleugenol and oestragole has been investigated, both in rat and human liver. The objectives were to provide a rational basis for interpretation of the relevance to humans of the high dose rodent carcinogenicity studies and to explore the possibility of inter-subject variation in metabolic bioactivation. The kinetics of 1′-hydroxylation of oestragole and methyleugenol were shown to differ markedly, with oestragole metabolized by a higher affinity enzyme system at a much faster rate at low concentrations. This suggests that oestragole may pose a greater toxic hazard than methyleugenol at low dietary exposures. 1′-Hydroxylation of methyleugenol in rat liver was shown to be catalysed by CYP2EI and CYP2C isozymes and dose-dependent autoinduction of this reaction was observed in livers from rats given methyleugenol over the dose range used in NTP carcinogenicity studies. This autoinduction of 1′-hydroxylation of methyleugenol was accompanied by a phenobarbital-like pattern of P_{450} enzyme induction, which raises the possibility of carcinogenicity via both epigenetic and genetic mechanisms. Analyses of a panel of human liver microsomes, and of cloned human P_{450}s expressed in yeast, revealed that 1′-hydroxylation of methyleugenol in humans is catalysed by CYP1A and CYP2C isozymes, but not by human CYP2EI, and may vary between individuals by greater than 30-fold, with activities ranging between 0.05- and 2-fold those seen in the rat. Moreover, a 30-fold variability in the rate of sulphation of 1′-hydroxymethyleugenol in a panel of human liver cytosols was observed, which could be attributed to metabolism by sulphotransferase isozyme MPST. These studies provide the first demonstration that the proposed route of rodent toxicity of the allylbenzenes (two step bioactivation by P_{450}s and sulphotransferases) is catalysed by human forms of the enzymes. Furthermore, they have established that metabolic variability is a major factor influencing the outcome of NTP carcinogenicity bioassays and also confounding extrapolation from animal models to the human situation.

The antibodies, specific for oestragole- and methyleugenol-adducts, have been used to demonstrate the formation of protein adducts in the liver, but not serum, of rats given these allylbenzenes. Interestingly, markedly different patterns of adducts derived from the two compounds were detected. Methyleugenol predominantly yielded a 44 kDA protein adduct which was shown to be a peripheral membrane protein concentrated in the microsomal and nuclear fractions and to be formed as a consequence of the 1′-hydroxymetabolite. In contrast, the predominant oestragole–protein adduct exhibited a molecular mass of 150 kDa and was a peripheral membrane protein present in the microsomal and cytosolic fractions. The levels of expression of the protein adducts indicate that they are formed more readily than allylbenzene–DNA adducts, while *in vitro* studies have shown that their formation required intact hepatocellular architecture. C

investigations of the nature of modified proteins and of the mechanism of adduct formation should provide important novel information on processes underlying the hepatotoxicity and/or carcinogenicity of the allylbenzenes. Moreover, immunochemical detection of the protein adducts may provide an alternative and highly promising approach for biomonitoring of exposure to their reactive metabolites.

Background levels of alkyl-DNA damage in relation to diet

A number of methylating agents have been used to induce experimental GI tract tumours such as *N*-methyl-*N'* -nitro-*N*-nitrosoguanidine (MNNG) and *N*-nitroso-*N*-methylurea (MNU) for gastric cancers (Preussmann and Stewart, 1984) and 1,2-dimethylhydrazine (DMH) for colorectal cancer (Druckrey *et al.* 1967). All of these agents either spontaneously generate methylating agents or do so after metabolic activation and result in the formation of DNA methyl adducts in the target organs of treated animals (Preussmann and Stewart 1984). In combination with this experimental data there are a number of reports that O^6-MedG levels are raised in human subjects at elevated risk of GI tract tumours (Kyrtopoulos *et al.* 1984; Hall *et al.* 1991, The EUROGAST Study Group 1994, Jackson *et al.* 1996) which suggests that methylating agents may be involved in the aetiology of these cancers. Furthermore, several studies have indicated that intragastric nitrosation (Sobala *et al.* 1991, Xu and Reed 1993) and intraintestinal nitrosation (Bingham *et al.* 1996) of dietary precursors may be significant sources of alkylating agents. However, it is unlikely that nitrosocompounds such as MNNG or MNU occur naturally in the human GI tract and, in fact, the endogenous nitrosation of dietary amino acids and peptides is a more likely reaction (Challis 1989, Shephard and Lutz 1989, Mirvish 1995).

A polyclonal antiserum against O^6-carboxymethyldeoxyguanosine (O^6-CMdG) (Harrison *et al.* 1997) and a monoclonal antibody against O^6-methyldeoxyguanosine (O^6-MedG) (Saffhill *et al.* 1982) have been used to prepare immunoaffinity columns for the selective purification and preconcentration of each adduct, prior to quantification by HPLC-fluorescence. Enzyme hydrolysates of treated DNA were applied first of all to one immunoaffinity column (O^6-CMdG) and the eluate from that column passed directly onto the second immunoaffinity column (O^6-MedG). The two columns were then washed and eluted separately and the eluates analysed. For the determination of O^6-CMdG and O^6-MedG in DNA, spiked DNA samples were used to construct calibration curves for O^6-CMGua and O^6-MeGua, both of which were linear, with limits of quantitation of 0.1 pmole O^6-CMGua mg^{-1} DNA and 0.05 pmol O^6-MeGua mg^{-1} DNA respectively.

In order to investigate the O^6-alkylation by *N*-carboxymethyl-*N*-nitrosocompounds and diazoacetic acid derivatives, DNA was treated with a range of concentrations of potassium diazoacetate (0–5 mM), APNG (0–50 mM) and azaserine (0–10 mM). In all cases, dose-dependent increases in the levels of O^6-CMGua and O^6-MeGua were seen. Interestingly, the initial results on the relative amounts of each adduct of O^6-methylguanine and O^6-carboxymethylguanine formed from these glycine derivatives show that there are large differences between azaserine, APNG and potassium diazoacetate. Potassium diazoacetate produces significantly more O^6-CMdG and O^6-MedG (413.14 pmol O^6-CMGua mg^{-1} DNA) than APNG (35.36 pmol O^6-CMGua mg^{-1} DNA) or azaserine (2.79 pmol O^6-CMGua mg^{-1} DNA) at equivalent doses (Harrison *et al.* 1997). The proportions of methyl adducts to carboxymethyl adducts formed is highest for APNG than for potassium diazoacetate and azaserine (1 methyl adduct in 10 carboxymethyl adducts for APNG compared with methyl adduct in 20 carboxymethyl adducts for potassium diazoacetate and 1 methyl adduct in 40 carboxymethyl adducts for azaserine). This recent discovery is being vigorously pursued as it may be an explanation for a number of studies which show that humans at high risk of gastric cancer have elevated levels of O^6-methylguanine in DNA from gastric tissue.

As glycine is one of the most abundant amino acids in nature it would seem likely that its nitrosation products would constitute a major source of alkylating agents. Thus, the findings that nitrosated glycine derivatives, either N-nitrosopeptides or dioazoacetic acid derivatives, decompose to give DNA-methylating agents lend considerable support to these hypotheses. Of additional interest is the finding that the major O^6-guanine adduct of nitrosated glycine derivatives, O^6-CMG, is not repaired by O^6-alkylguanine alkyl transferase (Margison and Shuker 1997) and is thus likely to accumulate in the DNA of GI tract tissues and be a promutagenic lesion.

Lipid peroxidation

Malondialdehyde (MDA) is the most abundant carbonyl compound and the major mutagenic and carcinogenic product generated by lipid peroxidation (Benimara *et al.* 1995). The major DNA adduct formed at neutral pH is the highly fluorescent pyrimidopurinone product from 2′-deoxyguanosine (M_1G, Figure 6; Seto *et al.* 1983, Basu *et al.* 1988). Recent evidence suggests that M_1G is present in human liver DNA at levels of 5–11 adducts per 10^7 bases (Chaudhary *et al.* 1994). The analytical method involved the isolation of M_1G base from DNA hydrolysates followed by conversion to a pentafluorbenzyl derivative which was quantified by NICI GC–MS (Chaudhary *et al.* 1994). The limit of sensitivity of the assay was approximately 2 adducts per 10^8 base pairs with 300 μg of DNA. Malonaldehyde–DNA adducts (including

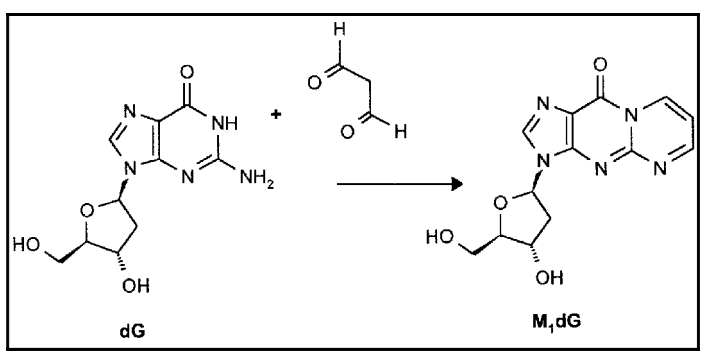

Figure 6. Reaction of malondialdehyde with 2′-

M_1dG) were also detected in normal and tumourous human breast tissues using ^{32}P-postlabelling at levels of 2–200 adducts per 10^9 normal bases (Wang *et al.* 1995).

More recently, an improved HPLC/^{32}P-postlabelling method for the measurement of the adduct in small amounts of DNA (μg) was developed (Leuratti *et al.*, unpublished observations). Standard MDA-3′-dGMP was synthesized for optimization of the method and very clean two dimensional TLC postlabelling maps were obtained using this improved procedure. The limit of detection for the synthetic standard was <1 fmol and for the adduct in DNA samples <1 adduct per 10^8 nucleotides. Mouse liver DNA and human lymphocyte DNA were analysed for the validation of the method and the results obtained in these studies are comparable to those reported in the recent literature.

In a recent paper (Fang *et al.* 1996), the average malondialdehyde–guanine adduct levels in individuals on a sunflower oil-based diet (rich in polyunsaturated fatty acids) was 3.6-fold higher than that found in individuals on a rapeseed oil-based diet (rich in monounsaturated fatty acids), thus supporting the idea of using MDA–dGMP as a biomarker of fat intake.

There has recently appeared a number of publications which suggest that lipid peroxidation gives rise to etheno adducts (Figure 7; Bartsch *et al.* 1994) and that this is relevant to the background levels of these adducts that have been observed in DNA from human tissues. Nair *et al.* (1995) found εA and εC (at levels of 2.5–25.9 adducts per 10^9 dA and 6.1–22.4 adducts per 10^9 dC, respectively) in DNA from human liver and similar observations were made in rodents. Subsequent work showed that the same adducts were formed in DNA *in vitro* when incubated with a microsomal system which generated lipid peroxides (El Ghissassi *et al.* 1995). εGua has also been detected in a human liver DNA sample (at a level of 7 adducts per 10^8 Gua) as well as in rodent liver DNA (Scheller *et al.* 1995). The studies described above have made use of sensitive analytical methods such as immunoaffinity clean-up/^{32}P postlabelling (Nair *et al.* 1995) and high resolution NICI GC–MS (Scheller *et al.* 1995) which enable unambiguous measurements to be carried out on small samples of DNA.

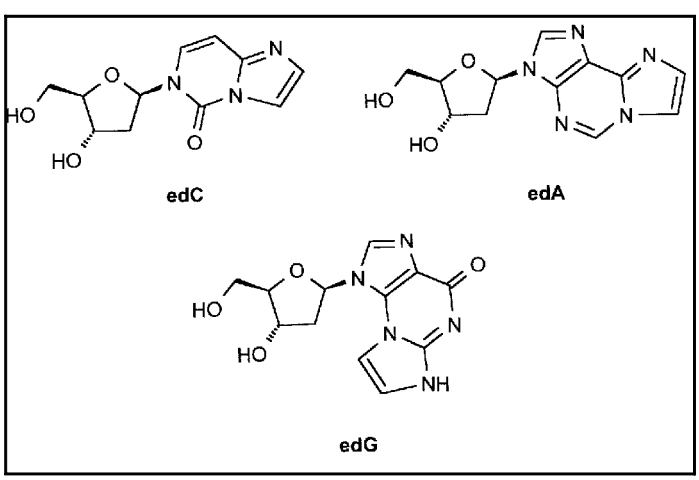

Figure 7. Structure of etheno (ε)-deoxynucleosides.

Oxidative DNA damage and diet

Superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO•) are all by-products of normal oxidative metabolism but it appears that HO• is the predominant reagent capable of oxidizing DNA. 8-Hydroxy-2′-deoxyguanosine (8-HOdG), thymidine glycol (dTG) and 5-hydroxymethyluracil (5-HOmU) are the major products of reaction with the hydroxyl radical and a number of groups have developed methods to analyse these adducts in DNA (Djuric *et al.* 1991a) or in urine (Shigenaga *et al.* 1989, Ames *et al.* 1993).

Djuric *et al.* (1991b, c) found that women (aged 18–67) who consumed at least 30% of their total caloric intake as fat had significantly higher levels of 5-HOmU in DNA from nucleated peripheral blood cells than those who consumed less than 15% dietary fat (9.29 ± 1.89 5-HOmU per 10^4 thymine vs 2.99 ± 0.56 5-HOmU per 10^4 thymine, $p = 0.004$). Experimental studies in female rats showed that both reduction in dietary fat and caloric restriction lowered 5-HOmU in DNA from the mammary gland and liver (Djuric *et al.* 1992, 1994, Djuric and Kritchevsky 1993).

In a study of 82 smokers and non-smokers in Denmark the excretion of urinary 8-HOdG was significantly higher in smokers (Loft *et al.* 1992). However, in both smokers and non-smokers there was an inverse relationship between 8-HOdG excretion and body mass index (BMI) which was attributed to the fact that a low BMI is associated with lean people who typically have a high metabolic rate. These results are consistent with experimental studies in different species where urinary 8-HOdG increases with metabolic rate (Adelman *et al.* 1988).

β-Carotene had no effect on urinary 8-HOdG in smokers in a randomized intervention study (van Poppel *et al.* 1995) and these data are consistent with the lack of correlation of intake of vitamins A, C and E on urinary 8-HOdG in the study by Loft *et al.* (1992). However, in non-smokers consumption of 300 g Brussels sprouts daily for 3 weeks did produce a 30% decline in urinary 8-HOdG (Verhagen *et al.* 1995).

Reactive nitrogen species

Nitric oxide (NO) is a relatively stable free radical gas which has been found to be an extremely important bioregulatory molecule having a number of physiological effects including control of blood pressure, antimicrobial and antitumour activity, neural signal transmission and platelet function (Moncada *et al.* 1991). These effects are usually mediated through the release of NO at very low concentrations (typically nanomolar) under tightly controlled conditions. However, during infection and inflammation, the concentrations of NO released can be several orders of magnitude higher (Marletta 1988). Under such conditions NO can be converted into a number of derivatives which have been termed 'reactive nitrogen species' (RNS) which are potentially genotoxic (Liu and Hotchkiss 1995).

Nitric oxide reacts quickly with very few compounds, but will react readily with O_2^- to form peroxynitrite ($ONOO^-$), which has been implicated as a mediator of oxidative stress-induced cellular damage. At physiological pH $ONOO^-$ is protonated to form peroxynitrous ac

initiate reactions characteristic of OH, nitryl cation (NO_2^+) and nitrogen dioxide radical ($NO_2^\bullet$). It may oxidize sulphydryl groups, induce lipid peroxidation and nitrate aromatic amino acids (tyrosine to 3-nitrotyrosine). The relatively long half-life of ONOOH (~1 s) may allow it to cross cell membranes and penetrate into the nucleus where it could induce DNA damage. Exposure of isolated calf thymus DNA and human skin epidermal keratinocytes to $ONOO^-$ or the $ONOO^-$ generator, 3-morpholinosydnonimine (SIN-1), led to extensive DNA base modification and DNA strand breakage. Large increases in xanthine and hypoxanthine (possible deamination products of guanine and adenine respectively), and in 8-nitroguanine were observed, but only small changes in some oxidized base products were seen. The pattern of DNA damage suggests that 'free' $^\bullet OH$ radicals were not major contributors to base modification caused by peroxynitrite, as $^\bullet OH$ is known to cause multiple oxidative modifications to all four bases. Instead, it seems that reactive nitrogen species play a much greater role in the mechanism of base damage, producing both nitration and deamination of purine bases when DNA or whole cells are exposed to peroxynitrite (Spencer *et al.* 1996). Future work will investigate whether this pattern of damage is unique to peroxynitrite and if it might act as a marker of cellular damage by this species *in vivo*.

Discussion

The last 10 years has seen great advances in the development of sensitive methods for the detection and quantitation of DNA damage. Many of these methods are capable of measuring, and indeed many have been used to measure food related DNA damage.

As more and more studies in humans are carried out, the phenomenon of 'background' levels of DNA damage becomes increasingly apparent. There is, however, an interesting gap between the exquisite sensitivity that can be achieved by some current analytical methods and the levels of the more common forms of DNA damage that have been detected. The latter are sometimes orders of magnitude *higher* than are capable of being detected by the former. However, some aspects of practicability need to be considered at this point. On the one hand, if only small amounts of tissue-derived DNA are available, such as from pinch biopsies or small blood samples, then clearly the absolute sensitivity of the assay needs to be very high. On the other hand, if large amounts of tissue (for example, autopsy samples) or body fluids (for example, urine) are available then methods with quite modest sensitivity are acceptable. This discussion leads directly to one of relevance – do high levels of oxidative, and certain kinds of alkylating, damage dominate the overall cumulative burden of DNA damage and does that manifest itself in overall risk of cancer? – or, do low levels of particular DNA adducts exert a disproportionate biological effect? It would appear the one approach to the resolution of this key problem is to conduct detailed studies in human populations. The suggestion of prospective cohort studies often meets with a lack of enthusiasm from many researchers because of the length of time that such studies take. However, several studies are currently underway on a vast scale (for instance, the EPIC study) with many tens of thousands of subjects recruited with samples collected and stored for eventual analysis. It is important to emphasize that no laboratory scientist is going to be expected to undertake the analysis of *all* the samples, except in very exceptional cases where an automated assay may be available. One of the most efficient and informative types of study design is a nested case-control study, in which incident cases of disease are identified within the cohort, matched with appropriate controls and the corresponding stored samples identified and analysed for some biomarker. This is exactly the strategy used in the Shanghai aflatoxin study which was described earlier. An important first step is to carry out small-scale studies in volunteers who are consuming diets which are well characterized and which can be varied in composition under strictly controlled conditions. There are a number of facilities for the conduct of such studies in the UK and suitable experimental protocols have been described (Bingham 1989).

The long term advantage of the conduct of both small-scale and large-scale studies in humans is that definitive answers will be obtained. The limits of traditional retrospective case-control studies of diet-related cancers are all too apparent and it is unlikely that the use of biomarkers of food-related DNA damage will have much to contribute except if characteristic mutational spectra can be detected in tumours which can then be related to food components consumed many years previously to the onset of the disease. As a model for this type of approach, a collaborative study is being limited in an attempt to establish whether the heterocyclic amines found in cooked meat comprise an important risk factor for colon cancer. This study will be conducted retrospectively in patients with colorectal cancer and, in particular, will investigate whether colorectal tumours that appear to be associated with heterocylic amine intake have a distinct 'mutational fingerprint' (Garner *et al.* unpublished).

In order to assess the importance of the low levels of DNA damage resulting from exogenous genotoxins, we need to estimate rates of 'background' mutations in which no specific exogenous agents are implicated. These have usually been monitored by phenotypic selection of mutants in lymphocytes, such as drug resistance in HPRT mutants or immunoselection of HLA mutants. This approach lacks sensitivity and requires the study of large populations in order to determine the contribution of endogenous genotoxins to the overall mutation rate and factors that influence their production. A new approach is to measure mutations in microsatellite sequences distributed throughout the human genome. Microsatellites are simple repeats of mono- to tetra-nucleotides, which can be detected by PCR-based methods, and their mutational spectra, such as point mutations, frameshifts, loss of heterozygosity and recombinational events can be identified. Once information has been obtained on the background mutation frequencies in health populations, it will be possible to establish whether the frequencies are modified by lifestyle factors such as diet (Davies *et al.*, unpublished).

The overall conclusion of the workshop was that, for the most part, current technology for the analysis of food-related DNA damage, particularly in the area

is sufficiently advanced that the next stage is to encourage the application of the methods in well designed small-scale human studies. However there is still some need for improvements in analytical methodology, particularly in the area of screening methods for complex dietary exposures.

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