A SHORT TERM *IN VIVO*
BIOASSAY FOR THE
ORGAN SPECIFICITY OF CARCINOGENS

by

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The possibility of using alkaline sucrose gradient analysis of the digestive tract tissue of mice to investigate the carcinogenic potential of organotropic compounds was examined. Young Swiss mice were injected with $^3$H-TdR to label the DNA of the epithelial cells of the digestive tract. Thirty h later they were force-fed carcinogenic or non-carcinogenic chemicals. Tissue samples were taken four h post-treatment and hydrolyzed on top of the alkaline sucrose gradient. Shifts in sedimentation profiles indicated that: (1) both cultured human fibroblasts and epithelial cells of the gastrointestinal system show a shift in sedimentation profile after treatment with the carcinogen MNNG that is taken to indicate repair; (2) the carcinogen 4-nitroquinoline 1-oxide (4NQO) and 6-methyl 4NQO cause DNA fragmentation in the epithelial cells of the gastrointestinal system while the non-carcinogen 6NQO lacks this capacity; (3) the ultimate carcinogen N-acetoxy 2AAF caused DNA fragmentation in esophagus and stomach cells while the precarcinogen 2AAF produced no significant effect; (4) only the carcinogenic nitrosation products of methylguanidine damaged the DNA of gastric epithelial cells; (5) the precarcinogens 2AAF and DMN produced DNA fragmentation in the main target organ - the liver - but had little effect on the epithelial cells of the stomach; and (6) extracts of the carcinogenic plant pteridium aquilinum (bracken fern) showed an organotropic DNA-fragmenting ability in vivo and in vitro that corresponded to its organ-specific tumour induction in cattle and rats. Treatment with heat appears to drastically reduce the DNA-fragmenting ability of the plant. The results suggest that the application of the sucrose gradient technique to the epithelial cells of esophagus, stomach and liver of pre-labelled ($^3$H-TdR) and force-fed young mice incorporates the advantages of in vitro short term bioassays with the completeness of tests using whole mammals.
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DNA Sedimentation Profiles of Mouse Pyloric Stomach and Esophageal Epithelial Cells Following Exposure to the Nitrosation Products of MG-HCl

DNA Sedimentation Profiles of Cultured Human Fibroblasts Following Exposure to Four Different Extracts of Bracken Fern

DNA Sedimentation Profiles of Mouse Cardiac Stomach Epithelial Cells Following Exposure to Four Different Extracts of Bracken Fern

DNA Sedimentation Profiles of Mouse Epithelial Cells from Various Gastrointestinal Areas Following Exposure to a DNA-fragmenting Bracken Fern Extract
I thank my research supervisor, Dr. H.F. Stich, for his stimulating interest and help in the preparation of this thesis. Without his nice balance of patience (to allow thought) and encouragement (to promote speed) it would never have been done.

Thanks also to my fellow researchers Bob, Lan, Richard, Paul, Brian and Charlee. They were tactful enough to pretend that the odour of the mice didn't matter.

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Among the agents that may or do cause tumours to arise in the human population, chemicals are implicated as among the most important. It is estimated that 70-90% of cancers are the result of environmental causes (Higginson, 1972; Wynder and Mabuchi, 1972). If such factors can be identified and eliminated, measures may be taken to minimize their impact. As long ago as 1775 a British surgeon named Percival Pott discovered that chimney sweeps exhibit cancer of the scrotum, apparently as a result of continual exposure to soot and tar (Pott, 1775). Hygienic measures taken to remove soot from clothing (taken in Sweden by the Sweeper's Guild, but not in Britain) dramatically lowered the incidence of these tumours. Another classic example of environmentally caused cancers is the development of nasopharyngeal tumours by employees in factories where the refining of nickel by a now obsolete process took place (Doll, 1970). In the synthetic dye industry, cancer of the urinary bladder was linked to exposure to certain aromatic amines and related chemicals (e.g., 2-naphthylamine) in a significant proportion of those contaminated (Friedell, 1969; Harper, 1969).

These are examples of occupational cancers. However, the total incidence of cancer suggests that these occupational cancers represent only a small proportion of total world tumours (Silverberg and Holleb, 1974; Eckardt, 1972). The real cancer problem lies in the genesis of those tumours which affect millions of people - gastric cancer in Japan and certain parts of Europe, liver cancer in Africa, and colorectal, breast and prostate cancer in the Western world (especially Anglo-Saxon countries).

In order to detect and eliminate the causes of these widespread tumours, epidemiological studies may provide a clue to the discovery of suspicious agents and circumstances that can give rise to cancers. Then the precise nature of that
agent or circumstance must be determined by using it to produce tumours in an animal used as a model (even though the same agent might not produce the same result in man).

Bioassays are the methods by which this purpose is carried out. They are specifically designed to detect carcinogenic chemicals, not only as a procedure for determining the cause of established tumours, but as a method for avoiding the spread of carcinogenic risk by the introduction of new chemicals which may have a potential for initiating human cancer. The powerful carcinogen 4-nitroquinoline-1-oxide (4NQO) was developed for use as a fungicide before it was discovered to cause lung tumours in test animals (Kawazoe, et al., 1967) and the risk of inadvertently causing tumours by medical use of this chemical was avoided by making its carcinogenic potential known.

Therefore, evaluating the carcinogenic potential of environmental chemicals concerns itself with two types of agents: synthetic chemicals either presently or potentially in use in the human environment, and naturally-occurring chemicals which may be responsible for existing cancers.

Bioassay systems must be made as sensitive, reliable, and specific as possible in the detection of carcinogenicity in chemicals. They must also be economical, fast, and as foolproof as possible (either in themselves or by cross-checking with other bioassays).

CARCINOGENIC PROCESS

A series of complex individual reactions and processes are thought to lead to the final overt cancer in man and animals that have the primary application of environmental chemicals as their cause. Each of these reactions and conditions may be subject to control by a number of possible modifying factors:

1) Upon application of the chemical carcinogen,
it may be activated to an ultimate carcinogen. This reaction may be modified by biochemical detoxification and elimination reactions.

2) The ultimate carcinogen may react with receptors in the cell. It is postulated that the relevant receptor is DNA, although RNA and protein receptors have not been ruled out. This interaction is subject to stereochemical conditions and competitive inhibition that are not yet well defined. The altered relevant macromolecule (e.g., DNA) may then be repaired and restored by repair enzyme systems whose susceptibility to error may play a significant part in altering the cell receptor responsible for conversion to the tumour state.

3) Duplication of the abnormal receptor so that it may be subsequently immune to the operation of repair systems.

4) Multiplication of the cells containing the abnormal receptor as latent tumour cells.

5) Growth of latent cells to form a well-differentiated tumour.

6) Conversion of the tumour by progression to an independent undifferentiated tumour.

The first two steps outlined above - biotransformation of precarcinogens to ultimate carcinogens, followed by attack of cellular or molecular receptors - may be generalized by noting that an electrophile is hypothesized as the ultimate reactant in every case (Miller, 1970). These electrophilic species will bind generally to electron-rich centers. These centers are found in proteins and nucleic acids, including both DNA and RNA. The reaction of electrophiles with electron-rich centers results in covalent bonding and, ultimately, an abnormal receptor in the cell.

With respect to DNA alkylation the most reactive site of all the DNA bases appears to be the nitrogen atom at the 7-position of guanine (the principal alkylating site of MNNG).
although it is only one of many sites available for alkylation. Other positions implicated as being particularly suspect in carcinogenesis are the 6-position oxygen and 3-position nitrogen of guanine, the 1, 3 and 7-position nitrogens of adenine, and the nitrogen at the 3-position of cytosine. In fact, almost all possible alkylatable sites are suspect since these sites have been reported to be attacked by carcinogens in vivo and in vitro (reviewed by Sarma, et al., 1974; Lawley, 1976).

In addition to alkylation of DNA, intercalation (insertion of a rigid, planar carcinogen between base pairs in the DNA double helix) and adlineation (external binding of charge-transfer complexes to bases perpendicular to the planes of the base pairs) may occur. In spite of the lack of strength of adlineation, it has been proposed as a model for interference with DNA by polycyclic aromatic hydrocarbons (Arcos and Argus, 1968), aflatoxins (Irving, 1973) and 4NQO (Paul and Montgomery, 1971). Both noncovalent interactions are much weaker than covalent linkage and this has prevented the development of firm evidence for the relevance of noncovalent binding in vivo (reviewed by Lawley, 1976).

The model system developed to explain liver carcinogenesis provides some insight into the events subsequent to DNA-chemical interaction that lead finally to cancer. The "initiation" process, or early rapid events occurring in hours or days, leads to the appearance of new cell populations and has been referred to as "neoplastic cellular evolution" (Farber, 1973). When a single dose of dimethylnitrosamine (DMN) is administered during liver cell division following partial hepatectomy, there is an eclipse period of several weeks between recovery from initial cell damage until the appearance of cellular alterations leading to progressive cell population changes and finally to liver cancer (reviewed by Farber, et al., 1974). Since a link must be found between the initial damage to the cell and final appearance of the tumour, DNA has been proposed as the relevant cellular acceptor of carcinogens. Specifically,
the somatic mutation theory of carcinogenesis proposes that chemical carcinogens are accepted by cellular DNA in such a way as to produce a nonlethal, heritable DNA damage expressed ultimately as a tumour when such cells are viewed as a group (reviewed by Foulds, 1969).

**DNA REPAIR**

When an interaction occurs between chemical carcinogens and DNA there may be a direct chemical depurination that leads to disruption of the sugar phosphate backbone and thus single-strand breaks. This can occur after treatment with alkylating agents that alter covalent and hydrophobic bonding characteristics of bases (e.g., nitrogen mustard). These agents bind alkyl groups to purine bases for the most part, but they will also alkylate pyrimidines (Lawley, 1976).

However, this "spontaneous" hydrolysis of DNA chains is not the only method for strand breakage. Enzyme-mediated removal of alkylated bases of DNA has also been proposed (Ikegami, et al., 1970). This cell-mediated excision of damaged DNA segments is thought to be part of a process of DNA repair - an attempt by the cell to maintain true DNA copies for immediate use and transmission to daughter cells. DNA repair may occur by a variety of methods. Photoreactivation repair requires a single enzyme to bind and photocatalytically cleave thymine dimers. Post-replication repair includes all those processes whereby errors in DNA are repaired after cell replication has taken place and damaged areas in DNA strands have been bypassed by the replication enzymes in such a way as to leave gaps in DNA strands. This process is largely theoretical in mammalian cells, and includes damage bypass that may take place by means of DNA recombination (reviewed by Hanawalt, 1975). Excision repair may occur according to the mechanism proposed in Figure 1. This is a process by which the cell repairs damage to DNA by removing the affected portion and replacing it with
distortion caused by bound chemical

a) damage-specific endonuclease incision, or
b) spontaneous hydrolytic fission

DNA polymerase fills gap
5' exonuclease degrades distorted DNA segment

polynucleotide ligase reforms 5' and 3' ends

replication

daughter strands
the correct structure before undergoing division.

The binding of chemical to DNA (either covalently or non-covalently) may lead to local distortion that can serve as a site for endonuclease attack, or as a position for "spontaneous" hydrolytic fission of the DNA chain (Paul and Montgomery, 1971). However, where coincident single strand breaks take place a faithful reconstruction of the original structure is unlikely, due to the absence of an intact complementary strand acting as a template for polymerase activity.

Excision repair may be monitored in cells by a variety of methods. Density labelling by radioactive 5-bromouracil or other density labels (¹³C, ¹⁵N, or ³H) and observation of newly synthesized DNA at the parental buoyant density in a CsCl equilibrium density gradient is one method (Hanawalt and Copper, 1971). Unscheduled synthesis of the DNA of cells radioactively labelled with thymine while out of S-phase has been taken to mean repair synthesis (Rasmussen and Painter, 1964, 1966; Painter and Cleaver, 1969; Stich, et al., 1970, 1973, 1977). The final rejoining step of excision repair as well as the initial incision or hydrolytic cleavage step can be monitored by examining the single-strand molecular weight distribution of DNA in alkaline sucrose gradient zone sedimentation (McGrath and Williams, 1966: Lett, et al., 1967; Laishes and Stich, 1973; Stich and Laishes, 1973; Koropatnick, et al., 1975; Abanobi, et al., 1977). This procedure will register breaks in the alkali-labile linkages in the backbone of the DNA structure, although DNA is stabilized in this respect due to a lack of 2'-hydroxyl groups on the ribose. However, the rejoining of breaks does not necessarily mean that distortions have been removed or that the original nucleotide sequence of the DNA has been restored. Also, degradation of a large amount of DNA may unmask a small amount of residual high molecular weight DNA to give the false impression that gaps have been repaired.
CHEMICAL CARCINOGENS

Chemical carcinogens may be divided into three major types according to current views: primary or ultimate carcinogens, secondary or procarcinogens, or cocarcinogens (promoting agents and factors).

PRIMARY OR ULTIMATE CARCINOGENS

These chemicals owe their biological reactivity to the chemical properties intrinsic to them when they enter the organism. They can interact directly with tissues and cell components to yield modified macromolecules which may lead to the genesis of preneoplastic cells. These are characteristic of changes leading to transformed cancer cells. Alkylating agents of various types are examples of these kinds of carcinogens, including nitrogens and sulphur mustards, sulphonic esters and sultones, ethylene imines and imides, strained or α,β-unsaturated lactones, epoxides, peroxides and chloroalkyl ethers (Miller, 1970). They are in their final reactive form as administered and generally take part in $S_{N2}$ (substitution, nucleophilic, bimolecular) reactions in which an alkylating electrophile combines with a nucleophile in the cellular target tissues or macromolecules (Price, et al., 1969; Ross, 1962; Shapiro, 1969). They are frequently not strong carcinogens, and may require multiple large doses at local tissue sites to exhibit carcinogenicity in test animals. This, presumably, is due to the presence of interfering nucleophiles (such as water and protein) which may dispose of much of the electrophile before its entry into target cells or reaction with target tissues (Miller, 1969; Miller, 1970). A small group of inorganic chemicals such as beryllium, cadmium, cobalt, nickel, lead, manganese, and chromium are, in their ionic forms, electrophiles and have been shown to be carcinogenic (Forst and Haro, 1969; Clayson, 1962). Generally, that class of chemicals that are...
electrophilic reagents, and possibly those with the ability to form free radicals, comprise the ultimate carcinogens.

SECONDARY OR PROCARCINOGENS

Differing widely in structure and charge, most carcinogenic chemicals fall into this class (Table 1). They include individual chemicals synthesized by man and complex mixtures that occur naturally. They are often chemically and biochemically inert (with respect to target macromolecules required for carcinogenesis) and spontaneous or host mediated and controlled activation reactions are required to convert procarcinogens to their ultimately reactive species.

When these procarcinogens are spontaneously converted to primary carcinogens by hydrolysis they may exhibit activity in a broad range of target species and organs due to the simplicity and universality of their "activation". On the other hand, where specific host-controlled biochemical activation is required there may be great diversity in activity from organ to organ, individual to individual, or species to species. Activation may depend on some specific enzyme system (Miller and Miller, 1971; Farber, 1973; Weisburger, 1973; Weisburger and Weisburger, 1973; Grover, et al., 1974; Oesch, 1972; Sugimura and Kawachi, 1973). This supplies a possible explanation for the observation that a chemical which is carcinogenic in some systems may show no carcinogenic activity at all in other species where the required activating enzyme or enzymes are absent.

COCARCINOGENS

These are agents that are unable to produce tumours by themselves but will potentiate the action of ultimate or procarcinogens. Complex mixtures such as tobacco smoke are now thought to contain large amounts of cocarcinogens, but rather small relative amounts of procarcinogens (Wynder and
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<td>Aromatic and heterocyclic amines and azo dyes</td>
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(adapted from Weisburger, 1976)
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<td>MNNG</td>
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</tr>
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<td></td>
<td>mouse</td>
<td>intestine, stomach, intestine</td>
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<tr>
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<td>S.G. hamster</td>
<td>glandular stomach, intestine</td>
</tr>
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<td>rabbit, dog</td>
<td>lung, stomach, intestine</td>
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<tr>
<td>4NQ0, 6-methyl 4NQ0</td>
<td>rat, mouse, hamster, guinea pig</td>
<td>lung, lung adenomas, leukemia, stomach, intestine</td>
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<td>3-methyl 4NQ0</td>
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<td>6NQO</td>
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<td>breast, liver, bladder, renal pelvis, acoustic duct, colon, lung, pancreas, bladder, kidney</td>
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<td></td>
<td>mouse</td>
<td>liver, lung, kidney</td>
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<td></td>
<td>S.G. hamster</td>
<td>liver, nasal cavities</td>
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<td>liver</td>
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<td>Bracken fern</td>
<td>cattle, rats</td>
<td>lower ileum, urinary bladder</td>
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Hoffmann, 1967; Van Durnen, et al., 1973; Saffiotti, 1969). The best known cocarcinogen is a croton oil, the extract of croton resin. It promotes mouse skin tumour formation after the application of a carcinogenic polycyclic aromatic hydrocarbon such as 2-methylcholanthrene (Hecker, 1971; Boutwell, 1974; Sivak and Van Durnen, 1971).

AROMATIC AMINES

An example of an ultimate carcinogen is the aromatic amine N-acetoxy-2 acetylaminofluorene (N-acetoxy-2AAF). This is the N-hydroxylation and subsequent esterification product of the procarcinogen 2-acetylaminofluorene (2AAF) (Cramer, et al., 1960; Miller, et al., 1961; Laishes and Stich, 1973).

Of the suitable candidates for the ultimate carcinogenic metabolite of 2AAF, N-acetoxy-2AAF shows high reactivity toward mammalian DNA, RNA and protein both in vivo and
in vitro (Miller and Miller, 1969). An oxidation reaction catalysed by the microsomal fractions found in liver, lung and bladder mucosal cells (Uehleke, 1966) is responsible for the N-hydroxylation reaction of aromatic amines to produce hydroxylamine derivatives such as the carcinogenic intermediate N-hydroxy-2AAF. Most tissues contain oxygenases, but certain cells (hepatic parenchymal cells are an example) contain in their endoplasmic reticulum the complete pathway of enzymes responsible for the hydroxylation of organic compounds. The general hydroxylation reaction may be written as:

\[
\text{R-CH}_3 + \text{NADPH} + \text{H}^+ + \text{O}_2 \rightarrow \text{R-CH}_2\text{OH} + \text{NADP}^+ + \text{H}_2\text{O}
\]

The responsible enzymes have been termed "mixed function oxygenases" (Mason, 1957) and, more recently, "mono-oxygenases" (Hayashi, 1969) and require NADPH and oxygen. They have been identified in intact animals, perfused organs, tissue slices and microsomes, and the many types of oxidation reactions and electron transport chain characteristics have been summarized (reviewed by Hutson, 1970). However, the N-hydroxylation product of 2AAF has very little ability to bind nucleic acids in vitro (Irving, et al., 1969) and a firm connection between in vitro reactivity and in vivo carcinogenicity could be established for 2AAF only when the hydroxyl group of N-hydroxy 2AAF was esterified. The "ultimate" carcinogen, N-acetoxy 2AAF, is able to bind, for example, to guanine in vitro, while 2AAF and N-hydroxy 2AAF cannot. Nucleic acid binding products isolated from the in vitro system have also been isolated from the livers of rats previously fed 2AAF (reviewed by Miller, 1970).

**NITROGEN COMPOUNDS**

Another directly acting carcinogen is the N-methyl-N-nitroso compound N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) which causes tumours in rats, mice, hamsters, rabbits and dogs.
It will alkylate the macromolecular constituents of target tissues, presumably by spontaneous N-demethylation and tautomerization in neutral aqueous media to produce transient methyldiazohydroxide (a source of methyldiazonium and methylcarbonium ions which can alkylate macromolecules). MNNG will also react with thiols to liberate methylating species, which is almost certainly a mode of activation in vivo (reviewed by Lawley, 1976).

**DIALKYLAMINES**

The N-nitroso dialkylamines are a large class of compounds, some of which are routinely detected in the environment (Hedler and Marquardt, 1968). More than 100 carcinogenic nitrosamines have been identified that can alkylate the macromolecular constituents of target tissues. The simplest of these compounds is dimethylnitrosamine (DMN) which is metabolized in vivo by a mechanism delineated by Heath in 1962. He showed that DMN undergoes N-demethylation to yield N-nitrosomethylamine and tautomerization to produce methyldiazohydroxide, which is a possible source of methyldiazonium and methylcarbonium ions—the same alkylating species produced by MNNG (see above). In this case, however, DMN will not react spontaneously and requires enzymic N-demethylation in order to be activated. Thus, it is a procarcinogen rather than an ultimately carcinogenic compound.

Unfortunately the enzymatically produced metabolites of DMN have so far been difficult to prepare synthetically, as well as isolate and store for specific analysis and testing. Therefore, the identification of relevant biotransformation
mechanisms remains largely speculative due to the instability of the metabolites (Laishes, 1974). DMN is a potent liver carcinogen in the rat, and is metabolized in human liver slices at close to the same rate as in rat liver slices (Montesano and Magee, 1970).

**NITROSATABLE COMPOUNDS**

Another example of the nitrosodialkylamines are the nitrosation products of methylguanidine, a naturally occurring compound which, when nitrosated, will produce at least two carcinogenic and mutagenic metabolites in a mixture of products. Methylnitrosoguanidine (MNNG) and methylnitrosourea (MNU) are these compounds (Endo, et al., 1974; Kapeller-Adler, 1930; Komarrow, 1929). Compounds such as methylguanidine are of importance to man, since (like many other naturally occurring or man-made substances) they can be nitrosated in the acid
conditions found in the human stomach (Endo, et al., 1974; Endo and Takahashi, 1973; Lane and Bailey, 1973) or at neutral pH by alimentary bacteria (Hawksworth and Hill, 1971). Because of the ubiquitous nature of these nitrosatable compounds they have been implicated in the genesis of human carcinomas (Sanders and Schweinsberg, 1972; Lo and Stich, 1975).

**NITROQUINOLINE N-OXIDES**

Among the procarcinogens are the nitroquinoline N-oxides, of which 4-nitroquinoline N-oxide (4NQO) is a good example. It must be enzymatically reduced to its ultimate carcinogenic state, 4-hydroxyaminoquinoline 1-oxide (4HAQO) in order to react with nucleic acid (Kawazoe, et al., 1972).

Because of its highly oxidized state, 4NQO has a low degree of electrophilic reactivity, so that it does not react covalently with DNA in vitro. However, it may intercalate and will certainly physically bind to DNA without activation. In this case 4NQO is associated with purine rather than pyrimidine bases.
On the other hand, 4NQO injected into rats bearing transplantable hepatoma cells in ascites form is found covalently bound to the DNA of those cells. Therefore, while 4NQO can become associated with DNA whether it is enzymically transformed or not, the overall evidence suggests that conversion to 4HAQ0 is necessary (reviewed by Clayson and Garner, 1976). It may be that the failure of 6NQO and 3-methyl 4NQO to be good carcinogenic compounds in animals is because of their inability to be enzymatically converted to this ultimate form (Kawazoe, et al., 1967).

**COMPLEX MIXTURES**

Although most laboratory testing is done with chemicals in as pure a state as can be obtained, most carcinogens in man's environment exist as parts of complex mixtures, and are exposed to him as such. A good example of these are carcinogens of plant origin - cycasin from cycad nuts, safrole from sassafras, and, in particular, the carcinogenic plant *pteridium aquilinum*, or bracken fern. By the late 19th century the lethal properties of this plant were scientifically recognized (Storier, 1893; Almond, 1894). The earliest intimation of a carcinogenic link with the plant came in 1960 (von Rosenberger and Heeschen, 1960) who described hematuria and polyp formation in urinary bladder mucosa of bracken-fed cattle. Work with rats and mice, corroborated by geographical and epidemiological data with Turkish bracken-fed cattle (Pamukcu, 1963), has shown that the carcinogenic agent or agents in bracken fern cause adenocarcinomas of the intestinal mucosa predominantly in the ileum and the urinary bladder (Evans and Mason, 1965; Evans and Widdop, 1966). Young animals are particularly susceptible (Evans, 1968, 1969, 1972).

Chemical studies involving the extraction of the plant with both organic and inorganic solvents have variously ascribed the carcinogenic effect of bracken fern to organic acids (Wang, et al., 1973), indanones (Kuroyanage, et al., 1974), pteroquilin (Kwasniewski, 1955), shikimic acid (Evans and Osman, 1974).
and tannin (Wang, et al., 1976). Definite identification of the carcinogenic or procarcinogenic agents on bracken fern has yet to take place.

The bracken carcinogen may or may not be hazardous to humans, but there are possibilities for danger which should not be overlooked. The contamination of milk and dairy products of cattle that are fed bracken fodder, or contamination of the water supply are the most likely possibilities. Bracken beer is brewed in some isolated areas of Norway and Siberia, but one suspects that this exposure is somewhat limited (Harrington, 1967). One unexplained, but very interesting, observation is that there is a marked regional prevalence of lip, mouth, esophagus and stomach cancer in southern Wales in comparison with the rest of the United Kingdom (Evans, 1976). Farmers are particularly vulnerable. If contamination of cow's milk is relevant, then the marginal farming and free range practices characteristic of much Welsh farming may be particularly significant. In Japan, which shares top place in the world for incidence of stomach cancer, immature bracken fern, or warabi, are considered a delicacy when steeped in hot water and sodium bicarbonate and then served cold. There is a marked regional distribution of these stomach tumours in Japan. The same vegetable is served in eastern and western Canada as "fiddlehead greens".

**ORGAN SPECIFICITY**

Chemical carcinogens are almost exclusively specific for various organs. For example, 4NQO causes primarily lung tumours (Kawazoe, et al., 1969) and DMN liver tumours in mice (Magee, 1972). Dimethylhydrazine induces colonic tumours in mice (Kanagalingam and Balis, 1975) and bracken fern ileum and bladder tumours in cattle (Pamukcu, 1955). There are several possible mechanisms to explain the observed organotropy:

1) The distribution of environmentally available
carcinogen may vary from tissue to tissue. In this way, the overwhelming majority of tumours caused by tobacco smoke will occur in the lung, where smoke arrives first. Similarly, directly acting carcinogens cause skin tumours at the site of application, and ingested compounds may cause gastro-intestinal tract tumours because this is the tissue first encountered by the carcinogen.

2) The activation of procarcinogenic compounds may vary from tissue to tissue. Certain organs contain higher levels of the P-450 cytochrome fraction that carries out the oxidation responsible for activation of mutagenic and carcinogenic polycyclic hydrocarbons (e.g., liver, kidney) (reviewed by Heidelberger, 1976). These organs may produce higher levels of ultimate carcinogens, and therefore exhibit greater susceptibility to tumours.

3) The inactivation of chemical carcinogens may vary from organ to organ. If activated carcinogens are circulated throughout the body, those organs which may inactivate the chemical efficiently may be less susceptible to tumour induction.

4) The repair of damage induced by carcinogens may vary between organs. It has been observed that repair of damage caused to DNA in one portion of the digestive tract is slower than repair of damage inflicted in another part (Kanagalingam and Balis, 1975).

5) The proliferative rates of cells in different organs may contribute to the initiation of tumours, since damage to critical macromolecules may be "fixed" more easily in rapidly dividing tissues where there is less time to repair damage inflicted by exogenous agents and enough divisions to exhibit the proliferation of cell types characteristic of the neoplastic cellular evolution in the first stage of tumour formation. For example, ethylnitrosourea is non-carcinogenic to adult brain but is carcinogenic to fetal brain (Druckrey, et al., 1970) and dimethylnitrosamine, given as a single dose, is carcinogenic to partially hepatectomized liver or liver pretreated with a single dose of carbon tetrachloride, but not to normal liver (Craddock, 1971; Pound, et al., 1973).
6) Genetic predilection for tumours in certain cell types might cause exogenous factors to produce tumours specifically in those cell types in affected individuals. Those affected by xeroderma pigmentosum, Fanconi's anemia and Bloom's syndrome show high levels of tumours of the skin (Poon, et al., 1974) and ataxia telangiectasia patients have a high level of nervous deterioration as well as stomach and other GI tumours.

The roles of these processes in organ-specificity of tumour induction is still a matter of conjecture (Magee, 1972). Also, attempts to relate sites of alkylation of DNA to organotropy have met with failure (Lijinsky, et al., 1970). Since tumour specificity may not be related to the site of binding of chemicals to DNA, it seems reasonable that the important initiating effect might be the amount of measurable DNA damage and repair that occurs in some tissues and not in others.

A variety of short term bioassays have been developed to assess the hazard these chemicals pose to humans. *In vitro* tests include transformation of cell cultures (Diploalo and Nelson, 1973; Heidelberger, 1974; Katsuta and Takota, 1972), mutagenicity tests (reviewed by Weisburger, 1975; Hollander, 1971), presence of foetal proteins or antigens (Kroes, et al., 1973; Nechaud and Uriel, 1973), the micronucleus test (Heddle, 1973), and autoradiographic assay for DNA repair (Stich and San, 1973; Cleaver, 1973). *In vivo* testing has been much more difficult owing to the complexity of the system, but work has been done to measure the amount of DNA damage and repair that occurs in target organs: *in vivo*. High levels of DNA damage have been shown to occur after the administration of chemicals known to be hepatocarcinogens (Cox, et al., 1973; Damjanov, et al., 1973; Laishes, et al., 1975; Abanobi, et al., 1977). It seems reasonable to use observation of DNA damage as a criterion for organ-specificity of carcinogen action in the various tissues of mice. Correlation between the sites of induced DNA damage and previously investigated tumour production could be investigated to determine what the link between the two is.
MATERIALS AND METHODS

CHEMICALS

Sucrose, EDTA (ethylenediaminetetraacetic acid), sodium chloride, sodium hydroxide and other common reagents were obtained from the Fisher Chemical Company, Vancouver, B.C. 2,5-diphenyloxazole (PPO) and 1,4-di(2(5-phenyloxazoyl))-benzene (POPOP) for preparation of scintillation fluid were purchased from Kent Laboratories, Vancouver, B.C.

RADIONUCLIDES

Thymidine-methyl-\(^{3}H\) (specific activity 20 Ci/mmole) was obtained from the New England Nuclear Corporation, Dorval, P.Q.

CHEMICAL CARCINOGENS

Dimethylnitrosamine (DNN) was purchased from K & K Laboratories, Plainview, N.Y. 4-nitroquinoline 1-oxide (4NQO), 6-nitroquinoline 1-oxide, and 3-methyl 4-NQO were purchased from the Daiichi Pure Chemical Company, Tokyo, Japan. N-methyl-N'-nitro-N-nitrosoguanidine was purchased from the Aldrich Chemical Company, Milwaukee, Wisconsin. 2-acetylaminofluorene (2AAF) and N-acetoxy-2AAF were kindly provided by Dr. James A. Miller, McArdle Laboratory for Cancer Research, Madison, Wisconsin.

NITROSATION OF METHYLGUANIDINE

The nitrosation procedure was a modification of that employed by Endo, et al., 1973. 218 mgm (2 mmole) of anhydrous methylguanidine hydrochloride (MG-HCl)(Sigma Chemical Co.)
was dissolved in 2 ml of distilled water. 414 mgm (6 mmole) of sodium nitrite was dissolved in another 2 ml of distilled water. 0.2 ml (0.2 mmole) of the MG-HCl, 0.2 ml (0.6 mmole) of the sodium nitrite, 0.1 ml of 10 N HCl and 0.5 ml of distilled water were mixed in a 9.5 X 1.5 cm test tube and incubated at 37° C for 1 h. Then, the pH of the solution was adjusted to 7.0 with 10 M NaOH and the volume to 2 ml with distilled water. This was the 0.1 M stock solution of nitrosation products of MG-HCl (Lo and Stich, 1975).

**EXTRACTION OF BRACKEF FERN**

The upper 10-20 cm of young bracken fern plants (*pteridium aquilinum*) that had attained a total height of approximately 30-40 cm were collected from the immediate surroundings of the James A. Mather building on the University of British Columbia campus. They were harvested in mid-June. The leaves were not yet present and the heads of the ferns were folded in the characteristic "fiddlehead" shape (*warabi* in Japanese). No attempt was made to distinguish between the several sub-species of fern.

The collected plants were extracted in 4 ways:

1) **Cold water extraction**

   i) The plants were mixed with distilled ethyl acetate (0.67 gm of plant per ml ethyl acetate) in an Osterizer blender until a fine puree was formed (30-60 sec at top speed). The solid phase was allowed to settle for several seconds and the relatively clear green supernatant was filtered through a double layer of cheesecloth and then vacuum-filtered through Celite (diatomaceous earth, Fisher Scientific Company, Vancouver, B.C.).

   ii) The filtered supernatant was washed with an equal volume of water and the water phase retained. This was the water extract of the ethyl acetate extract (extract no. 1).
2) Cold ethyl alcohol extraction

i) The plants were extracted in an Osterizer blender (0.67 gm plant per ml of distilled ethyl alcohol) by the same method as that employed for extract no. 1.

ii) The supernatant was filtered through cheesecloth and Celite. This was the cold ethanol extract (extract no. 2).

3) Sodium bicarbonate / hot water extraction

i) 0.03 gm sodium bicarbonate per gm fresh plant was sprinkled over the fresh bracken fern shoots.

ii) Freshly boiled distilled water was poured over this (4 ml per gm fresh plant) and let stand for 10 min.

iii) The brown sweet smelling infusion was poured off and retained. This was the sodium bicarbonate / hot water extract (extract no. 4).

4) Ethyl alcohol extraction of residue from extract no. 3

i) The extracted plant from process no 3 (i.e., the plants that were extracted using hot water and sodium bicarbonate) were washed 4 times, using 8 litres of fresh, cold tap water each time (in Vancouver this is "soft" water, with relatively few mineral solutes). A fifth aliquot of 8 litres was added and let stand overnight. This procedure was done in a stainless steel bucket.

ii) The fifth wash was poured off and the plants extracted with distilled ethyl alcohol as in extraction procedure no. 2 (i.e., 0.67 gm plant per ml distilled ethyl alcohol in an Osterizer blender).

iii) The resulting supernatant, filtered through doubled cheesecloth and Celite, was the ethyl alcohol extract of hot water / sodium bicarbonate extracted fern (extract no. 4).
The water-dissolved products of extraction procedures 1 to 4 were divided into aliquots of a maximum of 250 ml, frozen in liquid nitrogen and lyophilized under low pressure until dryness (this took, at most, 2 days). The resulting tarry powders were greenish-black and soluble in water.

EXPERIMENTAL ANIMALS

Outbred, 2 month old, male Swiss mice were obtained from the Animal Unit, Faculty of Medicine, University of British Columbia (origin: Connaught Laboratories, Willowdale, Ontario). They were maintained during experimentation on a diet of standard Purina Lab Chow and water ad libitum, and were subjected to a 12 h light cycle.

HUMAN CELL CULTURES

Skin punch biopsies were taken from the forearm of a 22 year old Caucasian female. The skin piece was teased into minute fragments with syringe needles and the pieces sandwiched between glass coverslips and incubated in MEM (minimal essential medium with 12-20% fetal calf serum) for 2 to 3 weeks at 37°C in a CO₂ incubator. Growth medium was changed every third day. When fibroblasts began to migrate from the tissue fragments the coverslips were opened and gross tissue fragments removed, leaving a partial monolayer of fibroblasts on the coverslips. These were incubated as above until the fibroblasts became a monolayer, at which point the cells were subcultured by standard techniques.

Cultures were maintained in a plateau phase at 37°C in a CO₂ incubator in plastic Petri dishes. Transfer passages 3 to 6 were used for all experiments. The cultures were routinely maintained in Eagle's MEM, supplemented with 15% fetal calf serum and antibiotics (200 units penicillin / ml, 40 microgm streptomycin / ml).
In order to obtain cells for alkaline sucrose gradient sedimentation analysis, approximately 60,000 cells were seeded in 5 cm plastic Petri dishes (without coverslips) and allowed to grow to confluency (7-9 days). The cells were used immediately.

**ADMINISTRATION OF CHEMICALS**

The carcinogenic and non-carcinogenic compounds were force-fed by esophageal intubation (under ether anaesthesia) in a vehicle of H₂O:DMSO (dimethylsulphoxide)(1:1) in a total volume of 0.1 ml with a 1 ml tuberculin syringe. After 4 h the animals were killed by cervical dislocation and exsanguination and tissue samples taken.

For tissue cultures, chemicals were dissolved in MEM (2.5% fetal calf serum) and added in a volume of 5 ml to the cultured fibroblasts. They were left for from 30 min to 2 h, at which time the media was removed and the cells used.

**ALKALINE SUCROSE GRADIENT ANALYSIS OF DNA DAMAGE**

a) **Labelling of gastric epithelium and liver tissue DNA:**

Young Swiss mice were injected intraperitoneally with $5 \times 10^{-5}$ Ci (0.05 ml) of $^{3}$H-TdR (specific activity, 20 Ci / mmole) to label the DNA of epithelial cells of the esophagus, stomach and liver. After 20 h the mice were injected intraperitoneally with 0.5 ml sterilized 0.9% NaCl to cause them to excrete as much free $^{3}$H label as possible from the tissues. The carcinogens and non-carcinogens were applied 30 h after the injection of $^{3}$H-TdR.

b) **Labelling of cultured human fibroblast DNA:**

When cultures of human diploid fibroblasts grown in 5 cm plastic Petri dishes without coverslips were 80 to 90% full, medium was removed and 5 ml of MEM (15% fetal calf serum) containing 2 microCi / ml (2 microliters of 20 Ci / mmole specific activity $^{3}$H-TdR per ml of MEM) was added. When the plates were
confluent (24 to 48 h later) the labelled medium was removed, the cells were rinsed 3 times with sterile, 37° c EDTA / saline buffer, and subjected immediately to the chemicals of interest.

c) Preparation of liver and gastro-intestinal epithelial tissue:

The method of Cox, et al., (1973) was followed in all of the essentials.

The animals were killed by cervical dislocation and decapitation to allow exsanguination. The liver was removed, stripped of connective tissue and gall bladder, and washed in ice-cold EDTA / saline buffer (0.024 M EDTA / 0.075 M NaCl, pH 7.4). The liver was dabbed on tissue to dry and placed in a Petri dish kept on crushed ice. 1 ml ice-cold EDTA / saline buffer was added for every gram of liver (wet weight) and the liver squashed with a spatula (3 to 5 min). The homogenate, without large pieces, was transferred to a small, cold centrifuge tube. Cell aggregates and tissue fragments were spun down at 1000 X g for 30 sec in pre-cooled centrifuge tubes. A diluted aliquot of supernatant was used to estimate the cell concentration in a hemacytometer chamber. 5 X 10^5 to 1 X 10^6 were routinely used in each alkaline sucrose gradient (ASG) sedimentation tube.

Pieces of esophagus, cardiac and pyloric stomach, duodenum, and intestine (including surface epithelium, the muscularis mucosae and submucosal layer) were removed and rinsed quickly in ice-cold EDTA / saline buffer to remove fecal or gastric contents and dabbed dry. A cold, new razor blade was used to remove a 1.0 to 1.5 mgm piece of tissue (sliced perpendicular to the direction of the sheet of tissue). 10 microl of ice cold buffer was added and the razor blade was used to mince the piece by chopping up and down 100 to 130 times while turning the Petri dish with the other hand. Tissue pieces should be able to pass through the bore of a 10 microl Corning disposable micro-sampling pipet. The tissue was then ready for layering.
d) Preparation of cultured human fibroblasts:

Fibroblasts were exposed to chemicals for from one-half to two hours, at which time the chemical was removed and the cells washed 3 times with ice-cold EDTA / saline buffer. 0.5 ml of cold EDTA / saline buffer was added and the cells were scrubbed away from the dish with a rubber policeman. The 0.5 ml cell suspension was placed in a 3 ml centrifuge tube and spun at 2600 r.p.m. in a clinical centrifuge for 5 min. The cell-free supernatant was removed and discarded. 100 microl cold EDTA / saline buffer was added and the cells were kept on an ice-bed in preparation for layering on gradients.

e) Estimation of DNA damage by alkaline sucrose gradient sedimentation:

Gradients were prepared an hour before use, according to the method of Cox, et al., 1973. Into nitrocellulose centrifuge tubes (Beckman Instrument Co., Vancouver, B.C.) was laid, in succession: 1 ml of 2.3 M sucrose; 5-20% alkaline sucrose gradient (0.9 M NaCl, 0.3 M NaOH); 0.3 ml lysing solution (0.3 M NaCl, 0.03 M EDTA, 0.1 M tris-HCl, 0.5% sodium dodecyl sulphate (SDS)); 5 X 10⁵ - 1 X 10⁶ cells or intact cell nuclei in a volume not exceeding 50 microl; 0.3 ml lysing solution; iso-octane to within 0.5 cm of the top of the tube. Gradients were placed in the buckets of a Beckman SW40 ultracentrifuge rotor and spun at 77,561 X g at an average radius of 11.10 cm (25,000 r.p.m.) for 30 min at 20⁰ C with the brake off in a Beckman L2B ultracentrifuge. Fifteen sequential fractions were taken from the bottoms of the pierced tubes, precipitated with 8-10% trichloroacetic acid (TCA) and collected on nitrocellulose membrane filters. Acid soluble radioactivity was removed by washing the filters with 8-10% TCA and ethanol. Acid insoluble radioactivity was counted by immersing the dried filters in toluene scintillation fluid (3 l toluene, 12 gm PPO, 0.3 gm
POPOP) and counting for 10 min per vial on the Nuclear Chicago Mark IIA liquid scintillation spectrophotometer.

f) Autoradiographic detection of $^3$H label in cellular DNA:

1) Mice were injected with 50 microCi $^3$H-TdR subcutaneously, as described above. After 30 h the animals were sacrificed and samples of liver, esophagus, cardiac and pyloric stomach, duodenum, colon, jejunum, urinary bladder and ileum were excised, washed in ice-cold EDTA / saline buffer, and dehydrated, cleared, embedded, sectioned, and stained (hematoxylin / eosin) by standard techniques.

2) Slides with tissue slices were dipped in Kodak NTB-3 emulsion (diluted 1:1 with distilled water), allowed to air dry for 30 min, stored at 4°C in light-tight boxes for 14 days, and developed by standard photographic procedures.
RESULTS

Cultured human cells have been shown to exhibit DNA repair synthesis following treatment with chemical carcinogens. This has been done by observation of unscheduled incorporation of $^3$H-TdR by autoradiography of the cells (reviewed by Stich, et al., 1977). Unfortunately, this repair mechanism reflects the resynthesis of excised regions of damaged DNA rather than assaying for the damaged regions themselves. With this in mind, it was attempted to show DNA repair by means of a decrease in the number of single-strand breaks over a period of time. The biophysical technique of alkaline sucrose gradient sedimentation has the advantage of being a direct demonstration of altered sedimentation properties of chemical carcinogen-treated DNA, presumably because of fragmentation of the DNA into smaller pieces. The alkaline sucrose gradient technique involves velocity sedimentation of DNA through a continuously increasing linear gradient of alkaline sucrose.

**IN VITRO**

When human fibroblasts were run on the gradient the sedimentation profile illustrated in Fig. 2 was observed. When the cells were incubated for one-half h with MNNG (5 X 10^{-6} M) in 2.5% MEM, the sedimentation profile shifted from the control level to that illustrated in Fig. 3. This demonstrates a decrease in molecular weight similar to a decrease observed previously with a variety of carcinogens (4NQO, N-acetoxy 2AAF, nitrosation products). However, when the MNNG was removed after one-half hour and the cells maintained with 10% MEM at 37° C for up to 30 h, the results shown in Fig. 4 were observed. DNA sedimented near the top of the gradient until approximately 16 to 18 h following carcinogen treatment. Then, from 18 to 30 h the DNA
Figure 2
DNA sedimentation profile of cultured human fibroblasts centrifuged through a 5-20% alkaline sucrose gradient. No carcinogens were applied.

Figure 3
DNA sedimentation profile of cultured human fibroblasts pretreated for 30 min with $5 \times 10^{-6}$ M MNNG in 2.5% MEM. The horizontal bar indicates the primary sedimentation peak of DNA from control cells.
Figure 4

DNA sedimentation profiles of cultured human fibroblasts treated for 30 min with 5 X 10^{-6} M MNNG in 2.5% MEM and then maintained in 10% MEM for: (A) 0 hr, (B) 12 hr, (C) 18 hr, (D) 30 hr. The horizontal bars indicate the primary sedimentation peaks of DNA from control cells.
gradually began to sediment at close to control levels, although the apparent molecular weight never quite reached that of normal untreated cells over the time period chosen.

**IN VIVO**

INCORPORATION OF LABELLED PRECURSOR INTO DNA

When autoradiographed slices of gastric, esophageal, duodenal, urinary bladder, and colonic tissue that had been pulse-labelled with 50 microCi \( ^3 \)H-TdR _in vivo_, it was observed that the cells incorporating label were the cells of the overlying epithelial layer of those tissues. Other cell types in the gastric tract appear to incorporate label at a much lower rate than the fast-proliferating epithelial cells. Tritium counts recovered from gross 1 to 2 mgm samples of esophagus sedimented through alkaline sucrose gradients were typically of the order of 50 to 100 disintegrations per minute (dpm) after subtraction of background counts, while similarly treated 1 to 2 mgm samples of other gastric tract tissues yielded 150 to 200 dpm after subtraction of background counts.

CONTROL SEDIMENTATION PROFILES

When alkaline sucrose gradient sedimentation was applied to esophagus, stomach and colonic tissues, sedimentation profiles such as those in Fig. 5 were observed. Gastric tract cells of animals force-fed the \( \text{H}_2\text{O:DMSO (1:1)} \) vehicle (0.1 ml per mouse) consistently gave sedimentation profiles taken to indicate no DNA damage (Fig. 5). On all runs, whether treated or untreated with chemical carcinogens, the gradients exhibited a slight amount of tissue debris just above the 2.3 M cushion after sedimentation. It might be argued that this detritus tends to sediment damaged DNA to give false control peaks, but there was little or no binding of \(^3\)H-TdR label to heavily sedimenting
Figure 5

DNA sedimentation profiles of Swiss mouse gastric tract epithelial cells derived from: (A) cardiac stomach, (B) pyloric stomach, (C) esophagus, (D) duodenum, (E) descending colon, and (F) urinary bladder. No carcinogens were applied to the mice.
% OF TOTAL $^3$H COUNTS

SEDIMENTATION
fragments of mouse lung (Laishes, et al., 1975).

TESTING OF CARCINOGENIC AND NON-CARCINOGENIC COMPOUNDS

The DNA-damaging capacity of highly oncogenic and non-oncogenic 4NQO derivatives was estimated by force-feeding rats with various nitroquinoline derivatives at equimolar concentrations and comparing the sedimentation profiles of DNA following centrifugation through an alkaline sucrose gradient.

Application of the strongly carcinogenic compounds 4NQO (1 X 10^{-4} gm / gm body weight) and 6-methyl 4NQO (1 X 10^{-4} gm / gm body weight) shifts the sedimentation profile of DNA released by cardiac and pyloric stomach tissue and esophagus to the right, indicating severe DNA fragmentation in the squamous epithelial cells (Fig. 6). The weakly carcinogenic 3-methyl 4NQO, at equimolar concentrations, elicited very little damage (Fig. 7). Application of the non-carcinogenic isomer 6NQO (2 X 10^{-4} gm / gm body weight) yielded a sedimentation profile (Fig. 8) similar to that of controls, as did epithelium of esophagus and pyloric stomach (Fig. 9).

DNA REPAIR

Mice force-fed the carcinogen MNNG (35 micrograms per gram mouse) and assayed for DNA damage in cardiac stomach epithelial cells from 4 to 30 h following showed a shift in sedimentation profile from fragmented to close to control levels (Fig. 9A), although control levels were not reached in the period of time of investigation.

PRECARCINOGENS AND ULTIMATE CARCINOGENS

Mice force-fed the precarcinogen 2AAF showed no significant change in the sedimentation profile of DNA released from the cardiac or pyloric part of the stomach (Fig. 10). However, mice treated with the ultimate carcinogen N-acetoxy 2AAF in concentrations equimolar to that of 2AAF (i.e., 1.1 X 10^{-5} gm / gm body weight) showed sedimentation profiles that indicated DNA damage (Fig. 11).
Fig. 10

DNA sedimentation profiles of cardiac stomach epithelial cells from Swiss mice force-fed 35 micrograms MNNG per gram of mouse and assayed at A) 4 h, B) 12 h, C) 18 h, and D) 30 h following administration. The horizontal bars indicate the primary sedimentation peak of DNA from control cells.
Figure 6

DNA sedimentation profiles of cardiac stomach epithelial cells from mice force-fed $1 \times 10^{-4}$ gm/gm body weight of: (A) 4NQO, or (B) 6-methyl 4NQO. Mice were sacrificed 4 hr after administration. The horizontal bars indicate the primary sedimentation peaks of DNA from control cells.

Figure 7

DNA sedimentation profile of cardiac stomach epithelial cells from mice force-fed $1 \times 10^{-4}$ gm/gm body weight of 3-methyl 4NQO. Mice were sacrificed 4 hr after administration. The horizontal bars indicate the primary sedimentation peaks of DNA from control cells.
Figure 8
DNA sedimentation profile of cardiac stomach epithelial cells derived from Swiss mice force-fed $2 \times 10^{-4}$ gm/gm body weight of 6NQO. Mice were sacrificed 4 hr after administration. The horizontal bar indicates the primary sedimentation peak of DNA from control cells.

Figure 9
DNA sedimentation profile of pyloric stomach epithelial cells derived from Swiss mice force-fed $2 \times 10^{-4}$ gm/gm body weight of 6NQO. Mice were sacrificed 4 hr after administration. The horizontal bar indicates the primary sedimentation peak of DNA from control cells.
DNA sedimentation profiles of cells from Swiss mice force-fed $8 \times 10^{-4}$ gm/gm body weight of 2AAF. (A) cardiac stomach epithelium, (B) pyloric stomach epithelium, and (C) liver. The mice were sacrificed 4 hr after administration. The horizontal bars indicate the primary sedimentation peak of DNA of tissues from control mice.
% OF TOTAL $^3$H COUNTS

SEDIMENTATION

A

B
DNA sedimentation profiles of cells of Swiss mice force-fed $1.01 \times 10^{-5}$ gm/gm body weight of N-acetoxy 2AAF. (A) cardiac stomach epithelium, and (B) liver. Mice were sacrificed 4 hr after administration. The horizontal bars indicate the primary sedimentation peaks of DNA of tissues from control mice.
It was found that application of the $^3$H-TdR aliquot sufficient to label gastric epithelial cells of 2 month old Swiss mice (50 microCi) also labelled some liver cells in the same 30 h period. These mice were not partially hepatectomized as indicated in the method of Cox, et al., 1975. The total recoverable, acid-precipitable $^3$H-labelled moiety from $10^5$ liver cells (counted with a hemacytometer) was 60 c.p.m. over background, as compared to 150 to 200 c.p.m. over background for similar gastric tract tissue. The simultaneous labelling of the DNA of liver and gastric tract epithelial cells permitted investigation of the action of precarcinogens and carcinogens of two tissues which differ in their susceptibility to DNA fragmentation by the same carcinogen.

An organ-specific effect becomes apparent if the action of the precarcinogen 2AAF is compared with that of the ultimate carcinogen N-acetoxy 2AAF. (Fig. 10 and 11). Both compounds induce DNA fragmentation in the liver but only the directly active N-acetoxy 2AAF appears to damage the epithelial cells of stomach or esophagus.

Similar results were obtained with the precarcinogen DMN and the alkylating carcinogen MNNG. Application of DMN (1.5 X 10^{-5} gm / mg body weight) by esophageal intubation resulted in sedimentation profiles similar to controls for cells of the cardiac stomach, but samples of liver from the same mouse gave sedimentation profiles indicating DNA damage (Fig. 12). The directly active MNNG produces DNA fragmentation in the stomach as well as in the liver (Fig. 13).

**NITROSATED COMPOUNDS**

Gastric cells of mice force-fed the mutagenic and carcinogenic products of methyl guanidine nitrosated by reaction in acidic solution showed DNA fragmentation of cardiac stomach.
Figure 12

DNA sedimentation profiles of cells from Swiss mice force-fed $1.5 \times 10^{-5}$ gm/gm body weight DMN. (A) cardiac stomach epithelium, and (B) liver. Mice were sacrificed 4 hr after administration. The horizontal bars indicate the primary sedimentation peaks of DNA of tissues from control mice.

Figure 13

DNA sedimentation profiles of cells from Swiss mice force-fed $3.5 \times 10^{-6}$ gm/gm body weight MNNG. (A) cardiac stomach epithelium, and (B) liver. Mice were sacrificed 4 hr after administration. The horizontal bars indicate the primary sedimentation peaks of DNA of tissues from control mice.
Figure 14

DNA sedimentation profiles of cardiac stomach epithelial cells of Swiss mice force-fed (A) $2.6 \times 10^{-4}$ gm/gm body weight of MG-HCl, or (B) the nitrosation product of $4.4 \times 10^{-5}$ gm/gm body weight of MG-HCl. Mice were sacrificed 4 hr after administration. The horizontal bars indicate the primary sedimentation peaks of DNA of tissues from control mice.

Figure 15

DNA sedimentation profiles of cells of Swiss mice force-fed the nitrosation product of $4.4 \times 10^{-5}$ gm/gm body weight of MG-HCl. (A) pyloric stomach epithelium, and (B) esophageal epithelium. Mice were sacrificed 4 hr after administration. The horizontal bars indicate the primary sedimentation peaks of DNA of tissues from control mice.
% OF TOTAL $^3$H COUNTS

SEDIMENTATION

A

B
cells by the alkaline sucrose gradient method (Fig. 14). The non-nitrosated precursor, MG-HCl, showed no detectable ability to change sedimentation velocity of DNA released from cardiac stomach cells. The MG-HCl was treated with acid and neutralized in the manner described by Lo and Stich (1975). Similar results were obtained from pyloric stomach and esophagus removed from the same mice (Fig. 15).

**COMPLEX MIXTURES**

While the scientist likes to test compounds in as pure a condition as he can obtain them, most carcinogens that appear in man's environment are in complex mixtures and he is exposed to them in that form. As an example of this pattern, plant carcinogens are a general example, and a specific one is the organotropic carcinogenic plant bracken fern (*pteridium aquilinum*). The extracts of this plant were dissolved in MEM and applied to cultured human fibroblasts for a period of 30 min (Fig. 16) or dissolved in 0.9% NaCl in distilled water and force-fed to $^3$H-labelled mice by esophageal intubation under ether anesthesia (Fig. 17). After 30 min for cultured fibroblasts or 4 h for mouse tissues samples were taken and analyzed for DNA fragmentation. The resulting sedimentation profiles indicate that extracts 1 and 2 have a DNA-damaging capacity, and that bracken fern shoots treated with boiling water and sodium bicarbonate lose a great deal of their detectable DNA-fragmenting ability.

When gastrointestinal samples of mice force-fed extract no.1 (4 mgm per gm mouse) were taken 8 h after intubation, DNA fragmentation appeared to be most pronounced in cardiac stomach, lower ileum and urinary bladder (Fig. 18), correlating well with observations of tumour specificity in cattle and rats (Hirono, *et al.*, 1970).
Figure 16

DNA sedimentation profiles of cultured human fibroblasts treated for 30 min with (A) 30 mg/ml of bracken fern extract no. 1 (cold water extract), or (B) 30 mg/ml of extract no. 3 (sodium bicarbonate/hot water extract), or (C) 30 mg/ml of extract no. 2 (cold ethyl alcohol extract), or (D) 30 mg/ml of extract no. 4 (ethyl alcohol extract of sodium bicarbonate/hot water extracted plant). All extracts were dissolved in 2.5% MEM. The horizontal bars indicate the primary sedimentation peaks of DNA of tissues from control mice.
Figure 17

DNA sedimentation profiles of cardiac stomach epithelium of Swiss mice force-fed (A) 4 mgm/gm mouse of bracken fern extract no.1 (cold water extract), or (B) 4 mgm/gm mouse of extract no. 3 (sodium bicarbonate/hot water extract of bracken fern), or (C) 4 mgm/gm mouse of extract no. 2 (cold ethyl alcohol extract), or (D) 4 mgm/gm mouse of extract no. 4 (ethyl alcohol extract of sodium bicarbonate/hot water extracted plant). Mice were sacrificed 4 hr after administration. The horizontal bars indicate the primary sedimentation peaks of DNA of tissues from control mice.
Figure 18

DNA sedimentation profiles of gastrointestinal cells from Swiss mice force-fed 4 mgm/ml of lyophilized cold ethanol extract of bracken fern dissolved in 0.5 ml of distilled water. Duplicate runs are illustrated for each tissue. Mice were sacrificed 8 hr after administration. The horizontal bars indicate the primary sedimentation region of DNA of tissues from control mice.
SEDIMENTATION

% OF TOTAL $^3$H COUNTS

CARDIAC STOMACH

UPPER ILEUM

LOWER ILEUM

ASCENDING COLON

DESCENDING COLON

URINARY BLADDER

Sedimentation
DISCUSSION

In this study the practicality of using DNA fragmentation as a short-term bioassay to study the ability of chemicals to damage macromolecules in the cell was investigated. In the past DNA fragmentation assays have been used primarily on human or animal cells cultured in vitro, as an alternative more relevant to the human system than the "classical" rodent tumour incidence tests. In the cultured cell system it has been shown that carcinogens may induce fragmentation and that an increase of apparent DNA molecular weight from damaged to near-control levels over 30 hours occurs, suggesting repair of the carcinogen-induced lesions. That repair takes place at all also suggests that these lesions are not the type that lead only to cell death, but rather those that can lead to mutations that might ultimately be expressed as tumours in vivo. However, these fast in vitro tests cannot completely replace the slower and more costly rodent tumour incidence tests, since studies employing cultured cells cannot be easily addressed to the problems of metabolic activation and inactivation, the complex interaction of carcinogens with numerous intra- and extracellular compounds and the multiplicity of cell types that may lead to the observed organotropy of many carcinogens. It would be highly desirable to have an in vivo test that could be used to investigate the initiation step of carcinogenesis, but does not ignore the process of carcinogenesis by observing only the direct incidence of tumours in the way that classical tests do. In the final analysis these rodent tests must be done to say definitely whether a compound can cause a tumour, but a fast, inexpensive intermediate in vivo assay would be useful to "prescreen" compounds to bring those with which suspicion lies down to a manageable number, and to investigate the various processes and modifying factors of tumour initiation. In short, a reliable indicator that can be measured within
a short period of application of the carcinogen is needed.

This study has investigated the use of DNA fragmentation, measured by a change in the velocity of sedimentation on an alkaline sucrose gradient, as one of these short-term bioassays to determine the in vivo action of carcinogens. The fragmentation of DNA (one of the presumed initiating steps in carcinogenesis) may be assayed for within a few hours following the application of carcinogens or precarcinogens, and can be completed within a two day period. The action of carcinogens in fragmenting DNA has already been employed by others working in vivo for liver, lung, kidney and jejeunum of rats or mice. However, all of these tests have employed the labelling of DNA with $^{3}H$-TdR over a two week period in the infancy of the test animals, followed by a six week waiting period for the mice to reach adulthood. Partial hepatectomy or nephrectomy may also be used in order to induce renewed growth of normally metaplastically dormant tissue, but this is restricted to tissues capable of regeneration.

The long prelabelling period required by these methods may be avoided by choosing gastric epithelial cells as a test system. These continue dividing into the adulthood of the animal, with the average turnover time of the cells being 20 to 40 hours. These tissues have an advantage in their use over fibroblasts in that they are epithelial and are the cells from which tumours normally arise. Also, they grow in a sheet over a basal layer that is much less likely to incorporate label. Therefore, epithelial cells may be selectively chosen as a relatively homogeneous population for study.

As a test for the effectiveness of using gastric epithelial cell DNA fragmentation as a screening assay for the carcinogenicity of certain chemicals, it was shown that:

1) Sedimentation profiles of the DNA of cells treated with strong carcinogens (4NQO, 6-methyl 4NQO) shifted far to the right (fragmented DNA), but the non-carcinogen 6NQO
was unable to fragment gastric cell DNA, even at higher concentrations. The weak carcinogen 3-methyl 4NQO was able to fragment DNA only partially. This indicates that shifts in sedimentation profiles, which are an indicator of DNA damage in vivo, may also accurately reflect the carcinogenicity of chemicals.

2) The ultimate carcinogen N-acetoxy 2AAF is able to induce DNA fragmentation in gastric epithelial cells while its precursor 2AAF is unable to do so at equimolar concentrations. Similarly, the ultimate carcinogen DMN was able to damage the DNA of liver (its main target organ in the formation of tumours), but did not elicit damage in the epithelial cells of the gastric tract, where primary tumours are not normally induced in test animals. The reason for this difference in capacity to allow DNA fragmentation may be one or more of several - differences in ability to activate the precarcinogen, inactivate the activated carcinogen, or the presence of other modifying factors - but the interesting observation is that the organ specificity that is evident in in vivo carcinogenicity tests may be paralleled in the short term by an assay that can be completed in hours or days.

3) When MG and its nitrosation products were fed to the mice, only the carcinogenic nitrosation products of the MG were able to damage the DNA of gastric epithelial cells. Precursors and carcinogenic products may also be differentiated using this technique.

4) When complex mixtures are fed to the mice, the sites of organ DNA damage correspond to the sites of tumour incidence observed epidemiologically. In the case of bracken fern shoots the ileum and urinary bladder showed the greatest amount of DNA fragmentation. This is also where tumours appeared in mice fed dried, milled bracken fern and in cattle grazed on fresh bracken fern. Also, the fern that was treated with boiling water and sodium bicarbonate prior to application to human cells in vitro or mouse gastric cells in vivo showed
a large decrease in ability to fragment DNA. Heat appears to be able to inactivate the DNA fragmenting properties of the bracken fern extract.

Fragmentation of DNA (as assayed by alkaline sucrose gradient sedimentation profiles) may be used in ascertaining the target organs of organotrophic carcinogens or precarcinogens. A good correlation between the site of greatest DNA damage (Laishos, et al., 1975) and DNA repair (Stich and Kieser, 1974) has already been observed. In support of this it may now be pointed out that 4NQ0 and MNNG cause epithelial cell damage in the stomach (where tumours appear when these carcinogens are applied to mice) and the precarcinogens 2AAF and DMN cause DNA fragmentation mainly in the liver, also the site of tumour formation.

Since an assessment of DNA fragmentation alone may indicate only the primary initiation step in carcinogenesis an in vivo assay of DNA repair is desirable. Kieser and Stich (1974) have accomplished this for liver and kidney, and Cox, et al., (1973) have assayed for DNA repair in vivo by monitoring the recovery of liver DNA to larger sizes following fragmentation with DMN. Cultured human fibroblasts in vitro or gastric epithelial cells in vivo also appear to increase the sedimentation velocity of their DNA following fragmentation by application of MNNG. Repair begins to take place at 16 to 18 hours following administration of chemical and DNA achieves a maximum rate of sedimentation following 24 to 30 hours of repair. Using the repair of gastric cell DNA after damage by MNNG as a control, it is easy to foresee the use of recovery of DNA sedimentation velocity as an assay for variations in repair both in rate and extent when individual organs or various chemicals are tested. If variability in either damage or repair of DNA (measured by a recovery of sedimentation velocity after damage) finds a parallel in incidence of tumours, measured in rodent tests or by human epidemiological data, then valuable information
might be gleaned concerning the importance of DNA damage or repair in tumour initiation.

Although a shift in DNA sedimentation profiles to indicate damage after treatment with carcinogens may be used as an indicator of organotropy, care must be exercised in the interpretation of such data. Alkaline sucrose gradient studies do not produce information on the cellular level, but on the molecular level. Therefore, other tests that produce information at the level of the cell must be employed to corroborate and amplify results. One profitable way of doing this appears to be by using autoradiographic analysis of DNA repair synthesis to allow recognition of the exact cell type that is damaged. This may help prove or disprove the idea that carcinogen-induced DNA lesions are restricted to target cells and tissues.

The use of DNA fragmentation in vivo, as measured by the alkaline sucrose gradient technique, appears to be a promising method for determining organotropy by measuring both DNA damage and repair. The use of liver tissue and gastric epithelial tissue combines the convenience of in vitro short term bioassays with the completeness of the metabolic system of the whole mammal.

Almond, N., J. Comp. Pathol. 7: 165 (1894)

Arcos, J.C., Argus, M.F., Advan. Cancer Res. 11: 305 (1968)


Damjanov, I., Cox, R., Sarma, S.D.R., Farber, E., Cancer Res. 33: 2122 (1973)


Eckardt, R.E., Environment and Cancer, Williams and Wilkins, Baltimore, (1972)


Endo, H., Takahashi, K., Aoyagi, H., Gann 65: 45-54 (1974)


Evans, I.A., Cancer Res. 28: 2252 (1968)

5: 178 (1972)

Evans, I.A., in: Chemical Carcinogens (ACS Monograph 173)
(C.E. Searle, ed.), American Chemical Society, Washington,
D.C., pp. 696-697, (1976)


Farber, E., Curr. Res. Oncol. Lect. 95 (1973)

Farber, E., D.S.R. Sarma, Rajalakshmi, S., Shinozuka, H.
in: Principles of Liver Disease (Becker, F.F., ed.), Marcel
Dekker, New York (1974)

(1969)


Furst, A., Haro, R.T., in: The Jerusalem Symposia on Quantum
Chemistry and Biochemistry - Physicochemical Mechanisms of
Carcinogenesis, vol. 1, pp 310-320, Jerusalem: Israel Academy
of Sciences and Humanities (1969)


Hanawalt, P., in: Molecular Mechanisms for Repair of DNA,
pp. 421-437, (P. Hanawalt, R.B. Setlow, eds.), Plenum Press,
New York (1975)


Hecker, E., Methods in Cancer Res. 6: 439 (1971)


Heidelberger, C., in: Carcinogenesis - a comprehensive survey,
(R.I. Freudenthal, P. Jones, eds.), p.1, Raven Press, New
York (1976)


Lo, L.W., Stich, H.F., Mutation Res. 30: 397-406 (1975).


Miller, J.A., Cancer Res. 30: 559-576 (1970)


Oesch, F., Xenobiotica 3: 305 (1972)

Paul, J.S., Montgomery, P.O'B., Louis, J.B., Cancer Res. 31: 413 (1971)
Pott, P., Chirurgical Works 5: 63 (1775)
Rasmussen, R.E., Painter, R.B., Nature 203: 1360 (1964)
Sanders, J., Schweinsberg, F., IARC Scientific Publ. 3: 97-103 (1972)


Storier, D.N., J. Comp. Pathol. 6: 276 (1893)

Sugimura, T., Kawachi, T., Methods in Cancer Res. 7: 245 (1973)

Uehleke, H., Life Sc. 5: 1489 (1966)


Von Rosenberger, G., Heeschen, W., Deut. Tierartzl. Wochschr. 67: 201 (1960)


Weisburger, J.H., Cancer Medicine, Holland and Frei, eds., Lea and Febiger, Philadelphia (1973)


Wynder, E.L., Mabuchi, K., Prev. Med. 1: 300 (1972)