INHIBITION OF DNA REPAIR BY SODIUM ASCORBATE IN VITRO AND IN VIVO

by

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ABSTRACT

Several short-term assays are in use to assess the carcinogenic hazard of chemicals. While the ability to induce initiating events that may lead to carcinogenesis is measured, compounds and conditions that might modify the ability of chemicals to cause those initiating events are not assessed by such tests. In addition, compounds that affect the ability of cells to react in a normal fashion to the damaging action of carcinogens are not detected by these methods.

Shifts in alkaline sucrose gradient profiles of centrifuged DNA (as an indication of DNA fragmentation) and formation of aryl and alkyl DNA adducts (as an indication of DNA modification) have been used as short-term assays for carcinogenic and mutagenic potential. Repair of DNA damage has been measured by restoration of near-control sedimentation profiles of DNA and the loss of aryl and alkyl adducts over time after damage or modification of DNA by carcinogens and mutagens.

In this study, the ability of sodium ascorbate to modify the DNA fragmenting and adduct-forming action of the carcinogens N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and benzo(a)pyrene (BP) was investigated. In addition, the ability of cells *in vivo* and *in vitro* to repair DNA in the presence of sodium ascorbate was assessed by the two methods described above.

It was found that sodium ascorbate inhibited repair *in vivo* and *in vitro*. In addition, sodium ascorbate was found to fragment DNA *in vivo* and *in vitro* in the presence of copper, and to inhibit the action of carcinogens *in vivo* and *in vitro* by nucleophilic

scavenging of electrophilic carcinogens.

Sodium ascorbate was also found to inhibit the binding of BP to DNA *in vivo* and *in vitro*. On the other hand, other reducing agents had other effects. Propyl gallate (a sulphydryl reducing compound) inhibited binding of BP to DNA *in vitro*, but enhanced binding of BP to DNA *in vivo*. The sulphydryl reducing agent glutathione enhanced binding of BP to DNA *in vivo* and *in vitro*.

Alkaline sucrose gradient analysis of DNA damage and recovery from that damage, and BP adduct formation in DNA and disappearance over time, appear to be suitable methods for assessment of the modifying properties of compounds and conditions on the initiating events that may lead to mutation or carcinogenesis.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

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INTRODUCTION

CHEMICAL CARCINOGENESIS

Although chemical carcinogenesis was first discovered in humans over 200 years ago (Miller, 1978) there are, today, only about 20 compounds or mixtures that are known to increase the risk of cancer in several organ sites in various subpopulations defined by their exposure in industrial, medical or societal situations. However, these are not the causes of the major burden of cancer (aside from the induction of primary bronchogenic carcinoma caused by cigarette smoke)(Doll, 1977). While the carcinogens responsible for the greater part of human cancers are unknown, much indirect evidence indicates that environmental factors (very probably chemicals for the most part) are involved in the genesis of these neoplasms (Doll, 1977). A large number of chemicals can cause cancer in various tissues of experimental animals, and humans are exposed to some of these carcinogens (generally at much lower concentration and sporadic administration). Generally, the concept is that a large number of cancers in the general population may be potentially preventable by the identification and elimination of these carcinogens.

In recent years the number of chemicals recorded in the literature has been a measure of the vast increase in chemical knowledge (Maugh, 1978), and the great majority of the approximately 4 million known chemical compounds are synthetic laboratory products (Miller and Miller, 1979). A certain number are useful enough to be used to the extent they have appeared in the human environment, and approximately 60,000 are estimated to be in "common use" in the U.S. (Maugh, 1978). The exact number may be disputed, but it is clear that at least several thousand

compounds are brought into contact with the human population in small amounts daily. A major source of contact of humans with naturally-occurring chemicals is in their daily intake of natural foods, which may contain several thousand low molecular weight, non-nutritive organic compounds among which are carcinogens and mutagens (Miller and Miller, 1979). Among these low molecular weight synthetic and naturally-occurring compounds existing in food, air, water, clothing, comsetics, etc., there exists a wide variety of structures which can produce a large range of effects in living systems. All of the compounds are toxic at sufficient dose, and toxic and pharmacologic information exists in large quantity on these compounds. However, due to the complexity, tedium, and expense of studies necessary to produce the information, knowledge of the mechanism of action is severely limited. Most information consists solely of gross data on the amount of chemical required to produce gross effects.

While the majority of toxic and pharmacological effects are due to non-covalent (and, therefore, reversible) interactions with cellular molecules (Goldstein, et al., 1974), recent years have shown that the toxic effect of chemical carcinogens, many mutagens, some allergens, and a few drugs are due to covalent interactions of these compounds and their metabolites in vivo with critical cellular molecules.

The great majority of chemical carcinogens are small organic compounds (of molecular weight less than 500) and are generally lipid-soluble and not very water soluble, although exceptions exist. There are some inorganic carcinogens, including metals (beryllium, cobalt, cadmium, chromium, and nickel compounds) as well as cis-platinum(II)diamine dichloride (Leopold, et al., 1970).

INTERACTION OF CARCINOGENS WITH INFORMATIONAL CELLULAR MACROMOLECULES IN VIVO

The transformation of normal cells to tumour cells appears to need, at least, a heritable, quasi-permanent alteration in phenotype that involves control of mitosis. This may be due to 1) heritable changes in DNA, or 2) quasi-permanent changes in genome transcription (analogous to the differential expressions of genomes of normal somatic cells). In either case informational molecules are involved, and chemical carcinogens must interact directly or indirectly with one or more informational macromolecules (DNA, RNA or protein) that have some effect on cell mitosis. Direct evidence has been provided for a link between DNA alterations and carcinogenesis by UV light (Hart, et al., 1977). However, for chemical carcinogens, the critical modification in any one of the macromolecules has not been demonstrated as the mechanism of action of any chemical or viral carcinogen. On the other hand, all adequately studied chemical carcinogens form covalently-bound derivatives with cellular macromolecules in vivo (with the exception of adriamycin, which binds tightly to DNA in vivo non-covalently (Marquardt, et al., 1977) and generates free radicals, but does not bind other macromolecules; and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is an inducer of mixed-function oxidases in liver (Poland and Kende, 1977) and so may potentiate the action of other carcinogens. Generally speaking, some binding interaction occurs between carcinogens and target macromolecules in cells to initiate carcinogenesis (Miller and Miller, 1979).

METABOLIC ACTIVATION TO REACTIVE ELECTROPHILES

The majority of chemical carcinogens require activation - metabolism to reactive forms - to react covalently with nucleic acids and proteins in vivo.

The covalent interaction is by means of non-enzymatic nucleophilic substitution. The carcinogens are termed precarcinogens, and must be converted to their final reactive form - ultimate carcinogens - in a conversion that is usually catalyzed by enzymes, and certain intermediate metabolites (proximate carcinogens) may be generated in the process.

The ultimate carcinogenic form of most, if not all, chemical carcinogens is a strong electrophilic reactant (Poland and Kende, 1977; Miller, 1970) which is able to acquire electrons from nucleophilic atoms in cellular components (especially the informational macromolecules such as nucleic acids and proteins). This is in contrast to the fact that the precarcinogenic forms of substances able to induce cancers may have very little in common in the way of structural features. The common factor is the strong electrophilicity of the ultimate reactant to which they are converted.

Some carcinogenic alkylating and acylating agents do <u>not</u> require metabolic activation, since they are strong electrophilic reactants as such, and require only dissolution in water to produce carcinogenic species. Some chemicals do not give rise to ultimate electrophilic derivatives, but appear to be able to cause development of tumours in experimental animals nonetheless. These compounds may not be initiators of tumours in a strict sense, but modifiers of secondary responses to initiation events (promotion, immune response) that permit the development of tumours from previously initiated cells.

In addition to the common ultimate electrophilicity of the diverse pantheon of chemical carcinogens, it has been shown that whenever the <u>reactive</u> form of the carcinogen can be brought into contact with DNA in mutagenicity test systems, they show mutagenic activity. When metabolic activation is taken into consideration, approximately 90-95% of chemical carcinogens exhibit mutagenicity in a variety of test systems (Hollaender,

1978; McCann, et al., 1975; McCann and Ames, 1976; Purchase, et al., 1978; Sugimura, et al., 1976). Most non-carcinogenic analogs and metabolites have little or no mutagenicity, and thus there is a strongly formal positive relationship between chemical carcinogenicity and mutagenicity.

SITES OF ELECTROPHILIC ATTACK IN VIVO

One goal of cancer research is to determine what the macromolecular target of chemical carcinogens is with respect to the cell. Because of the strong relationship between carcinogenicity and mutagenicity, the initiation step is generally regarded as involving a mutation or mutations in DNA, and these mutations may be prevented, or they may be "fixed" by various DNA repair mechanisms. Because of the rapidity of the initiation step and the persistence of the initiated state, the role of DNA is especially attractive.

However, chemical carcinogens bind to RNA and protein in vivo as well, so that epigenetic mechanisms of initiation can be invoked (i.e., stable, heritable states of aberrant cell behaviour without precursor lesions in DNA (Mintz, 1978)). The scenario is that a carcinogen could bind to a critical site in DNA, and that RNA and protein-bound carcinogen derivatives could then induce promotion (Berenblum, 1974; Scribner and Boutwell, 1972). While there is no experimental evidence to indicate that this is true, phorbol myristate acetate (a potent promoter) does not bind to cellular macromolecules in vivo (Weinstein, et al., 1979). There is very little data to explain the pleiotropic responsed to this agent in mouse skin or cell cultures, and the mechanisms of promotion. Therefore, both genetic and epigenetic mechanisms (with the possible participation of viral information) must be considered in studies on initiation and promotion in chemical carcinogenesis.

In the case of nucleic acids, many nucleophilic sites are open to attack by carcinogens *in vivo*. The most nucleophilic base is guanine, and may be electrophilically substituted at the N-3, N-7, N^2, O^6 and C-8 atoms. Adenine, although less preferential a site, is attacked at N-1, N-3 and N-7, and cytosine at N-3 and O^4. Thymine may be substituted at O^2 and O^4. Virtually any site is open to attack, and even the nucleic acid backbone may be substituted at phosphate oxygen atoms. In each case, a covalent bond is formed between the electrophilic carbon or nitrogen radical present in the ultimate carcinogen and the nucleophilic site in the nucleic acid.

In proteins, the sulphur atoms of methionine and cysteine, the ring nitrogen of histidine, and tyrosine (at two positions) may be substituted by carcinogenic electrophiles (reviewed by Miller and Miller, 1979).

CARCINOGENIC PROCESS

The induction of malignant tumours is a multifactorial process that has a multi-step evolution. There is a progression of complex individual reactions and processes that are thought to lead to the final overt cancer in man and animals. Each of these reactions and conditions may be subject to control by a number of possible modifying factors.

1) A chemical carcinogen introduced into an *in vivo* system may be activated to an ultimate carcinogen, either non-specifically or by specific enzyme systems. This reaction may be modified by biochemical detoxification and elimination reactions.

2) The ultimate carcinogen may react with targets in the cell. Although RNA and protein targets have not been ruled out, it is postulated that the relevant target is DNA. This interaction is subject to stereochemical conditions and competitive inhibition that are not yet

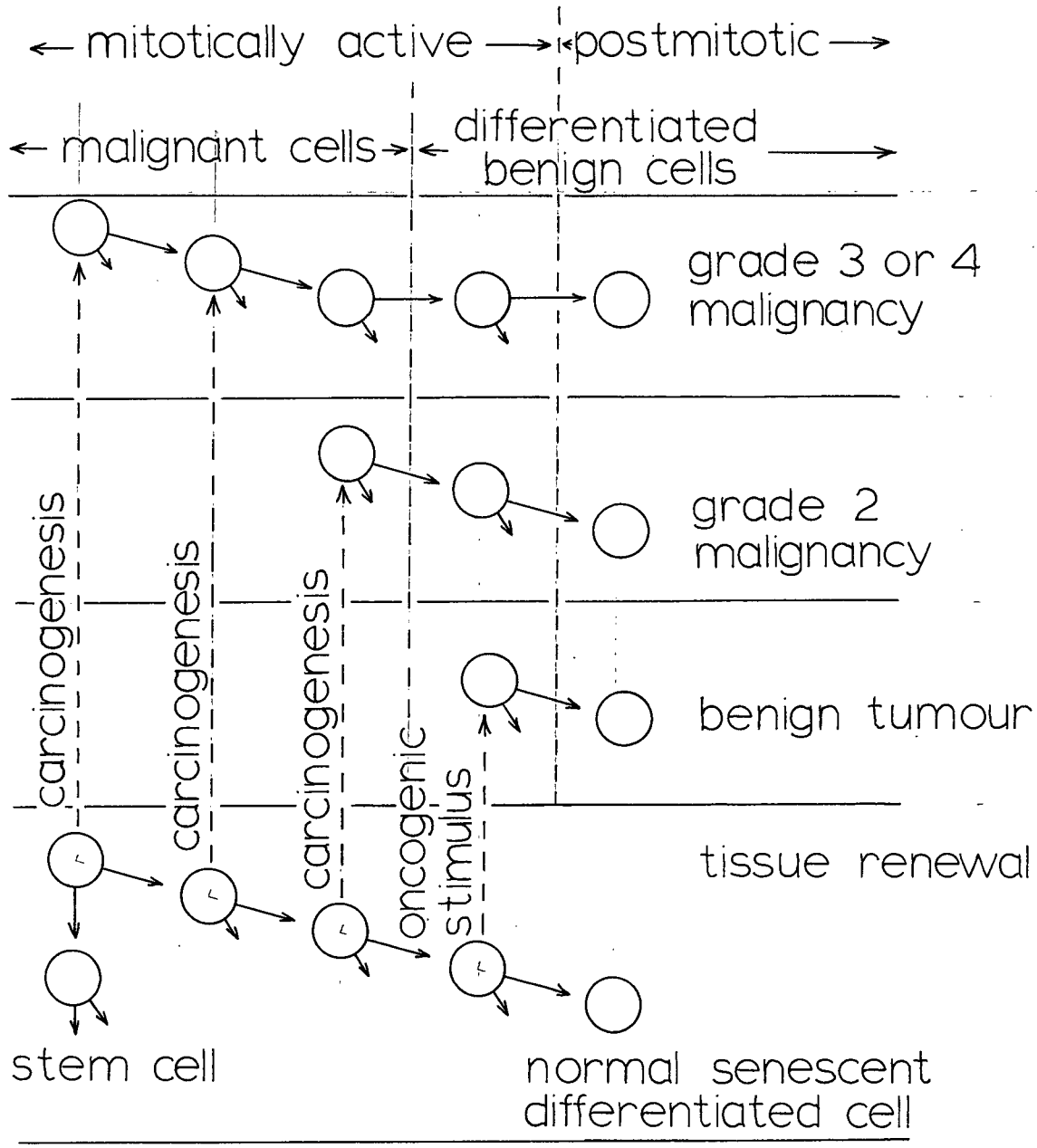
well-defined. The altered relevant macromolecule (e.g., DNA) may then be repaired and restored by repair enzyme systems whose susceptibility to error may play a significant part in altering the cell's conversion to the malignant state.

3) The altered carcinogen receptor is duplicated so that it becomes subsequently immune to the operation of repair systems.

4) Cells containing the abnormal receptor will divide to form extremely malignant tumours, or benign tumours, depending on the initial cellular targets of the carcinogenic stimulus. If primordial stem cells are the target of carcinogenic stimuli, then relatively malignant tumours (i.e., metastasizing tumours, relatively independent of control by surrounding tissue) will result. If relatively well-differentiated cells undergoing only the final stages of maturation are subjected to the carcinogenic stimulus, then relatively benign tumours (i.e., non-metastasizing, slow-dividing tumours relatively dependent on the influence of surrounding tissues) will likely result. These benign tumours would contain no cells less differentiated than their normal counterpart tissue (i.e., that tissue targeted by the carcinogenic stimulus). Tumours of intermediate levels of differentiation would be the expected result if cells of intermediate differentiation were the targets of oncogenic stimulus (Pierce, 1974; Pierce, et al., 1978)(Fig. 1).

Benign tumours would tend to appear earlier than malignant tumours, since, being more differentiated than stem cells, they would not be so "foreign" to the tissue in which they appear, and a threshold number would not need to accumulate to produce a tumour (Grobstein and Zwilling, 1953; Pierce, et al., 1978). Undifferentiated malignant stem cells would have a prolonged latent period, but would be largely refractory to environmental stimulus when a critical number had been produced.

Figure 1 Differentiation and Neoplasia



- from Pierce, et al., 1978

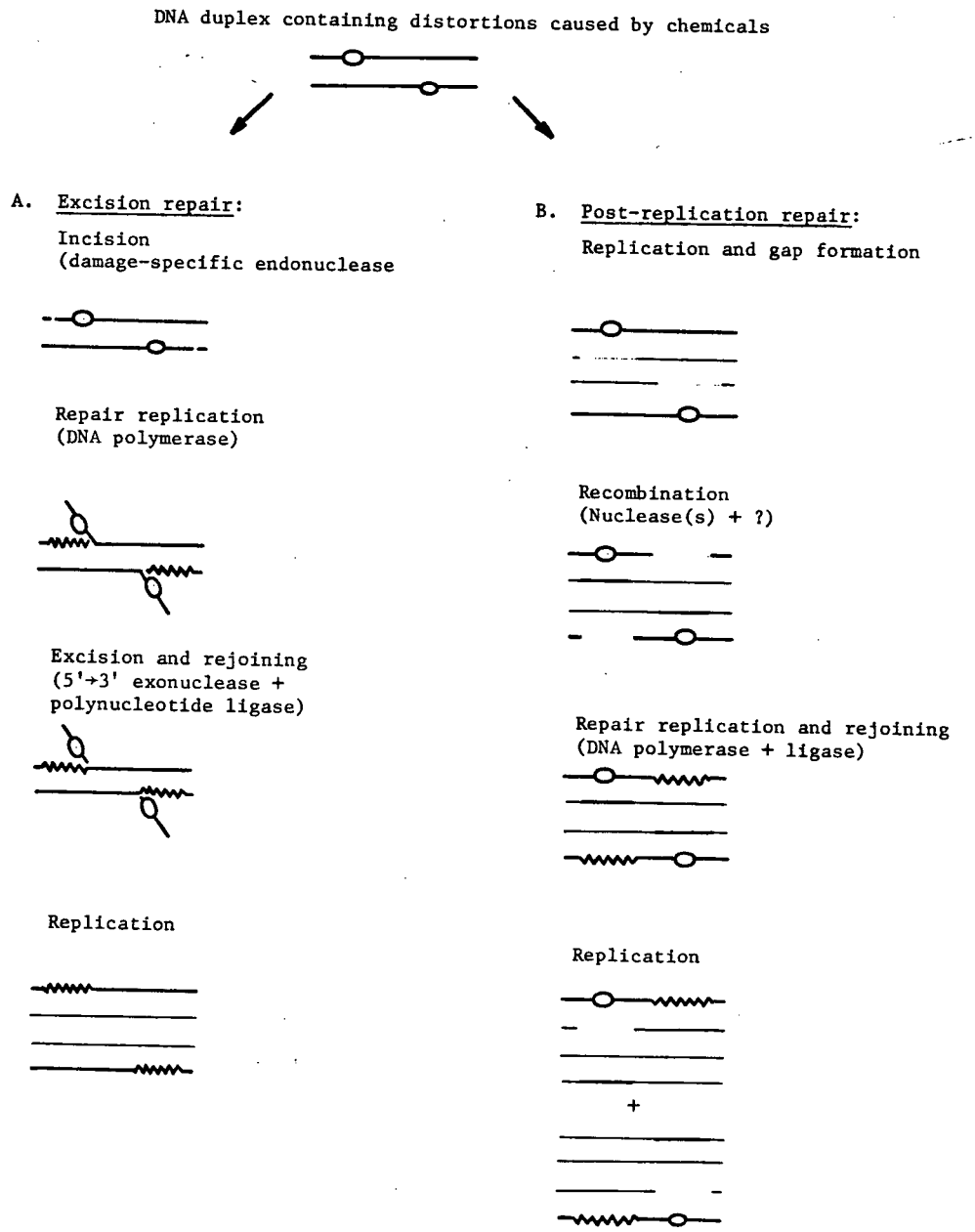
DNA-chemical interaction is, as shown here, far removed from the final observation of gross tumours. The model system developed to explain liver carcinogenesis proposed that the "initiation" process (or early, rapid events occurring in hours or days) leads to the appearance of new cell populations. This model system has been referred to as "neoplastic cellular evolution" (Farber, et al., 1974). A single dose of the potent liver carcinogen diethylnitrosamine (DEN) administered during liver cell division following partial hepatectomy will not induce cellular alterations leading to progressive cell populations, and hence to liver cancer, until several weeks following initial cell damage (Solt and Farber, 1977; Ogawa, et al., 1979; Fiala, et al., 1972; Laishes, et al., 1978). Since a link must be found between the initiated damage to the cell and final appearance of the tumour, DNA has been proposed as the relevant cellular target of carcinogens. In general, the somatic mutation theory of carcinogenesis proposes that chemical carcinogens modify cellular DNA in such a way as to produce a non-lethal, heritable DNA change expressed ultimately as a tumour when such cells are viewed as a group (Foulds, 1969; Weinstein, et al., 1979).

DNA REPAIR

When chemical carcinogens interact with DNA there may be a direct chemical depurination, either spontaneous or enzyme-mediated, that leads to disruption of the sugar-phosphate backbone and thus single-strand breaks. This may occur after treatment with alkylating agents that alter covalent and hydrophobic binding characteristics of bases (e.g., nitrogen mustard). For the most part, purine bases are alkylated, although pyrimidines may be alkylated to a lesser extent (Lawley, 1976).

However, this "spontaneous" hydrolysis of DNA strands is not the

FIGURE 2


DNA duplex containing distortions caused by chemicals
A. Excision repair:
Incision
(damage-specific endonuclease
Repair replication
(DNA polymerase)
Excision and rejoining
(5'→3' exonuclease +
polynucleotide ligase)
Replication
B. Post-replication repair:
Replication and gap formation
Recombination
(Nuclease(s) + ?)
Repair replication and rejoining
(DNA polymerase + ligase)
Replication

only method for strand breakage. Enzyme-mediated removal of alkylated bases from 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. In most eukaryote cells there are three principal mechanisms for the repair of DNA damage: photo-reactivation, excision repair and post-replication repair (Fig. 2). Photo-reactivation repair requires a single enzyme to bind and photocatalytically cleave thymine dimers. Post-replication repair includes all those processes whereby errors in DNA that persist after nuclear 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 daughter strands. The process is largely theoretical in mammalian cells. Damage may be bypassed and "left behind"by means of DNA recombination (Hanawalt, 1975). Excision repair is an enzyme-mediated process by which the cell repairs damage to DNA by removing the affected portion and replacing it with the correct structure before undergoing division.

The covalent or non-covalent binding of chemical to DNA 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, et al., 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.

DNA REPAIR INHIBITORS

The process of inhibition of DNA repair in mammalian cells is still

rather poorly understood. According to Cleaver (1974) caffeine is an outstanding inhibitor of excision repair in bacteria, along with chloroquine, quinacrine, chloramphenicol and acriflavine. In the bacterial case, this is due to inhibition of repair replication at concentrations that do not affect normal semi-conservative repair. The precise method by which this is done is unknown, although binding to single-stranded DNA as well as inhibition of DNA polymerases, endonucleases and exonucleases has been implicated, and some cell-mediated binding of the inhibitor to DNA may be the necessary prerequisite (reviewed by Kihlman, 1977). Interestingly, caffeine is not included among those agents that are able to inhibit DNA repair replication or excision of UV dimers in mammalian cells (although its effect on the excision of other types of chemical lesions in not known). Compounds observed to inhibit repair replication in mammalian cells will also inhibit semi-conservative DNA replication. These compounds are acriflavine, crystal violet, actinomycin D, chloroquine and iodoacetate, and they appear to act by some mechanism that includes covalent or non-covalent binding to DNA (Kihlman, 1977).

However, caffeine has been shown to inhibit the gap-filling associated with post-replication repair in rodent cells at concentrations that have little effect on semi-conservative DNA replication (Cleaver and Thomas, 1969; Fujiwara, 1975; Nilsson and Lehmann, 1975). In human cells, caffeine has little effect on normal human fibroblasts, but strongly inhibits gap-filling (Buhl and Regan, 1975; Lehmann, et al., 1975) and the ability to synthesize DNA segments of normal size at long times after irradiation (Buhl and Regan, 1974) in cultured xeroderma pigmentosum skin fibroblasts.

MEASUREMENT OF DNA DAMAGE AND REPAIR

The only true test for carcinogenicity of chemicals is the induction of tumours in whole animals. Because of the high cost in time and effort involved in assays of this type, short-term in vivo and in vitro bioassays have been developed to assess carcinogenic risk by measuring the ability of compounds to induce primary events in cells where those primary events can be linked to the causal, initiating events of carcinogenesis. DNA damage and its subsequent repair have been implicated as just such events (Miller and Miller, 1969; Kihlman, 1977; Weinstein, et al., 1979; Foulds, 1969).

1) Alkaline sucrose gradient sedimentation:

The final DNA ligation and chromosomal reorganization steps of DNA repair, as well as the original carcinogen or enzyme-mediated cleavage step, can be monitored by examining the single-strand molecular weight distribution of DNA in alkaline sucrose gradient zone sedimentation profiles. Originally adapted to detect DNA fragmentation in cultured cells by ionizing radiation (Lett, et al., 1967; McGrath and Williams, 1966) it has been modified to assay chemical-induced fragmentation in cultured cells (Laishes and Stich, 1973; Stich and Laishes, 1973; Andoh and Ide, 1972; Coyle and Strauss, 1970) and mammalian cells in vivo (Cox, et al., 1973; Laishes, et al., 1975; Koropatnick and Stich, 1976; 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. We have recently developed a system for in vivo determination of DNA damage and repair by

chemical agents using mouse gastric epithelial cells as a test system (Koropatnick and Stich, 1976; Stich and Koropatnick, 1977). In this method the terms "single-strand break" and "fragmentation of DNA" are used operationally and refer to DNA that sediments slowly in comparison with control DNA sedimented under identical conditions (Abanobi, et al., 1977). Repair of DNA is measured by a shift in sedimentation profile peaks, from regions indicating DNA fragmentation to those approximating profile peaks derived from untreated control DNA, in the period following chemical administration. This repair involves, not only the rejoining of single DNA strands, but also repair-type processes that involve considerably larger DNA segments than those concerned with single-strand rejoining, possibly reorganization of chromatin (Elkind and Kamper, 1970). Therefore, substances that may be shown to inhibit repair (as monitored on gradient profiles) may inhibit complex chromatin reorganizational steps as well as simple ligation.

2) Excision of aryl and alkyl adducts from DNA:

Arylation or alkylation of macromolecular targets in cells occurs with almost all carcinogenic species (see above). Both alkylating and arylating agents will modify DNA to produce modified bases, which may then be excised by enzymatic or non-enzymatic processes within the cell. Benzo(a)pyrene adducts are excised from DNA in vivo in the case of primary hamster embryo cells (Ivanovic, et al., 1978), human lung cell cultures (Feldman, et al., 1978) and mouse embryo fibroblasts (Brown, et al., 1979). In addition, methylated sites in mammalian DNA produced by a variety of chemical agents administered in vivo have been shown to be excised over a period of 24 to 72 hours, although a significant amount (10-30% of

original methylated sites) appears to be resistant to excision (Pegg, 1978; Montesano, et al., 1979; Swann and Mace, 1980; Kleihues and Margison, 1974, 1976; Goth and Rajewsky, 1974; Nicoll, et al., 1977; Thorgiersson, et al., 1980). In general, cultured cells are treated with radioactively-labelled aryl or alkylating agents by incubation in medium for from 12 to 24 hours (Ivanovic, et al., 1978) and mammals are injected subcutaneously or intraperitoneally and allowed to remain untreated for 12 to 18 hours (Thorgeirsson, et al., 1980). DNA is isolated from cells or tissues of interest at up to 72 hours following the procedures described, and the amount of radioactive label covalently bound to the DNA is used to estimate the level of aryl or alkylation of DNA. Since O-6 aryl and alkyl guanines may be more important than other adducts in mutagenic and carcinogenic effects (Loveless, 1969; Gerchman and Ludlum, 1973) a refinement of this procedure has been to hydrolyse DNA by acid or enzymes, and separate purine bases on Sephadex G-10 columns to determine the extent of O-6 methylation specifically (Kleihues and Magee, 1973).

FACTORS AFFECTING CHEMICAL CARCINOGENESIS

In view of the multi-step nature of mammalian induction of cancer, modification of the ability of chemical carcinogens to cause tumours may take place at two different levels. First, environmental factors may affect the primary "initiation" steps in carcinogenesis that take place during the period immediately following exposure to carcinogens (up to several days) and, second, they may affect the behaviour of cells that already carry the critical change that places them on the path toward becoming tumour cells.

The factors falling into the first group include those that affect the

activation of precarcinogens, the interaction of ultimate carcinogens with macromolecular targets in cells, and those that alter the cellular response to interaction of carcinogens (e.g., cell division, DNA repair). The factors that are included in the second group are those that modulate programs of gene expression to induce clonal growth of initiated cells (Weinstein, et al., 1979) or inhibit their growth (Higginson, 1979) and a host of ill-defined factors, including sociological characteristics (e.g., "age at first intercourse" or "age at first marriage" with respect to the incidence of adult mammary tumours) that have a complex effect on tumour induction (reviewed by Higginson, 1979).

1) Factors affecting the initiating action of carcinogens

A number of studies have demonstrated that it is possible to protect against the carcinogenic effects of chemical carcinogens by inducing increased mixed function oxygenase activity. Polycyclic hydrocarbons (either carcinogenic or non-carcinogenic) have been shown to inhibit the occurrence of hepatic cancer resulting from the feeding of 3'-methyl-4-dimethylaminobenzene (Richardson, et al., 1952; Miller, et al., 1958). Also, polyaromatic hydrocarbon (PAH) inducers of mixed function oxygenases have been shown to markedly reduce the incidence of tumours of the liver, mammary gland, ear duct, and small intestine in rats fed 2-acetylamino-fluorene or 7-fluoro-2-acetylaminofluorene (Miller, et al., 1958). Recently, it has been shown that it is possible to protect against the carcinogenic effects of a number of other carcinogens, including urethane, dimethylbenz(a) anthracene (DMBA), aflatoxin, bracken fern, and benzo(a)pyrene (Table 1). In each case, the modifying compounds used are potent inducers of mixed function oxygenase activity.

Table 1

Inhibition of carcinogenesis by induction of microsomal enzyme activity

Carcinogen	Inducer	Species	Organ	References
3'-methyl-4-dimethyl-aminoazobenzene	Polycyclic hydrocarbons α-benzene hydrochloride	rat	liver	Miller, et al., 1958 Richardson, et al., 1972
Urethane	β-naphthoflavone	mouse	lung	Yamamoto, et al., 1971
7,12-dimethylbenz-(a)anthracene	Polycyclic hydrocarbons	rat	breast	Huggins, et al., 1964
Aflatoxin	Phenobarbitol	rat	breast	McLean and Marshall, 1971
Bracken fern carcinogen	Phenothiazine	rat	small intestine bladder	Pamukcu, et al., 1971
Benzo(a)pyrene	β-naphthoflavone	mouse	lung, skin	Wattenberg and Leong, 1970

The paradoxic nature of this induction effect resulting in reduced carcinogenesis requires some explanation. It can be argued that if a compound is activated by an enzyme system to a carcinogenic form, then enhancement of that system would result in greater induction of cancers. This would be the case in situations involving an effect in which there is a substantial "threshold level" for the carcinogenic agent, below which tumours would not be initiated, irrespective of length of exposure. However, there appears, in the case of chemical carcinogens, to be either no threshold or a very low threshold (Dipaolo, et al., 1971). Therefore, it might be expected that slow activation would result in greater carcinogenic effect than rapid activation. It could be that the carcinogenic species must be applied at a critical time or times in the cell cycle. Also, there is less likelihood of loss of activated species (due to carcinogen interaction with non-specific cellular targets) which might be expected to occur when an excess amount of carcinogenic species is produced over that most effective for the number of critical binding sites (Wattenberg, 1974). In addition, the ultimately reactive carcinogenic species are only transient compounds in a pathway employed by the cell to detoxify those compounds. Thus, inducers of the mixed function oxygenases induce detoxification (usually by ring hydroxylation) as well as activation (usually by nitrogen hydroxylation) as is the case for aromatic amines (Miller and Miller, 1969).

In addition to enhancement of mixed function oxygenase activity, certain conditions may decrease enzyme activity. Administration of some chemicals depresses enzyme action (diethyldithiocarbamate to reduce metabolism of dimethylnitrosamine (Abanobi, et al., 1977) and coumarin or a-angelicalactone to reduce metabolism of benzo(a)pyrene and benzo(a)pyrene-induced neoplasia of forestomach (Wattenberg, et al., 1979). Also, nutritional states may also decrease enzyme activity -

starvation of Sprague-Dawley rats for 24 hours results in almost total loss of mixed function oxygenase activity in small intestine (Wattenberg, 1971) as is the case for rats fed a fat-free diet (Wattenberg, 1974). Purified diets also resulted in loss of activation ability in small intestine (Wattenberg, 1974). Thus, most, if not all, of the mixed function oxygenase activity of the small intestine is caused by an exogenous inducer or inducers.

The ability to enhance the production of ultimate carcinogens or to stimulate their deactivation is not necessarily confined to separate modifying compounds. The pyrolysis products of tryptophan -norharman (9H-pyrido-(3,4-b)-indole), harman, and 3-methyl indole (skatole) - are capable of inhibiting the whole metabolic pathway of BP, including both the conversion of BP to ultimate carcinogenic form, and production of water soluble metabolites from the ultimate carcinogenic species (Fujino, et al., 1978; Matsumoto, et al., 1977). On the other hand, in in vitro systems employing an artificial activation mixture with S-9 microsomes derived from rat liver, high concentrations of harman or norharman result in a decrease in mutagenic potential, while low concentrations produce an increase in mutagenic potential (Fujino, et al., 1978). This is due to a differential inhibition of activating or deactivating enzyme systems, since harman and norharman are more active in depressing the deactivating ability of enzymes that convert hydrophobic metabolites to hydrophilic ones (R_1)(Matsumoto, et al., 1977) than in depressing the activating ability of enzymes that convert the parent compound to the oxygenated intermediate (R_2). Low concentrations of inhibitor may therefore exert partial inhibition of R_1 and complete inhibition of R_2, resulting in accumulation of carcinogenic BP* intermediates:

$$BP \xrightarrow{R_1} BP^* \xrightarrow{R_2} \text{BP conjugates}$$

2) Inhibition of non-enzymatic formation of ultimate carcinogens:

The best known example of a compound preventing the non-enzymatic production of carcinogenic species is the case of nitrosation of nitrosatable compounds in acid conditions. Methylguanidine may be reacted with nitrite at low pH to produce mutagenic and carcinogenic compounds, including methylnitrosourea and N-methyl-N'-nitro-N'-nitrosoguanidine (Lo and Stich, 1978). Ascorbic acid is able to prevent nitrosation by preferentially reducing the nitrous acid (Mirvish, et al., 1972; Synnot, et al., 1975) in a reaction that is well understood (Dahn, et al., 1960). Other reducing agents, including cysteine, cysteamine, and propyl gallate are also able to inhibit the formation of these nitrosation products, which have been implicated as being biologically available by reaction of nitrite with various compounds in the acid conditions of the stomach (Lo and Stich, 1978).

3) Inhibition of action of ultimate carcinogens:

Once the reactive form of a carcinogenic species has been created, agents that alter the activation of precarcinogens by enzymatic means will have little effect on carcinogenic potential. If the reactive species is sufficiently electrophilic, weak nucleophiles (such as water and protein) may exert a protective effect by acting as non-critical "targets" (Miller and Miller, 1979). However, carcinogens of lesser electropotential may be inhibited in their action by "trapping" of the reactive electrophilic species through administration of agents that a carcinogen reduces preferentially over critical cellular macromolecules (Bartsch, et al., 1972 1973;

Miller and Miller, 1969, 1974, 1976; Miller, 1970; Scribner and Naimy, 1973). Reducing agents such as butylated hydroxyanisole and butylated hydroxytoluene (Grantham, et al., 1973), ascorbic acid (Guttenplan, 1977; Pipkin, et al., 1969; Schlegel, et al., 1969), selenium (Jacobs, et al., 1977), cysteamine (Marguardt, et al., 1974) and disulfiram (Schmall and Kruger, 1972) have also been shown to inhibit carcinogenesis in animal test systems, or mutagenesis in bacterial asseys, if they are applied concurrently with carcinogens. Chemical carcinogens of every type capable of producing reactive electrophiles are susceptible (Rosin and Stich, 1978).

Sodium Ascorbate

While synthetic chemicals and those found in exotic foods attract most attention when carcinogenic and mutagenic hazards of environmental agents are assessed, chemicals which are an integral part of cellular metabolism or are of vital nutritional value are often passed over.

Ascorbic acid (vitamin C) is consumed in large doses by humans (Lewin, 1976; Pauling, 1970). It has been widely used, and is generally regarded as safe, as a food additive to prevent formation of carcinogenic nitrosation products, and to prevent "browning" by air oxidation (Cardesa, et al., 1974; Fiddler, et al., 1978; Lo, et al., 1978; Mirvish, et al., 1975, 1976). Sodium ascorbate has a capacity for inactivating bacteriophage (Murata, 1975) and mammalian viruses (Jungeblut, 1935,1939), possibly by the liberation of peroxide from oxidation of ascorbate since catalase provides protection (Schwerdt and Schwerdt, 1975; Wong, et al., 1974), or by the action of monodehydroascorbate radical produced during the oxidation process (Bielski, et al., 1975). Sodium ascorbate has been shown to inhibit the formation of ultimate carcinogens by acting as a

preferred site of oxidation in the oxidation-reduction reaction that reduces peroxide by the action of peroxidase and an electron donor (Floyd, et al., 1976) as well as acting as a "trapping" agent by preferential reduction of ultimate electrophiles (see above). In addition, sodium ascorbate has been shown to have antimutagenic properties (Guttenplan, 1978; Rosin and Stich, 1979; Marquardt, et al., 1977) and anti-cancer properties (Cameron and Campbell, 1974; Pipkin, et al., 1969; Raineri and Weisburger, 1975).

However, ascorbic acid has been reported to exert effects other than the protective ones outlined above. Sodium ascorbate has been shown to increase the frequency of sister chromatid exchanges (a sensitive indicator of DNA damage) (Speit, et al., 1980). It has been shown to fragment DNA and induce chromosome aberrations in cultured cells (Stich et al., 1976) as well as mutations in S. Typhimurium (Stich, et al., 1978). Copper(II) as well as Mn(II) and Fe(II) and (III) enhance the chromosome-damaging effect of ascorbate by catalyzing the autoxidation of ascorbate to form H_2O_2, since catalase reduces or abolishes the effects (Stich, et al., 1979). Ascorbate will also inhibit mitosis (Stich, et al., 1979).

Sodium ascorbate has also been shown to convey an increased sensitivity to cells to treatment with carcinogens and mutagens, causing cell death and decreased incorporation of thymidine by unscheduled DNA synthesis at lower carcinogen and mutation concentrations than those normally required (Stich, et al., 1978, 1979). Sodium ascorbate will also enhance the mutagenicity of N-hydroxy-2-acetylaminofluorene (Thorgeirsson, et al., 1980).

TYPES OF CARCINOGENS

Chemical carcinogens may be divided into three classes: primary

or ultimate carcinogens, secondary or precarcinogens, and cocarcinogens (promoting agents and factors).

1) Primary carcinogens:

These chemicals are biologically active carcinogens without recourse to metabolic activation. They may interact directly with tissues and all components to yield modified macromolecules that may produce preneoplastic cells. Primary carcinogens include various types of alkylating agents, such as nitrogen and sulphur mustards, sulphonic esters and sulfones, ethylene imines and imides, strained or α, β-unsaturated lactones, epoxides, peroxides and chloroalkyl ethers (Miller, 1978). They are in their final reactive form as administered, and take part in S_N2 (substitution, nucleophilic, bimolecular) reactions with critical macromolecules in vivo (Price, et al., 1969; Ross, 1962; Shapiro, 1969). They are not generally strong carcinogens - due, presumably, to the interference of non-critical nucleophiles (water, proteins) in vivo which makes it necessary to administer large doses at local tissue sites to induce tumours (Miller, 1978). Much of the electrophile may be disposed of before entry into target cells.

Most of the inorganic carcinogens (e.g., metals) are electrophiles in their ionic forms (Forst and Haro, 1969).

2) Precarcinogens:

Most carcinogenic chemicals, synthetic or natural, fall into this class, so structures differ widely. They are usually chemically and biologically inert with respect to target macromolecules, and require spontaneous

or host-mediated and controlled activation reactions to convert them to their ultimate reactive species. Where they are spontaneously converted to primary carcinogens by hydrolysis, they may be active for a broad range of organs and species, due to the simplicity and universality of their activation.

However, when specific host-controlled biochemical activation is required, there can be great diversity in activity from organ to organ, individual to individual or species to species. Activation depends on specific enzyme systems (Kasper, 1974; Mukhtar, et al., 1979; Grover, et al., 1974). This is a possible reason why a chemical which may be carcinogenic or mutagenic in one system may be non-carcinogenic or mutagenic in an organism or system where the required activating system is absent.

3) Cocarcinogens:

These are agents that cannot be used to induce tumours, but will potentiate the tumour-inducing action of ultimate or precarcinogens. Complex mixtures such as tobacco smoke are thought to contain large amounts of cocarcinogens, but small relative amounts or precarcinogens (Wynder and Hoffman, 1967; Van Duurnen, et al., 1973; Saffiotti, 1969). Croton oil is the best known cocarcinogen (and extract of croton resin). It promotes skin tumour formation after the application of a carcinogenic polycyclic aromatic hydrocarbon, such as 2-methylcholanthrene (Boutwell, 1974; Sivak and Van Duurnen, 1971).

SPECIFIC CARCINOGENS

1) Nitrogen compounds:

Two compounds that are potential alkylating agents are methylnitrosourea (MNU) and N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). Both are potent carcinogens (Table 2). As a class, the alkylnitrosamides are impressive because of the wide variety of tissues in which they induce tumours, and by the susceptibility to low doses of most of the species in which they have been tested (Lowry and Richardson, 1976; Magee, et al., 1976). This is presumably due to the fact that both require only reaction with certain ubiquitous nucleophiles (water, thiols, amino groups) to be converted into electrophiles that can successfully alkylate critical cellular nucleophiles (Fig. 3). Both of these compounds are found among the nitrosation products of methylguanidine which may be produced by reaction in the acid conditions found in human stomach (Hedler and Marquardt, 1968; Heath, 1962; Montesarro and Magee, 1970) or at neutral pH by alimentary bacteria (Badger, 1962). Because of the ubiquitous nature of these nitrosatable compounds, they have been implicated in the production of human carcinomas (Kotin and Falk, 1963; Lijinsky and Ross, 1967).

2) Dialkylnitrosamines:

The N-nitrosodialkylamines comprise a large class of compounds, some of which are routinely detectable in the environment (Hedler and Marquardt, 1968). Dimethylnitrosamine (DMN) is the simplest of these compounds. They are less versatile than the nitrosoamides because enzymic N-demethylation is the first step in production of the ultimate carcinogenic

FIGURE 3

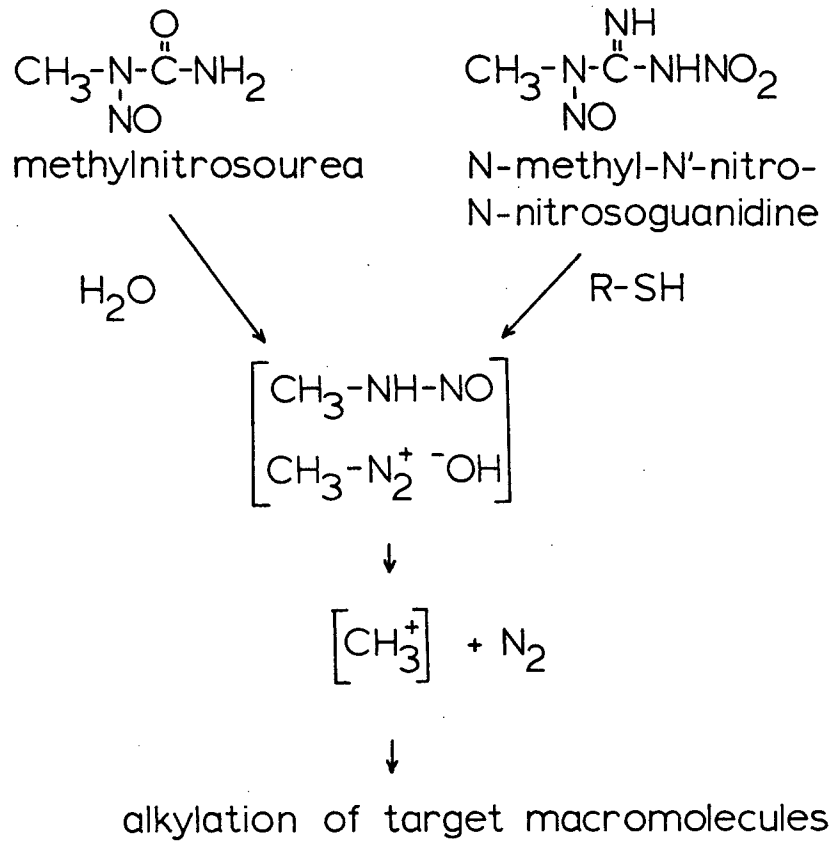
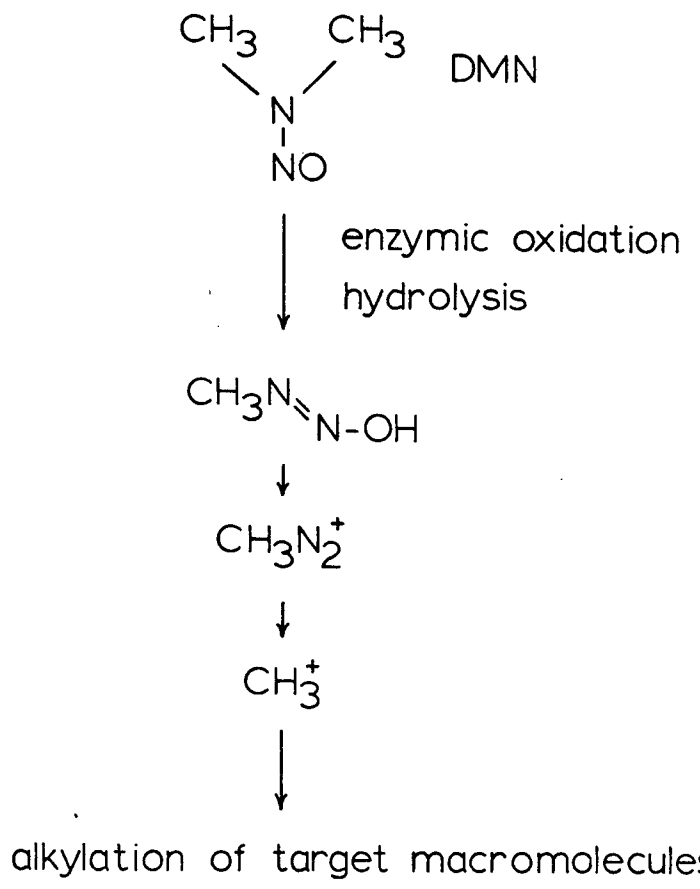

methylnitrosourea
N-methyl-N'-nitro-
N-nitrosoguanidine
H_2O
R-SH
$[CH_3-NH-NO]$
$[CH_3-N_2^+ \ ^-OH]$
$[CH_3^+]$ + N_2
alkylation of target macromolecules

FIGURE 4


CH3
CH3
DMN
N
NO
enzymic oxidation
hydrolysis
CH3N=N-OH
CH3N2+
CH3+
alkylation of target macromolecules

species, and these compounds are, therefore, precarcinogens (Heath, 1962). The enzymatically produced metabolites of DMN are difficult to prepare synthetically, and are too short-lived to store for analysis and testing. The exact nature of the alkylating species is unknown, but a mechanism of action has been hypothesized (Fig. 4) (Heath, 1962). DMN is a potent liver carcinogen in the rat (Table 2) and is metabolized in human liver slices at close to the same rate as in rat liver slices (Montesano and Magee, 1970).

3) Polycyclic aromatic hydrocarbons (PAH)

PAH are formed during the incomplete combustion of organic matter (e.g., fossil fuels) and man is therefore exposed to them in a variety of ways (Badger, 1962). PAH have been detected in air, water, soil, vegetation and food (Kotin and Falk, 1963; Lyjinski and Ross, 1967; Shabad, et al., 1971; anon., 1973). Benzo(a)pyrene (BP) is an example that has been shown to be a potent carcinogen (Table 2). BP was once thought to be carcinogenic as such, since it was commonly able to induce tumours at the site of application. However, this observation might better be explained by the low aqueous solubility of the compound which makes a single application into a chronic exposure. The metabolism of BP, as with other PAH, is carried out by "mixed function oxygenases", enzymes that are NADPH-dependent and catalyze the incorporation of molecular oxygen into the substrate molecules (Holtzman, et al., 1967) and which contain multiple forms of the cytochrome P-450 (Fig.5). The oxygenases are also termed aryl hydrocarbon hydroxylase (AHH) and serve to induce the formation of epoxides at the 4,5:7,8: and 9,10 positions. Although the 4,5-epoxide is the most stable, the 9,10-epoxide is considered to be the precursor most

FIGURE 5

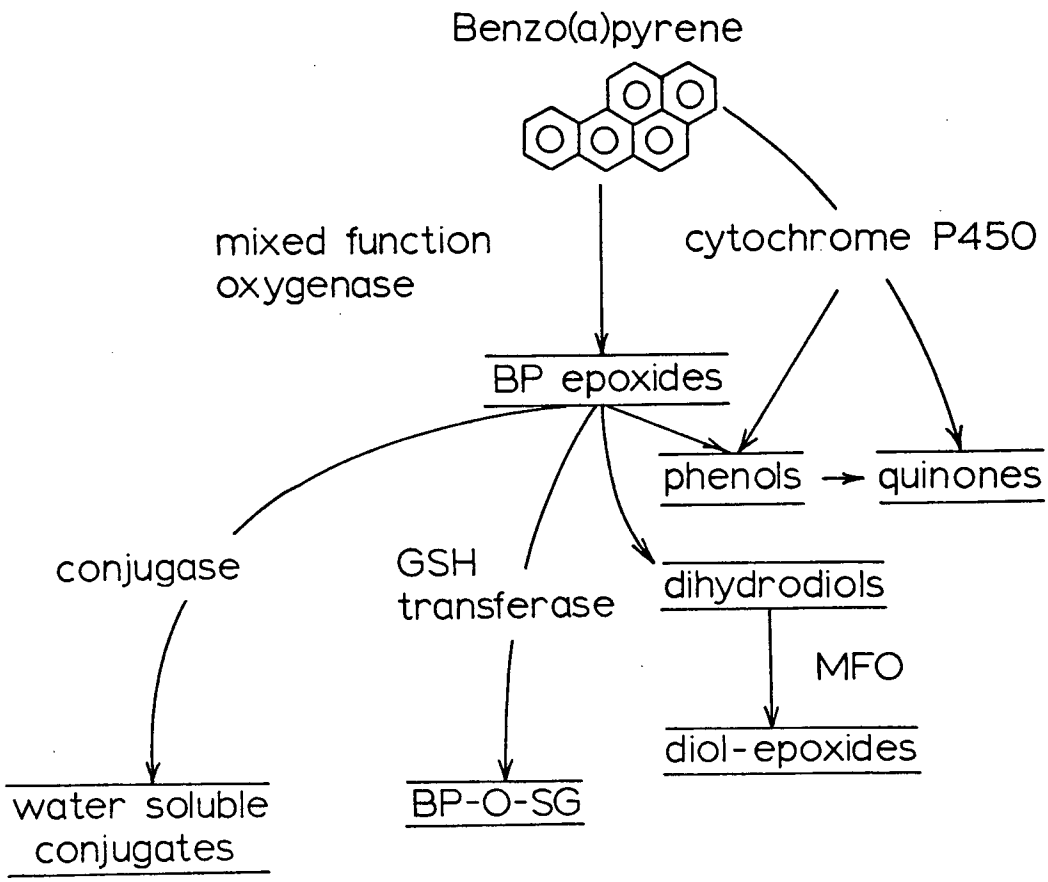

Benzo(a)pyrene
mixed function oxygenase
cytochrome P450
BP epoxides
phenols
quinones
conjugase
GSH transferase
dihydrodiols
MFO
diol-epoxides
water soluble conjugates
BP-O-SG

TABLE 2

Carcinogenicity of Some Chemicals

Carcinogen	Animal	Sites of tumour formation
MNNG	rat	glandular stomach, forestomach, intestine, subcutaneous (site of injection
	mouse	intestine, forestomach, skin (site of injection)
	S.G. hamster	glandular stomach, intestine
	rabbit	lung
	dog	stomach, intestine
MNU	rat, mouse	central and peripheral nervous system, intestine, kidney, forestomach, glandular stomach, skin and annexes, jaw, bladder, uterus, vagina, lung, liver, pharynx, esophagus, trachea, bronchi, oral cavity, pancreas, ear ducts
DMN	rat	liver, kidney, nasal cavities
	mouse	liver, lung, kidney
	S.G. hamster	liver, nasal cavities
	rabbit	liver
B(a)P	mouse	cervix, hematological tumours, skin, respiratory system, mammary tumours, liver, lung, forestomach (when fed B(a)P)
	rats	subcutaneous, mammary, lung, ovaries
	hamsters	subcutaneous, stomach, trachea, skin
	rabbits	skin
	newts	subcutaneous

-from "Survey of Compounds Which Have Been Tested For Carcinogenicity" DHEW Publication No. (NIH) 73-35, Public Health Service Publication No. 149, U.S. Dept. of Health, Education and Welfare, U.S.A. (1969)

important for carcinogenicity and mutagenicity (Baird, et al., 1975). The reactions to form these epoxides go on in the endoplasmic reticulum, although cytochrome P-450's and epoxide hydratases also occur at similar specific activities in the nuclear membrane (Kasper, 1974; Mukhtar, et al., 1979). Although this membrane is much smaller than the endoplasmic reticulum (and so absolute amounts of enzyme are small) the proximity of the membrane to DNA might make the metabolism of PAH that goes on there disproportionately important. Epoxide hydratases do not require NADPH or other cofactors (Oesch, et al., 1971) and have the capacity to convert BP-epoxides to dihydrodiols (Kapitulnik, et al., 1977). Glutathione-S-transferase (GSHT), a soluble fraction enzyme, converts epoxides to water soluble conjugates (Nemata and Gelboin, 1975) although non-enzymatic conjugation of glutathione has been demonstrated (Mukhtar and Bresnick, 1976). Conjugases have also been described which may incorporate BP-epoxides into water soluble conjugates (Nemata and Takayama, 1977).

OUTLINE OF THE PROBLEM

A wide variety of tests have been divised to assay the ability of environmental compounds to induce cancer. These tests are both in vivo and in vitro, as well as short-term (to measure pre-cancerous lesions) and long-term (to measure the induction of resultant tumours). Both pure compounds and complex mixtures have been and are to be tested.

While these tests are useful in investigating chemicals that may cause cancer independently, they are not generally aimed at detecting those chemicals or conditions which may modify the induction of cancer.

At present, only those agents important in promoting the production

of tumours after the initiating events in carcinogenesis are complete are being widely investigated. The aim of this thesis is to demonstrate that modification of two proposed initiation steps in carcinogenesis (DNA damage by fragmentation or carcinogen-adduct formation, and DNA repair) may be brought about by the addition of chemical reducing agents (sodium ascorbate, propyl gallate, glutathione). The ability of certain chemicals or conditions to modify DNA damage and repair will go undetected in short and long-term carcinogenesis assays as they are generally applied. It becomes necessary for these tests to address themselves to such compounds and conditions by setting standard carcinogenesis conditions (i.e., MNNG fragmentation of DNA, BP-adduct formation in DNA) and testing compounds for their ability to modify them. In this way, a better correlation between cancer incidence and environmental cause may ultimately be achieved.

MATERIALS AND METHODS

CHEMICALS

Sucrose, EDTA (ethylene diaminetetraacetic acid, sodium chloride, sodium hydroxide and other common reagents and solvents were obtained from the Fisher Chemical Co., Vancouver, B.C.

RADIONUCLIDES

Thymidine-methyl-^{3}H (1 mCi/ml, specific activity: 20 Ci/mmole) was obtained from the New England Nuclear Corporation, Dorval, P.Q. (G-^{3}H) benzo(a)pyrene (5 mCi/ml, specific activity: 37 Ci/mmole) was obtained from Amersham Corp., Ltd., Oakville, Ontario.

CHEMICAL CARCINOGENS

Dimethylnitrosamine (DMN) was purchased from K and K Laboratories, Plainview, N.Y. N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was purchased from the Aldrich Chemical Comapny, Milwaukee, Wisconsin.

REDUCING AGENTS

L-ascorbic acid (sodium salt), glutathione (reduced) and propyl-gallate were obtained from the Sigma Chemical Company, St. Louis, MD, and were stored in a desiccator at 4^{o} C to minimize air oxidation.

METAL SALT SOLUTIONS

A solution of 0.1 M cupric sulphate was prepared in distilled water, as was glycine at a concentration of 0.5 M. Stock solutions of glycine complexed copper were made by mixing appropriate amounts of glycine stock with metal stock, followed by dilution with 2.5% minimal essential medium (MEM plus 2.5% fetal calf serum). The molar ratio of copper to glycine was 1:10.

NITROSATION OF METHYLGUANIDINE

The nitrosation of 1-methyl-guanidine sulphate (Endo and Takahashi, 1974) with modifications made by Lo and Stich (1978) was followed. A 1 ml reaction mixture (0.2 M 1-methyl-guanidine sulphate (Sigma Chemical Co.), 0.6 M $NaNO_2$ and 0.6 M sodium ascorbate) was incubated for one hour at 37^{o} C. pH was adjusted to 7.4 with 2 N sodium carbonate and the volume brought to 2 ml by addition of distilled water. Serial dilution with MEM without fetal calf serum was used to lower the concentration of the original methylguanidine to 5×10^{-4} M.

Alternatively, the above procedure was carried out with the omission of sodium ascorbate in the reaction mixture.

EXPERIMENTAL ANIMALS

Outbred, 4 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 hr light cycle.

Food, but not water, was removed from cages at 5:00 p.m. on the day before carcinogen administration.

In the case of density gradient sedimentation analysis of DNA fragmentation, mice were injected intraperitoneally with 5×10^{-5} Ci (0.05 ml) of (^{3}H)TdR in distilled water at 72, 24 and 16 hrs prior to use, to label mucosal cells of the stomach (Koropatnick and Stich, 1976).

CELL CULTURES

Cultured human fibroblasts were grown from skin punch biopsies taken from the forearm of a 22 year old Caucasian female. The skin 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 CO_2 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 complete monolayer, at which point the cells were subcultured by standard techniques.

Cultures were maintained in plateau phase at 37° C in a CO_2 incubator in plastic Petri dishes. Transfer passages 3 to 6 were used in all experiments. The cultures were routinely maintained in Eagle's MEM, supplemented with 15% fetal calf serum and antibiotics (200 units penicillin/ml, 4 μg streptomycin/ml).

A line of Chinese hamster cells (CHO)(kindly supplied by the laboratory of Dr. L. Skarsgard (Palcic and Skarsgard, 1978) were grown in

MEM (Grand Island Biological Co.) supplemented with 15% fetal calf serum, antibiotics (streptomicin sulfate (29.6 μg/ml), penicillin G (204 μg/ml), kanamycin (100 μg/ml), and fungizone (2.5 μg/ml)) and 7.5% sodium bicarbonate (10 ml/800 ml medium). The stock cultures were maintained in 250 ml plastic culture flasks (Falcon plastics) and kept in MEM with 15% fetal calf serum at 37° C in a water-saturated CO_2 incubator.

For experiments where damage to DNA, but not repair of that damage, was to be determined in human fibroblasts or CHO cells, approximately 1.6 X 10^5 cells were seeded into 55 mm plastic dishes (Falcon plastics) and kept in MEM with 15% fetal calf serum at 37° C for 2 to 3 days. Experiments were begun when cells were 70-80% confluent.

Where DNA repair was to be determined in human or CHO cells, fibroblasts were grown in the plastic dishes in 15% MEM until about 50% confluent. In order to inhibit cell division after reaching 70-80% confluence, the cells were then transferred to arginine-deficient medium (ADM) supplemented with 2.5% fetal calf serum and incubated for another 3 to 4 days, at which point they were 70-80% confluent and ready for use. Since these cells were not undergoing scheduled DNA synthesis, this process was not confused with repair synthesis of DNA.

In the case of density gradient sedimentation analysis of DNA fragmentation, DNA of cultured cells was prelabelled by incubation with 5 ml of 10% MEM supplemented with ^{3}H-TdR (0.6 μCi/ml) for 24 hrs immediately prior to use.

PREPARATION OF S9 ACTIVATION MIXTURE

Adult male Swiss mice were killed by decapitation and bleeding. The livers were quickly removed and minced with ice-cold Dulbecco's phosphate-buffered saline (PBS) with 0.25 M sucrose, homogenized by a Sorvall teflon pestle torsion homogenizer at 1000 rpm and centrifuged at 9000 X g for 10 min at 4^{o} C (Garner and Hanson, 1971). The resulting postmitochondrial supernatant fraction was frozen in liquid nitrogen and stored in a Revco freezer at -71^{o} C.

The activation mixture consisted of 4 μmoles NADPH, 25 μmoles $MgCl_2$, 20 μmoles glucose-6-phosphate, 0.1 ml NaOH (0.2 N) and 0.4 ml of postmitochondrial liver supernatant fraction (which contains approximately 240 mg of organ wet-weight (Laishes and Stich, 1973)). Ingredients were mixed and 0.25 ml of S9 activation mixture added to cell cultures within 5 min.

ADMINISTRATION OF CHEMICALS

Dimethylnitrosamine:

To each plate of cultured human fibroblasts was added 1) 4.5 ml of MEM without fetal calf serum, 2) 0.25 ml of DMN dissolved in MEM without fetal calf serum (0.1 M)(sufficient to produce a final DMN concentration of 5×10^{-3} M on the plate), and 3) 0.5 ml of S9 activation mixture.

In the case of sodium ascorbate inhibition of nitrosation product formation, sufficient sodium ascorbate (dissolved in MEM without fetal calf serum) was added to produce a concentration of 1×10^{-2} M immediately prior to addition of S9 activation mixture.

Cells were exposed at 37° C for 1 hr and washed three times with PBS solution (8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.2 g KH_2PO_4 in 1000 ml of distilled water: pH 7.45) and used for alkaline sucrose gradient analysis.

Nitrosation products of methylguanidine:

Serial dilution of the nitrosation reaction mixture with MEM without fetal calf serum was made to reduce the starting concentration of methylguanidine to 5×10^{-4} M. Cells were treated for 1 hr and prepared for alkaline sucrose gradient (ASG) analysis in the manner described above.

MNNG:

1) In vitro: MNNG was dissolved in 1 ml of dimethyl sulphoxide (DMSO) and diluted to the appropriate concentration by addition of 99 ml of MEM with 2.5% fetal calf serum. Cells were treated for 0.5 hr at 37° C in a CO_2 incubator followed by preparation for ASG analysis in the manner described above.

2) In vivo: MNNG was force-fed to experimental animals under ether anaesthesia by esophageal intubation in a total volume of 0.5 ml. The 0.5 ml contained 0.1 ml DMSO; 0.4 ml distilled water; MNNG. After 4 hr the animals were killed by cervical dislocation and bleeding and tissue samples taken.

Sodium ascorbate:

Sodium ascorbate was dissolved at twice the desired concentration in MEM without fetal calf serum. Glycine/$CuSO_4 \cdot 5H_2O$ solutions were made as described above at twice the desired concentration. The two solutions were mixed in equal volumes and added to cells within 10 sec.

In cases where sodium ascorbate or $CuSO_4 \cdot 5H_2O$/glycine were administered alone, the concentrated solutions were mixed with MEM (without fetal calf serum) alone and added to cells.

Cells were incubated *in vitro* with mixtures for 0.5 min at 37^{o} C. Cells were washed with PBS and used in ASG analysis of DNA damage.

Mice under light ether anaesthesia were force-fed 0.5 ml of mixtures of the desired concentration. After 4 hr the mice were killed and tissues excised for ASG analysis of DNA damage.

Benzo(a)pyrene:

1) *In vitro*: Sufficient ^{3}H-BP in toluene was placed in a 1 litre glass bottle to produce 4.16×10^{-7} M BP when diluted to stock volume. 2 ml of DMSO was added to the ^{3}H-BP and air blown over the surface of the fluid with an air pump and Pasteur pipet for 15 min under the fume hood to remove volatile toluene. 498 ml of MEM with 5% fetal calf serum was added to the mixture, shaken, and allowed to sit 10 min before use. The mixture was used within 1 hr.

Sodium ascorbate, propyl gallate or glutathione was made up in MEM without fetal calf serum and used within 10 min of mixing.

The pH of glutathione mixtures was adjusted to 7.0 with concentrated NaOH. All concentrations were 2.083 times greater than that required in the final mixture, since dilution took place in cell culture dishes.

2.4 ml of reducing agent, 2.4 ml of BP mixture and 0.2 ml of S9 activation mixture were added to the cells, in that order.

Cells were incubated at 37° C in a CO_2 incubator for 2 hr. The cells were then washed 2 times with 5 ml of ice-cold PBS and the DNA isolated for BP adduct analysis.

2) In vivo: ^{3}H-BP in toluene was added to DMSO in the ratio of 1 µg BP to 0.1 ml DMSO. Toluene was volatilized in the same way as described above. Enough MEM with 2.5% fetal calf serum was added to produce a final ^{3}H-BP concentration of 1 X 10^{-6} g/ml (sufficient to deliver 100 ng to each mouse).

Mice which had been deprived of food, but not water, at 5:00 p.m. the night before were force-fed 0.5 ml of the BP mixture (500 ng) under light ether anaesthesia between 10:00 a.m. and 1:00 p.m. the following day. Mice were killed and stomachs excised at varying times following this prodedure, the DNA extracted, and BP adduct analysis carried out.

Alkaline sucrose gradient analysis of DNA damage and repair:

1) In vivo: The method of Cox, et al. (1973) was followed in all of the essentials. The animal was killed by cervical dislocation and bleeding. The stomach was excised proximal to the pyloric sphincter and distal to the esophagus, opened along its anterior aspect and rinsed twice in ice-cold PBS. Gastric surface mucosa was removed by scraping with a cold glass microscope slide. The scrapings were mixed with

3.0 ml of ice-cold PBS and spun at 1000 rpm in a Dynac clinical centrifuge for 3 min to sediment cells. The cells were mixed with 3.0 ml PBS, spun down again, and resuspended in 0.5 ml of ice-cold PBS. A 25 µlitre aliquot was layered on the gradient.

2) In vitro: Chemically treated cells were rinsed 3 times with ice-cold PBS. 0.5 ml of cold PBS was added and the cells were scrubbed away from the dish with a rubber policeman. The cell suspension (0.5 ml) was placed in a 3 ml centrifuge tube and spun at 2600 rpm in a clinical centrifuge for 5 min. The cell-free supernatant was removed and discarded. 100 µlitres of cold PBS was added and the cells were kept on an ice-bed in preparation for layering on gradients.

Alkaline sucrose gradients:

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); 1 X 10^5-5 X 10^5 cells or intact cell nuclei in a volume not exceeding 50 µlitres; 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 SW 40 ultracentrifuge rotor and spun at 77,561 X g at an average radius of 11.10 cm (25,000 rpm) for 30 min at 20° 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 and collected on nitrocellulose membrane filters.

Acid soluble radioactivity was removed by washing the filters with 8-10% trichloroacetic acid and ethanol. Acid insoluble radioactivity was counted by immersing the dried filters in toluene scintillation fluid and counting for 10 min per vial on the Searle Delta 300 liquid scintillation counter.

BP adduct measurement in DNA:

DNA isolation prodedure was a modification of that used by Diamond, et al. (1967).

1) Tissues: Mouse stomach was excised as described for ASG analysis, rinsed by holding with forceps and plunging in two washes of ice-cold PBS, and the whole stomach placed in a 25 ml Pyrex glass test tube with 5 ml of 1% sodium dodecyl sulphate/25 mM EDTA. The stomach was dispersed using a Polytron tissue homogenizer at setting 5, number 2 head, for 20 sec. The tissue solution was transferred to a 15 ml teflon-capped glass centrifuge tube.

2) Cells: CHO cells (approximately 6 X 10^6 cells per plate) were treated with activated BP for 2 hr as described above and were rinsed 2 times with 5 ml aliquots of PBS. 2.5 ml of 1% SDS/25 mM EDTA was added to each plate and the cells scraped off with a rubber policeman. Cells from two plates were transferred to a 15 ml teflon-capped glass centrifuge tube.

DNA isolation:

1) Solutions were extracted 4 times with 5 ml tris-equilibrated phenol (90% phenol, equilibrated with equal volumes of 0.5 M tris-

HCl (adjusted to pH 8.0 with concentrated HCl)).

2) Solutions were then extracted 2 times with 5 ml ethyl ether.

3) 1 ml of beef pancreatic RNAase (200 μg/ml, Sigma Chemical Corp.) was added and solutions incubated 1 hr at 37° C.

4) 1 ml of 0.02% pronase (Sigma Chemical Corp.) was added and solutions incubated overnight at 37° C.

5) Solutions were extracted 4 times with 5 ml of chloroform/ isoamyl alcohol (24:1 v/v).

6) Solutions were extracted three times with 5 ml ether, and the last ether traces were boiled off by placing the open tubes in warm tap water (approximately 60° C) for 15 min.

7) 6 ml of 2% sodium acetate in 99% ethanol was added to precipitate DNA, and the tubes left at -4° C overnight.

8) DNA was precipitated by spinning in a Dynac clinical centrifuge at setting 100 for 20 min (approximately 2000 rpm).

9) Supernatant was discarded and the DNA pellet dried and resuspended in 4 ml of 0.03 M sodium acetate.

DNA concentration determination:

Purified calf thymus DNA (Sigma Chemical Corp.) was dissolved in a 0.03 M sodium acetate solution and serially diluted to produce a curve of DNA concentration versus absorbance at 260 nm. It was found that:

$$\frac{A_{260}}{0.024} = \text{DNA concentration } (\mu g \cdot ml^{-1})$$

All samples were measured in 1 ml quartz cuvettes in a Bausch and Lomb

Spectronic 21 UV spectrophotometer. DNA solutions were diluted, if necessary, to ensure that absorbance readings fell between 0.1 and 0.8 absorbance units (the range in which the graph was most linear). The absorbance of each sample at 260 and 280 nm was taken and any samples with A_{260}/A_{280} falling below 2.00 were discarded. 1.0 ml of each sample was used for DNA concentration determination.

Measurement of radioactivity:

2.5 ml of DNA solution was placed in a 25 ml plastic scintillation vial and 15 ml of PCS water-miscible scintillation fluid (Amersham Corp.) was added and mixed by shaking. The tubes were kept in the dark at room temperature for 24 hr to minimize chemiluminescence.

Samples were counted for ^{3}H radioactivity on the Searle Delta 300 liquid scintillation counter for a sufficient period to allow no more than 1% error in count-reading. Disintegrations per minute were calculated for each sample using channels-ratio quench correction.

Using the specific activity of the BP, the BP concentration in the DNA solution was calculated, and the result expressed as "ng of benzo(a)pyrene per g of DNA".

RESULTS

Inhibition of DNA repair by sodium ascorbate

1) Alkaline sucrose gradient analysis of DNA damage and repair:

a) In vitro: DNA from cultured human fibroblasts, when released by cell lysis on alkaline sucrose gradients, has been shown to sediment to the saturated sucrose cushion (Fig. 6) (Stich, et al., 1979,a,b). When cultured human fibroblasts were treated with MNNG (1×10^{-5} M) for 30 min, and sampled immediately (i.e., placed on ice within 5 min and then into lysing solution within 20 min) a shift in the peak of DNA concentration from the regions occupied by fast-sedimenting DNA to slow-sedimenting DNA was seen, between fractions 10 and 15 (Fig. 7A). However, cells treated identically, but switched to MEM with 5% fetal calf serum after treatment with MNNG and then allowed to incubate for 30 hr at 37^{o} showed DNA sedimenting at close to control regions (Fig. 7B). This shift in DNA profiles from damage to repair regions was taken to indicate repair.

On the other hand, cells treated with MNNG for 0.5 hr (Fig. 8A) but which have a solution of sodium ascorbate (1×10^{-3} M) in the 5% MEM bathing medium in which they repaired during the following 30 hr showed decreased ability to approach control DNA profiles after 30 hr (Fig. 8B).

When cells were treated with MNNG as above, but were allowed to repair in 5% MEM supplemented with sodium ascorbate (1×10^{-3} M) which was renewed every 4 hr up to 20 hr after MNNG treatment

Figure 6

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts.

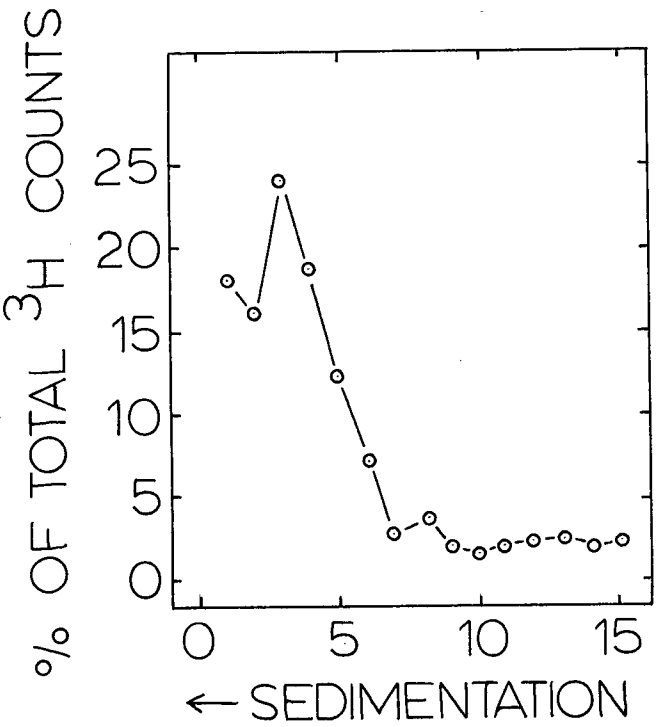

% OF TOTAL ^{3}H COUNTS
0
5
10
15
20
25
0
5
10
15
← SEDIMENTATION

Figure 7A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to MNNG (1×10^{-5} M) for 0.5 hr.

Figure 7B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to MNNG (1×10^{-5} M) for 0.5 hr followed by incubation in 5% MEM for 30 hr at 37^{o} C.

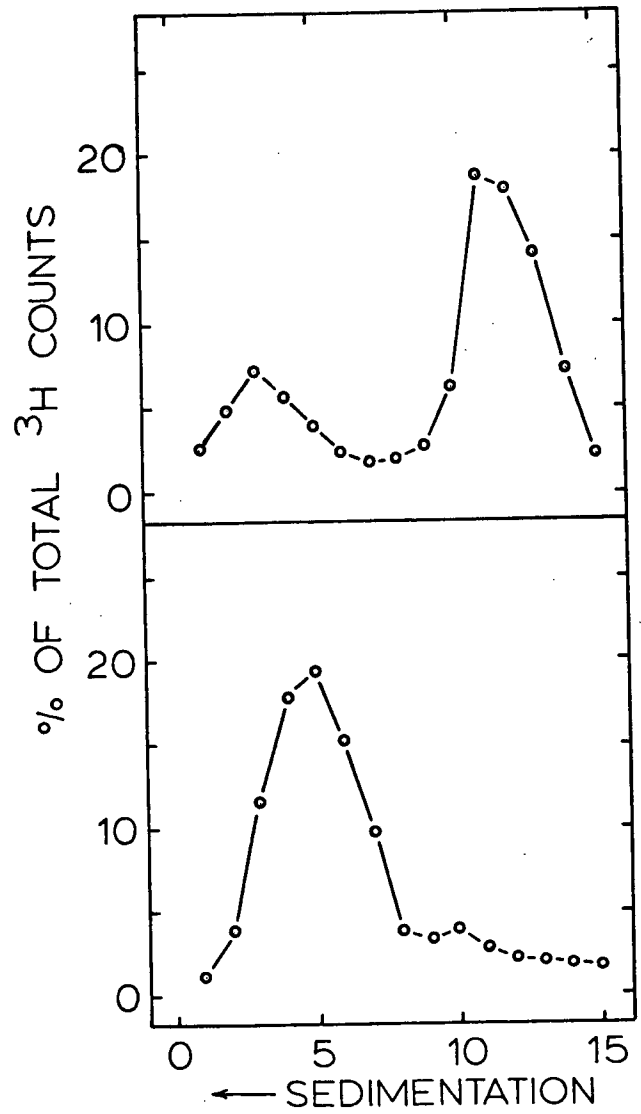
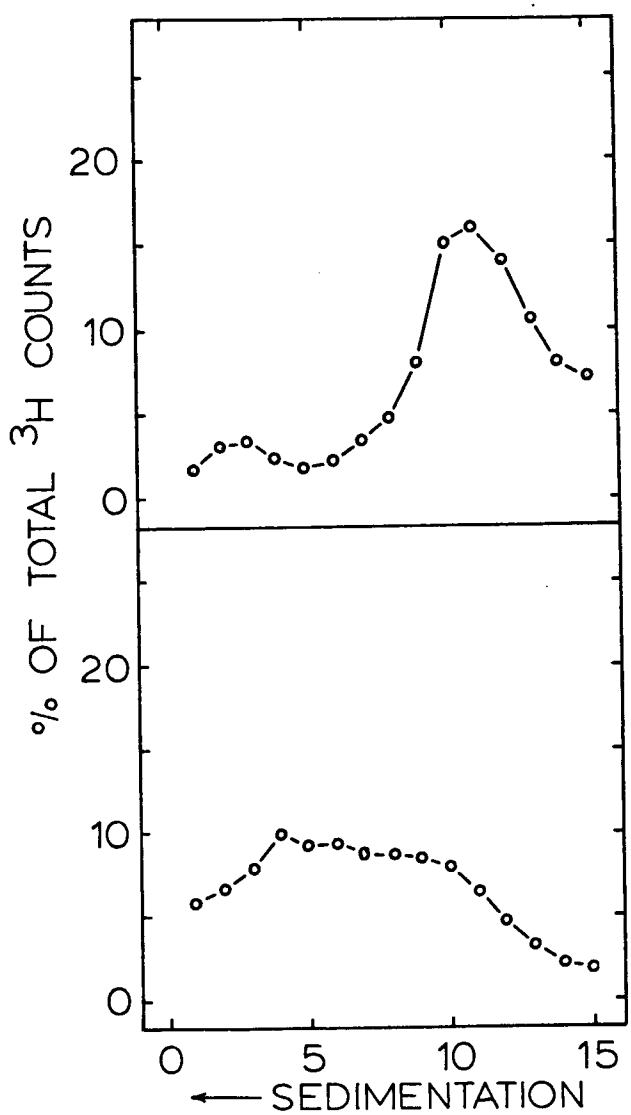

% OF TOTAL ^{3}H COUNTS
20
10
0
20
10
0
0
5
10
15
← SEDIMENTATION

Figure 8A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to MNNG (1×10^{-5} M) for 0.5 hr.

Figure 8B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to MNNG (1×10^{-5} M) for 0.5 hr followed by incubation in 5% MEM supplemented with sodium ascorbate (1×10^{-3} M) for 30 hr at 37^{o} C.


% OF TOTAL ^{3}H COUNTS
20
10
0
20
10
0
0
5
10
15
SEDIMENTATION

(5 medium changes in all), then restoration of control DNA profiles was even more inhibited (Fig. 9A). This indicated that continued incubation of sodium ascorbate may inhibit its repair-inhibiting properties, either by autoxidation to other products, or by cellular inactivation of the repair-inhibiting species.

Because apparent lack of repair on alkaline sucrose gradients might be due to cell death, it was necessary to demonstrate that sodium ascorbate had not inflicted sufficient injury on the fibroblast cells to prevent them from being able to recover. Therefore, cells were treated with MNNG, then with 5 doses of sodium ascorbate over a 20 hr period, and then allowed to repair in a medium containing 5% MEM up to 72 hr following MNNG treatment (Fig. 9B). DNA from these cells once again sedimented in a manner that approached that of control DNA.

It might be argued that sodium ascorbate contributes to cell killing such that certain cells die while others are able to go on to repair DNA to restore its fast-sedimenting ability. A measure of this was the amount of radioactive ^{3}H label recoverable from the gradients. Cells that have undergone repair should have decreased recovery of ^{3}H label (a measure of the amount of DNA) if some cells had been unable to recover and had been lysed. Table 3 shows results obtained from the experiment illustrated in Fig. 9. The results of three separate experiments are shown. Of the 5 results, two came from the first experiment, two from the second, and one sample from the third experiment. In this case, cells that had undergone repair after MNNG and sodium ascorbate treatment still retained 70-93% of the ^{3}H label recoverable from control cells untreated with MNNG or sodium ascorbate. If higher concentrations of sodium ascorbate were used,

Figure 9A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to MNNG (1 X 10^{-5} M) for 0.5 hr followed by incubation in 5% MEM supplemented with sodium ascorbate (1 X 10^{-3} M) for 30 hr at 37° C. The MEM with sodium ascorbate was replaced with freshly-mixed sodium ascorbate solution every 4 hr for the first 20 hr after MNNG treatment.

Figure 9B

Alkaline sucrose gradient sedimentation prodile of ^{3}H-DNA from cultured human fibroblasts exposed to MNNG (1 X 10^{-5} M) for 0.5 hr followed by incubation in 5% MEM supplemented with sodium ascorbate (1 X 10^{-3} M) for 30 hr at 37° C. The MEM with sodium ascorbate was replaced with freshly-mixed sodium ascorbate solution every 4 hr for the first 20 hr after MNNG treatment. At 3 hr post-MNNG treatment, the sodium ascorbate solution was removed and replaced with 5% MEM without sodium ascorbate, incubated for a further 72 hr at 37° C, and sampled.

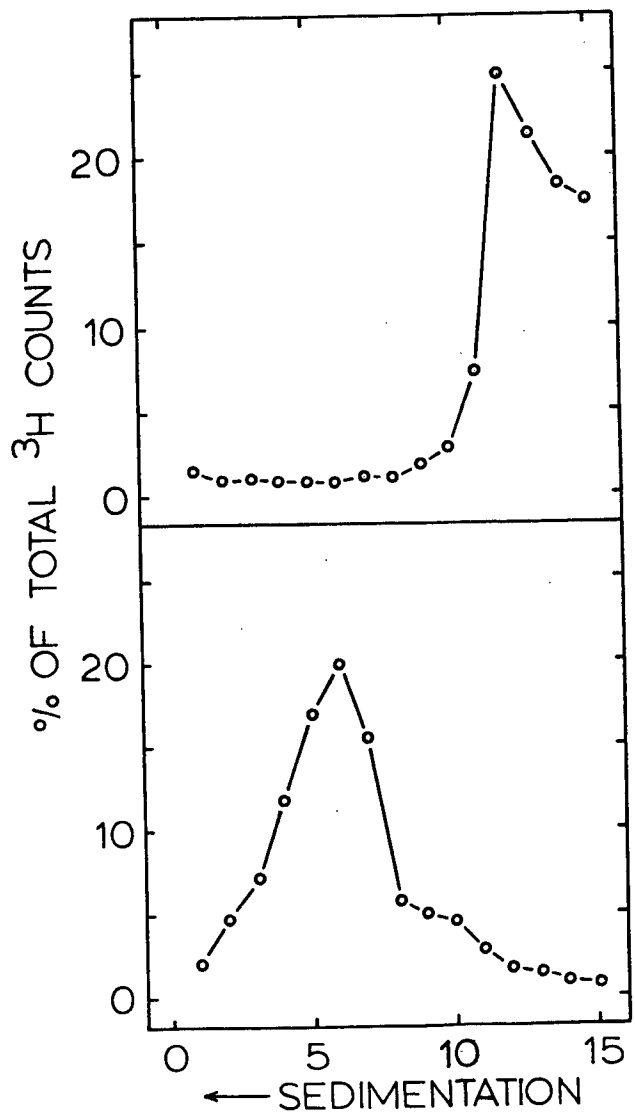

% OF TOTAL ^{3}H COUNTS
20
10
0
20
10
0
0
5
10
15
SEDIMENTATION

TABLE 3

Recovery of radioactivity from alkaline sucrose gradients

Source of DNA	c.p.m. (mean of 5 samples ± standard error)	% of radioactivity in control cells recoverable from gradients
^{3}H-treated control cells (200,000 cells)	1392 ± 320	100%
MNNG treatment followed by incubation with 5 sodium ascorbate treatments over 20 hours	1580 ± 107	114%
MNNG treatment followed by sodium ascorbate treatment followed by repair in MEM without sodium ascorbate up to 72 hours following MNNG treatment	1220 ± 66	88%

the recoverability of ^{3}H counts tended to decrease, although the shape of the alkaline sucrose gradient profiles was not found to change. Therefore, sodium ascorbate concentrations for treatment of cells were chosen such that they were as high as possible without decreasing ^{3}H recoverability to any great extent. MNNG concentrations chosen for use were those great enough to cause the mass of ^{3}H label to accumulate between fractions 9 and 13, but no greater. In this way, the lowest possible MNNG concentrations (those just causing enough damage to observe repair) and the highest possible sodium ascorbate concentrations (those that did not contribute to enhanced cell killing) were used.

Sodium ascorbate treatment alone did not fragment DNA (Fig. 10).

b) In vivo: DNA from gastric mucosal cells of mice force-fed MNNG (40 mg/kg body weight) waas shown to be damaged 4 hr after force-feeding (Fig. 11A). However, repair occurred that returns DNA to near control sedimentation profiles by 30 hr following force-feeding (Fig. 11B).

When, on the other hand, MNNG was force-fed to mice in the same concentration (Fig. 12A) followed by force-feeding sodium ascorbate (20 mg/kg body weight at 15 min, 4 hr, 8 hr, 12 hr, 16 hr, 20 hr and 24 hr following MNNG treatment) no evidence of repair was apparent by 30 hr and sedimentation of DNA did not approach near-control profiles, but remained slow-sedimenting (Fig. 12B). Sodium ascorbate treatment alone did not fragment DNA (Fig.10, 13A).

In the same manner as cultured human fibroblasts in vitro, gastric mucosal cells in vivo retained the ability to repair DNA (as measured by their ability to restore fast-sedimenting properties to DNA)

Figure 10

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to sodium ascorbate (1×10^{-3} M) for 20 hr. The sodium ascorbate solution was replaced with freshly mixed solution every 4 hr for the first 20 hr after the beginning of treatment.

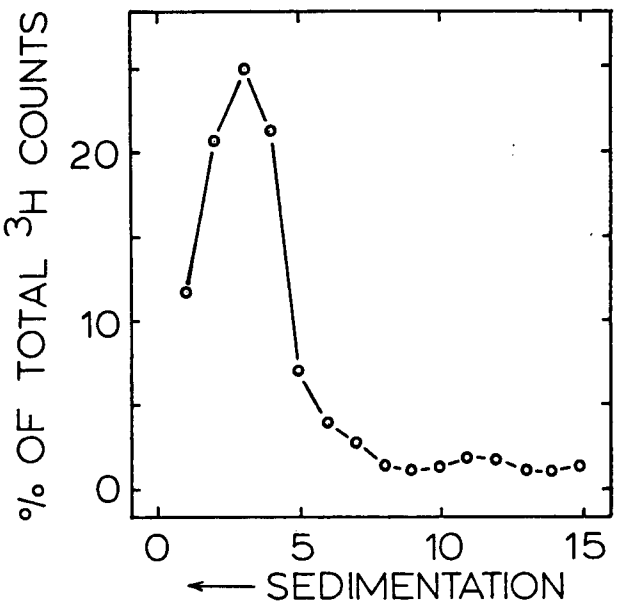

% OF TOTAL ^{3}H COUNTS
20
10
0
0
5
10
15
SEDIMENTATION

Figure 11A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed MNNG (40 mg per kg body weight) at 0 hr and sampled at 4 hr.

Figure 11B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed MNNG (40 mg per kg body weight) at 0 hr and sampled at 30 hr.

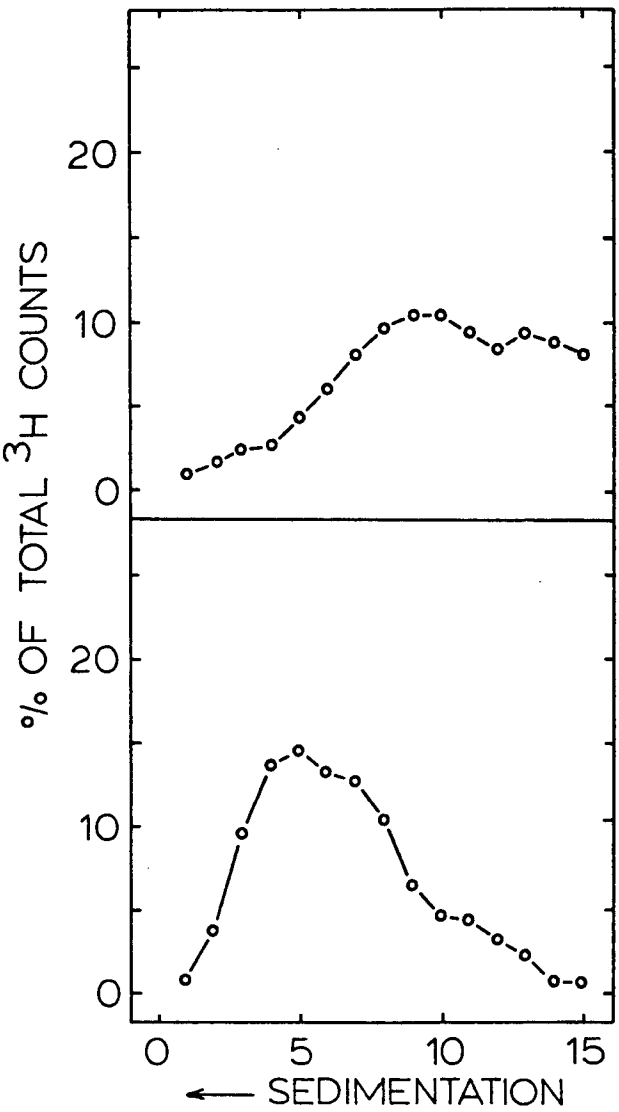
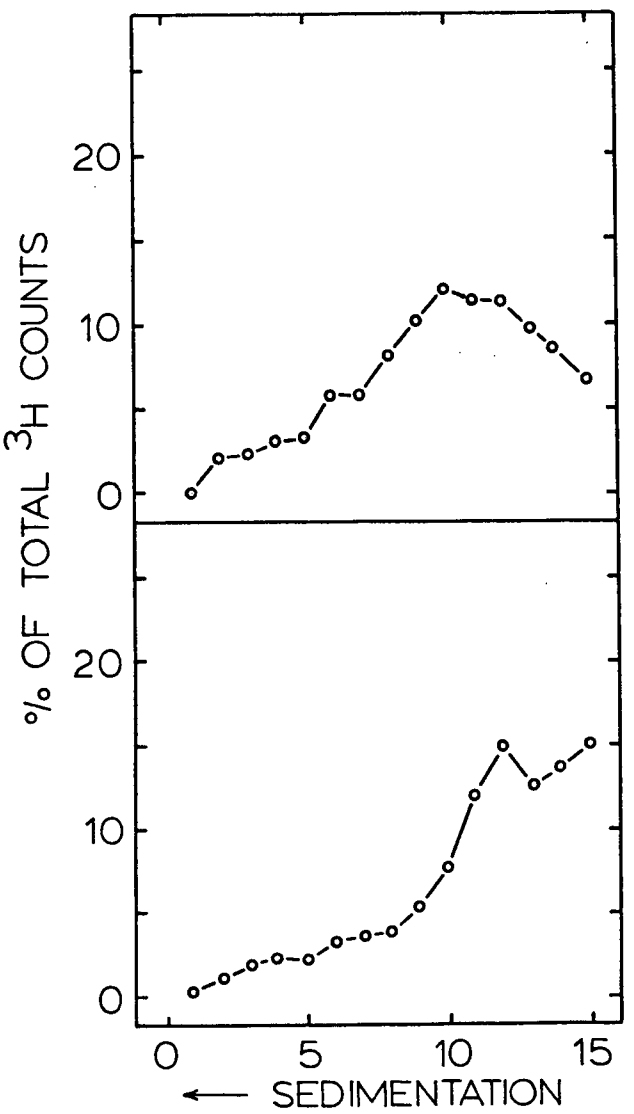

% OF TOTAL ^{3}H COUNTS
20
10
0
20
10
0
0
5
10
15
SEDIMENTATION

Figure 12A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed MNNG (40 mg per kg body weight) at 0 hr and sampled at 4 hr.

Figure 12B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed MNNG (40 mg per kg body weight) at 0 hr followed by force-feeding of sodium ascorbate (20 mg per kg body weight) at 15 min, 4 hr, 8 hr, 12 hr, 16 hr, 20 hr and 24 hr, and sampled at 30 hr.


% OF TOTAL ^{3}H COUNTS
20
10
0
20
10
0
0
5
10
15
SEDIMENTATION

since fast sedimentation was restored when mice that had been force-fed MNNG, 7 doses of sodium ascorbate (as in Fig. 12) and were allowed to repair without further sodium ascorbate treatment up to 72 hr after MNNG administration (Fig. 13B).

2) BP-DNA adduct analysis of DNA repair:

a) In vitro:

i) One of the problems associated with the administration of polycyclic aromatic hydrocarbons to cultured cells or mammalian systems is the hydrophobicity associated with them. BP tends to leave water solutions by attachment to the glass or plastic container, or by separation of the non-polar solvent carrying the BP from the water solution. In order to solve this problem, ^{3}H-BP in toluene was dissolved in DMSO, the toluene blown off by a stream of air, and the resulting water-miscible DMSO/BP solution dissolved in MEM supplemented with 5% fetal calf serum. BP in this solution (presumably bound to the protein component of fetal calf serum) remains available in solution for at least 70 min following solution, while BP treated identically, but dissolved in MEM without FCS, rapidly becomes unavailable in the water solution (Fig. 14). BP in solution was measured by scintillation counting of an aliquot of solution.

ii) BP binding to DNA was determined for 2 hr incubations of concentrations ranging from 0.1×10^{-7} M to 3×10^{-7} M produced by dilution of a single parent BP stock with MEM with 5% fetal calf serum (Fig. 15). Since the 2×10^{-7} M concentration falls in a region of the curve with constant slope, it was deemed an appropriate concentration to use routinely for binding and repair studies.

Figure 13A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed sodium ascorbate (20 mg per kg body weight) at 15 min, 4 hr, 8 hr, 12 hr, 16 hr, and 20 hr and sampled at 24 hr.

Figure 13B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed MNNG (40 mg per kg body weight) at 0 hr followed by force-feeding of sodium ascorbate (20 mg per kg body weight) force-fed at 15 min, 4 hr, 8 hr, 12 hr, 16 hr, 20 hr and 24 hr and sampled at 72 hr.

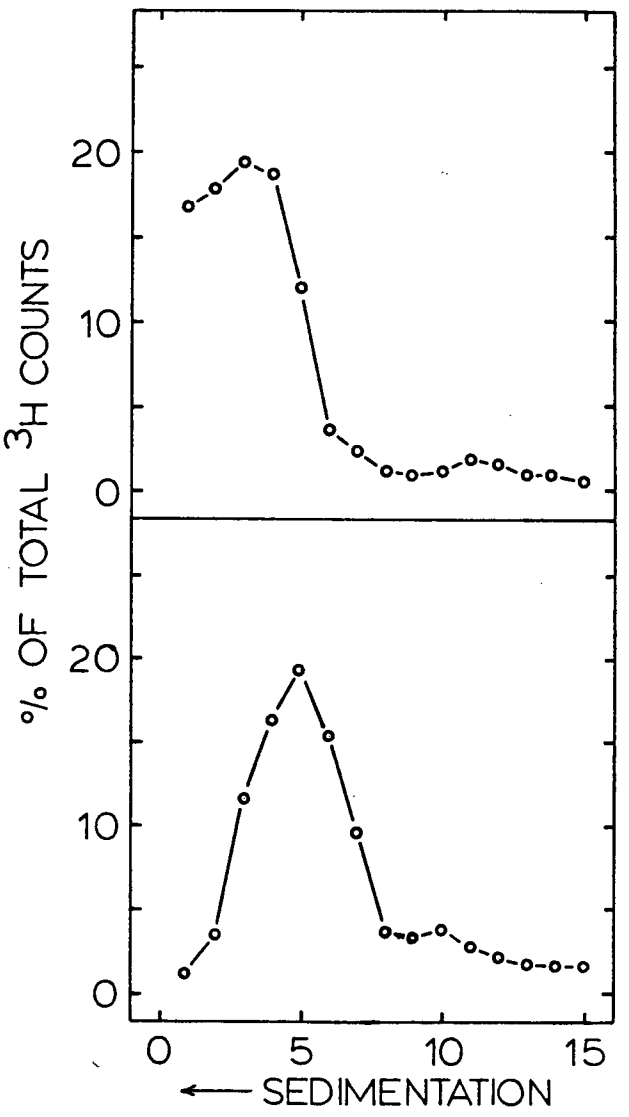

% OF TOTAL ^{3}H COUNTS
20
10
0
20
10
0
0
5
10
15
SEDIMENTATION

Figure 14

^{3}H-BP available in solution at various times after mixing with MEM without fetal calf serum (●) or with MEM with 5% fetal calf serum (○). ^{3}H-BP in a 0.5 ml aliquot was measured by scintillation counting in water-miscible scintillation fluid at the times indicated. A single sample is plotted for each time.

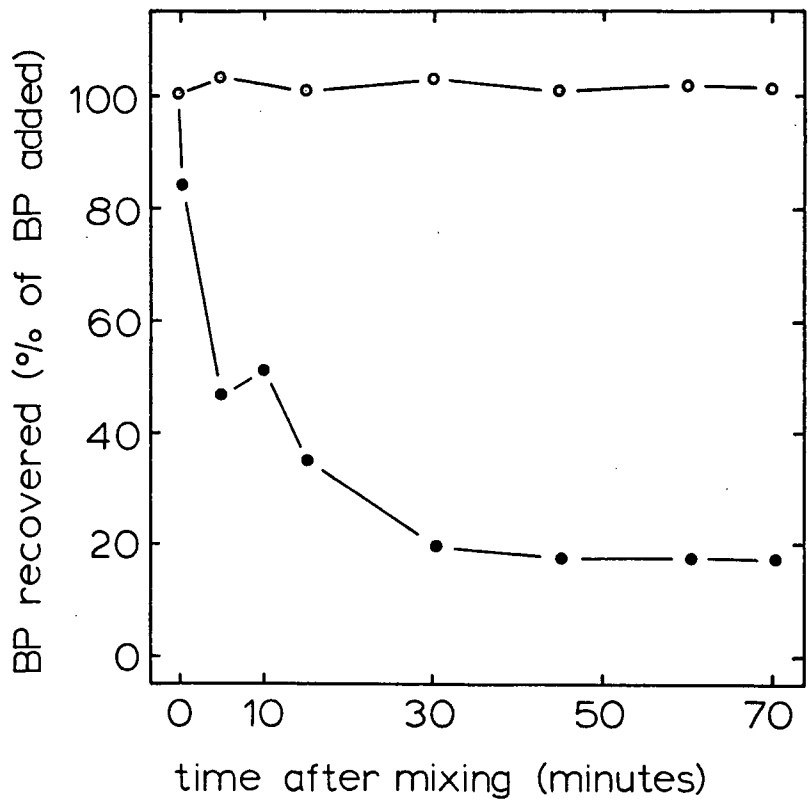
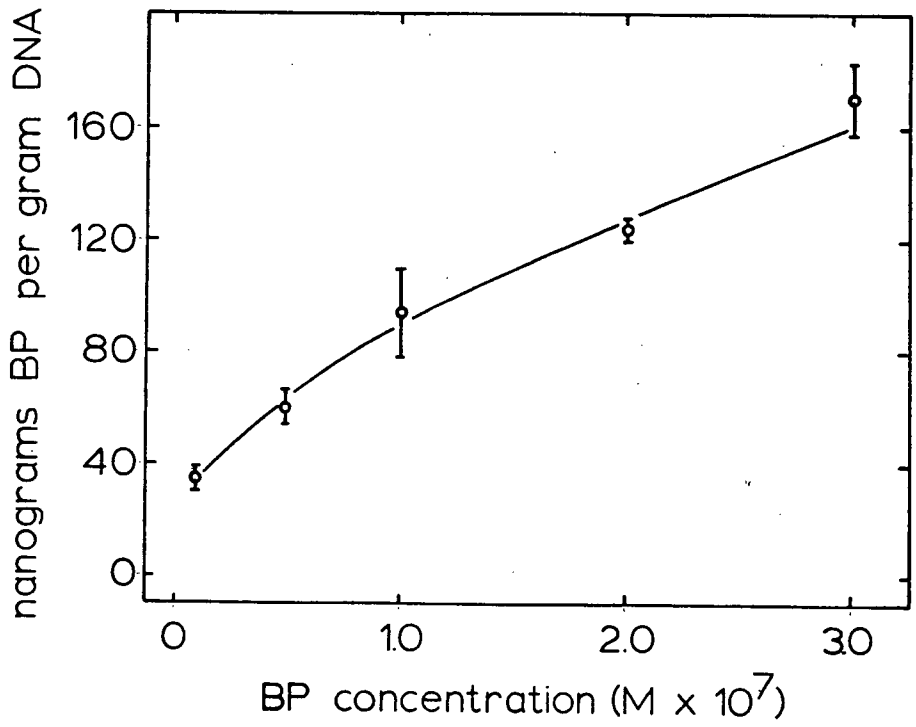

BP recovered (% of BP added)
100
80
60
40
20
0
0
10
30
50
70
time after mixing (minutes)

Figure 15

Binding of ^{3}H-BP to DNA of cultured CHO cells exposed to various concentrations of ^{3}H-BP in combination with an S9 activation system for 2 hr and sampled immediately. The mean of 10 samples is plotted for each concentration ± standard error.


nanograms BP per gram DNA
160
120
80
40
0
0
1.0
2.0
3.0
BP concentration (M x 10^7)

iii) To follow the disappearance of covalently bound ^{3}H-BP from DNA, CHO cells that had been inhibited in the G_1 stage of cell division by incubation in arginine-deficient medium(ADM)(Stich and San, 1970) were treated with BP (in 5% ADM), rinsed twice with ADM without fetal calf serum, and then allowed to repair in 5% ADM without BP. CHO cells rather than human fibroblasts were employed because greater rates of cell division allowed larger numbers of cells to be used. BP binding to DNA was determined at various times after reaction, up to 72 hr (Fig. 16). There was a slight rise in total BP adducts between 0 and 1.5 hr after ^{3}H-BP reaction, which may be due to binding of BP that had not been completely removed from the system (Ivanovic, et al., 1978). Thereafter there is a rapid decline in adducts up to 12 hr, at which time about 30% of the initial bound ^{3}H label remained. Up to 72 hr, the rate of decrease in bound BP was much less and about 70% of the bound ^{3}H label at 24 hr remained at 72 hr.

iv) When CHO cells were treated identically to those described above, but were subjected to sodium ascorbate (1-5 X 10^{-3} M) dissolved in the post-BP incubation medium, loss of bound ^{3}H label was significantly inhibited (Fig. 17). At 5 X 10^{-3} M, the rapid decrease in bound BP was lost altogether. DNA-bound ^{3}H label remained relatively constant up to 24 hr, at which time it began to drop off to control levels. 1 X 10^{-3} M sodium ascorbate induced less rapid initial loss of bound ^{3}H label. By 24 hr the bound ^{3}H label reached the same level as control samples taken at the same time. It appears that sodium ascorbate inhibits the early, rapid excision of bound BP products to DNA. However, the relatively rapid autoxidation of sodium ascorbate to dehydroascorbate in solution might explain the return to normal excision levels by 24 to 48 hr.

Figure 16

Binding of ^{3}H-BP to the DNA of non-dividing cultured CHO cells exposed to ^{3}H-BP (2×10^{-7} M) in combination with an S9 activation system for 2 hr followed by rinsing with PBS and addition of 5% ADM. Samples were taken at up to 72 hr after exposure to ^{3}H-BP. Three identical runs are plotted for each time.

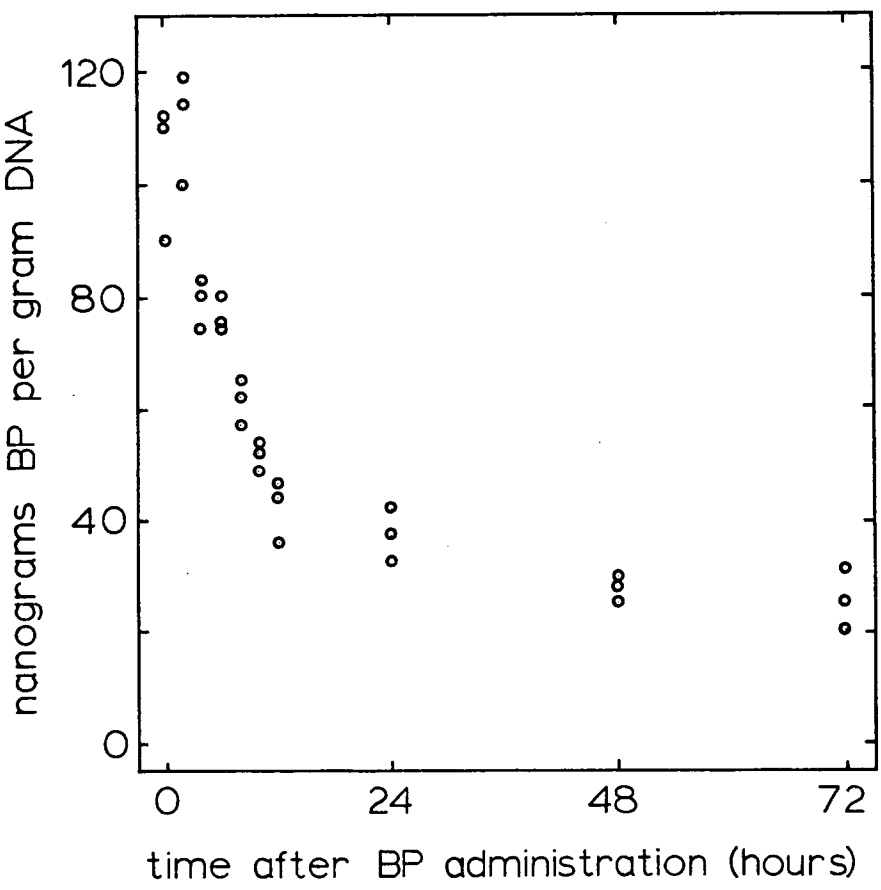
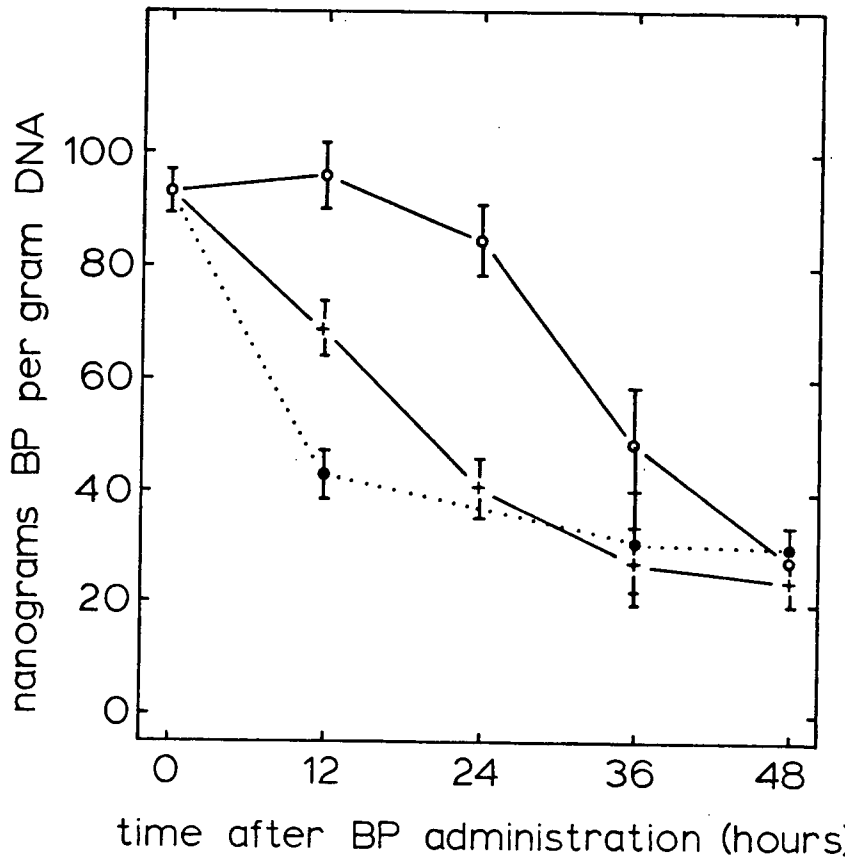

120
80
40
0
nanograms BP per gram DNA
0
24
48
72
time after BP administration (hours)

Figure 17

Binding of ^{3}H-BP to the DNA of non-dividing cultured CHO cells exposed to ^{3}H-BP (2×10^{-7} M) in combination with an S9 activation system for 2 hr followed by rinsing with PBS and addition of 5% MEM supplemented with sodium ascorbate, 5×10^{-3} M (o) or 1×10^{-3} M (+), or 5% MEM without sodium ascorbate (●). The mean of 5 samples is plotted for each time, ± standard error.


nanograms BP per gram DNA
100
80
60
40
20
0
0
12
24
36
48
time after BP administration (hours)

v) Although sodium ascorbate could be used to inhibit repair, the sulphydryl reducing agent cysteine did not apparently inhibit repair of BP adducts (Fig. 18).

b) In vivo:

i) BP binding to mouse gastric mucosal cell DNA was determined for various amounts of ^{3}H-BP force-fed to mice, and the results plotted as the mean of 8-10 trials ± standard error (Fig. 19). The curve is relatively linear from 100 ng per mouse to 750 ng per mouse, with a steeper rise up to 1000 ng per mouse. In order to use a ^{3}H-BP concentration for further experiments that fell into the region of the graph where slope varied the least, a concentration of 500 ng per mouse was chosen as a standard for further experiments.

ii) In order to determine the time course of ^{3}H-BP binding to mouse gastric cell DNA after force-feeding 500 ng of ^{3}H-BP, stomach samples were taken 1.5 to 72 hr after administration and the amount of BP bound to DNA calculated (Fig. 20). BP binding increased up to 12 hr, followed by a relatively rapid decrease to approximately 50% of the 12 hr binding by 48 hr. The 48 hr binding was not significantly reduced by 72 hr.

iii) In order to determine whether sodium ascorbate had an effect on the loss of ^{3}H-BP label in vivo, mice were force-fed 500 ng of ^{3}H-BP at 0 hr followed by 100 mg of sodium ascorbate per g body weight (in a total volume of 0.5 ml of MEM) at 15 min, 4, 8, 12, 24 and 36 hr (Fig. 21). ^{3}H label remained constant from 12 to 36 hr while DNA from control mice lost approximately 50% of ^{3}H label. Following cessation of force-feeding of sodium ascorbate, ^{3}H label fell rapidly from 40 hr to 72 hr, by which time it had reached the same level as that observed in control mice sampled 72 hr after BP force-feeding.

Figure 18

Binding of ^{3}H-BP to the DNA of non-dividing cultured CHO cells exposed to ^{3}H-BP (2×10^{-7} M) in combination with an S9 activation system for 2 hr followed by rinsing with PBS and addition of 5% MEM supplemented with cysteine (5×10^{-3} M)(o) or 5% MEM without cysteine (●). The mean of 5 samples is plotted for each time, ± standard error.

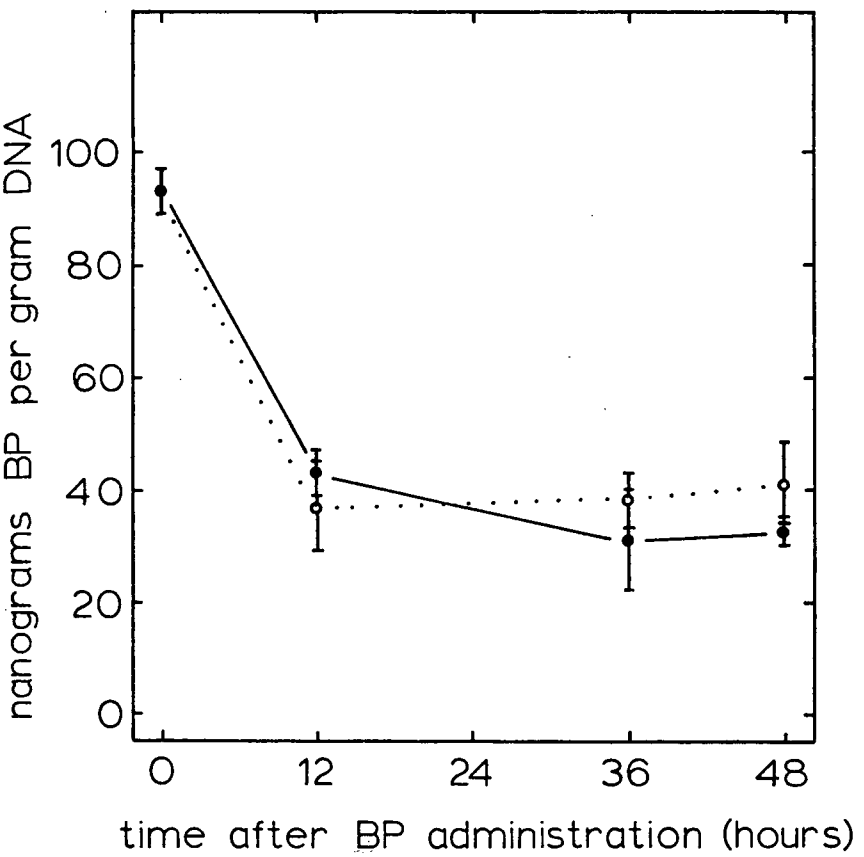

nanograms BP per gram DNA
100
80
60
40
20
0
0
12
24
36
48
time after BP administration (hours)

Figure 19

Binding of ^{3}H-BP to DNA from gastric cells of mice force-fed BP of various concentrations at 0 hr and sampled at 18 hr. The mean of values from 10 mice is plotted for each concentration, ± standard error.

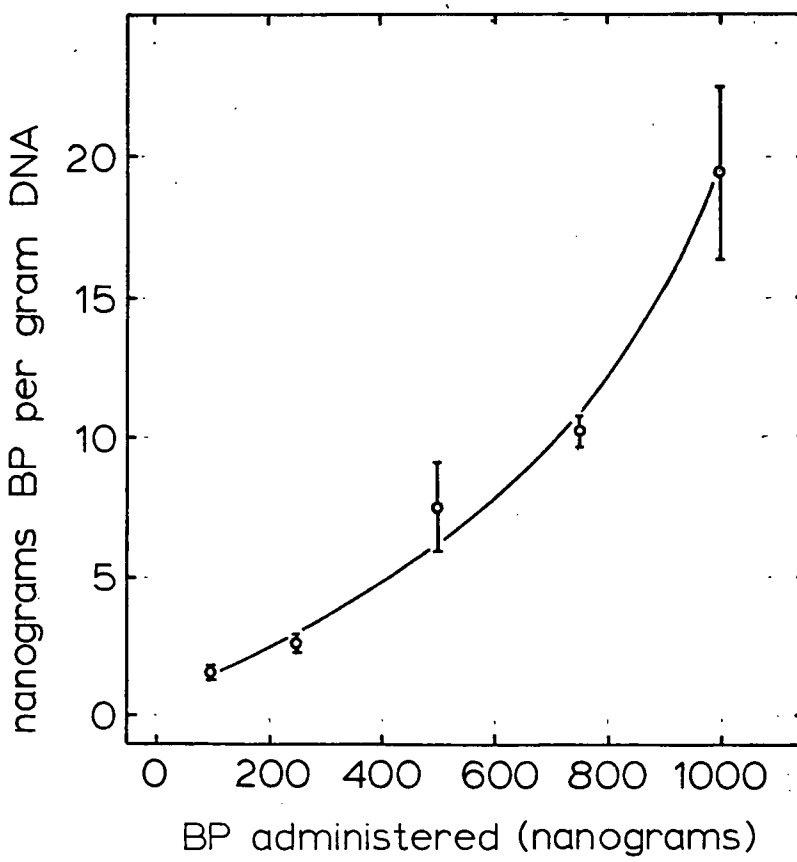

nanograms BP per gram DNA
0
5
10
15
20
0
200
400
600
800
1000
BP administered (nanograms)

Figure 20

Binding of ^{3}H-BP to DNA from gastric cells of mice force-fed ^{3}H-BP (500 ng per mouse) at 0 hr and sampled at various times thereafter. The mean of values from 10 mice is plotted for each time, ± standard error.

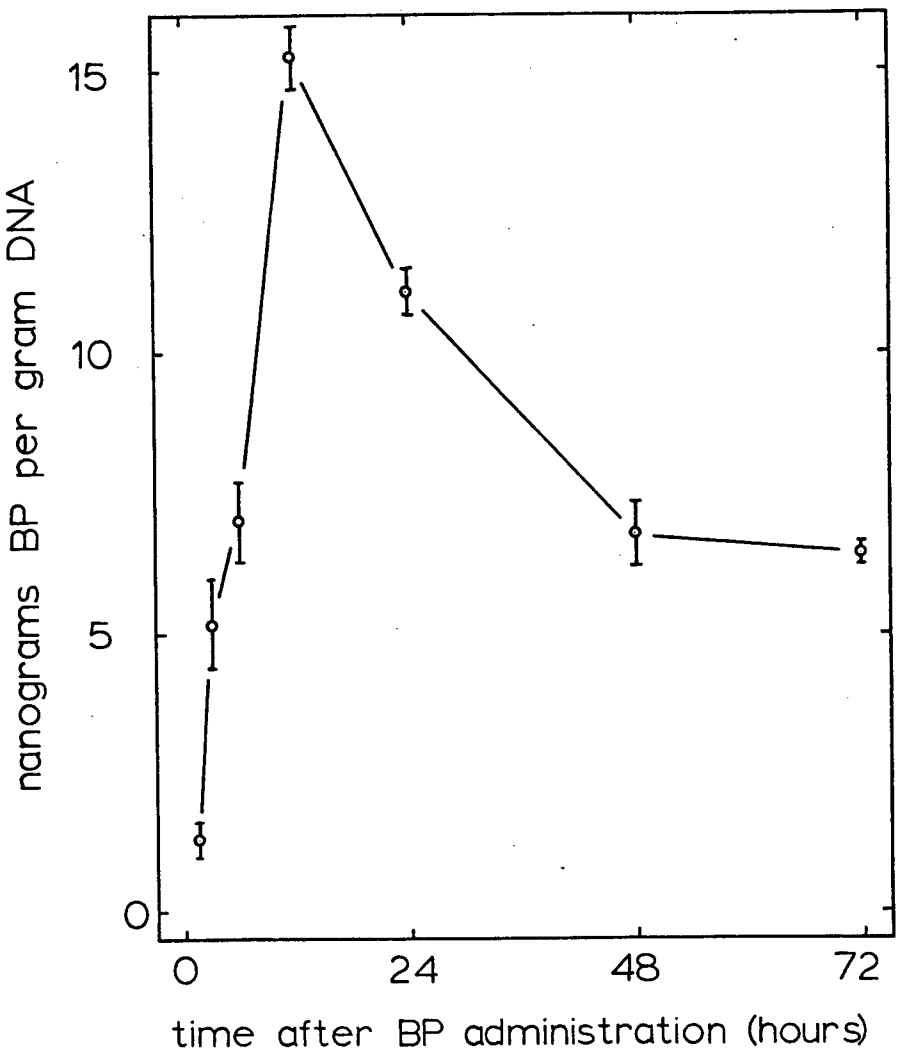
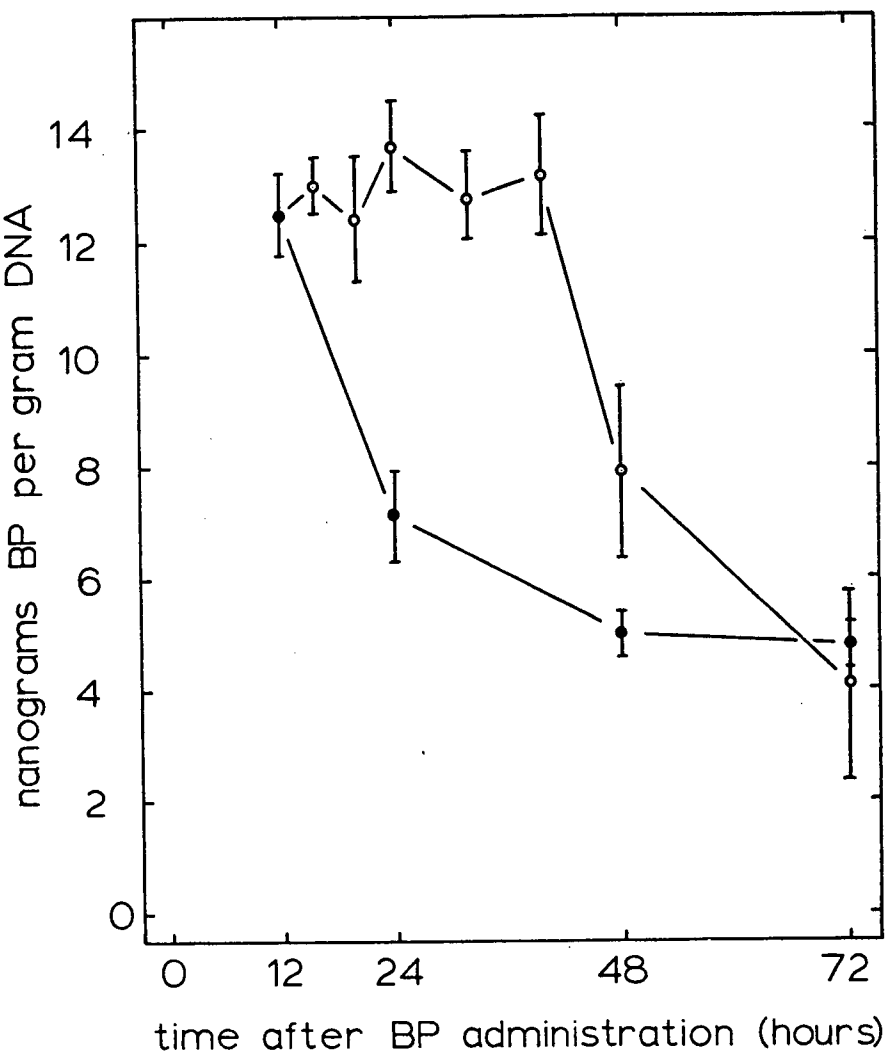

15
10
5
0
nanograms BP per gram DNA
0
24
48
72
time after BP administration (hours)

Figure 21

Binding of ^{3}H-BP to DNA from gastric cells of mice force-fed ^{3}H-BP (500 ng per mouse) at 0 hr followed by force-feeding sodium ascorbate (100 mg per kg mouse) at 15 min, 4, 8, 12, 24 and 36 hr (O) or by no further treatment (●). The mean of values from 10 mice is plotted for each time, ± standard error.


nanograms BP per gram DNA
0
2
4
6
8
10
12
14
0
12
24
48
72
time after BP administration (hours)

Other effects of sodium ascorbate

While sodium ascorbate was able to inhibit the repair of damage inflicted on DNA by MNNG and BP, it was able to produce other clastogenic or protective effects both *in vitro* and *in vivo*.

1) Sodium ascorbate fragmented DNA of cultured human fibroblasts in the presence of copper (Fig. 22B), while $CuSO_4 \cdot 5H_2O$ alone exerted no detectable effect (Fig. 22A). In a similar way, sodium ascorbate (0.5 ml of 0.15 M solution) or $CuSO_4 \cdot 5H_2O$/glycine complex force-fed to mice did not, in isolation from each other, fragment gastric mucosal cell DNA (Fig. 23A). However, when identical amounts of sodium ascorbate and copper/glycine complex were force-fed together, DNA fragmentation in gastric cells was apparent by 4 hr (Fig. 23B). These damaged cells were able to repair DNA so that it sedimented at near-control levels by 48 hr post-treatment (Fig. 23B).

2) Sodium ascorbate has been used as a "trapping" agent to scavenge reactive electrophiles (see introduction). This was demonstrated by the ability of sodium ascorbate to inhibit the action of activated DMN. Cultured human fibroblasts exposed to DMN alone (Fig. 24) showed no DNA fragmentation, while addition of S9 activation system induced considerable breakage (Fig. 25A). Only 12% of recoverable ^3H counts were found in fractions 1-5 (the region into which 80-90% of undamaged DNA normally falls). When sodium ascorbate (1×10^{-2} M) was added to cells immediately prior to addition of the S9 activation system, *less* fragmentation of DNA was observed. Approximately 40% of recoverable ^3H counts were found in fractions 1-5 (Fig. 25B). The decrease in fragmentation was probably not due to inhibition of the

Figure 22A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to $CuSO_4 \cdot 5H_2O$ (1.8×10^{-5} M) for 0.5 hr.

Figure 22B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to sodium ascorbate (1.8×10^{-3}M) in the presence of $CuSO_4 \cdot 5H_2O$ (1.8×10^{-5} M) for 0.5 hr.

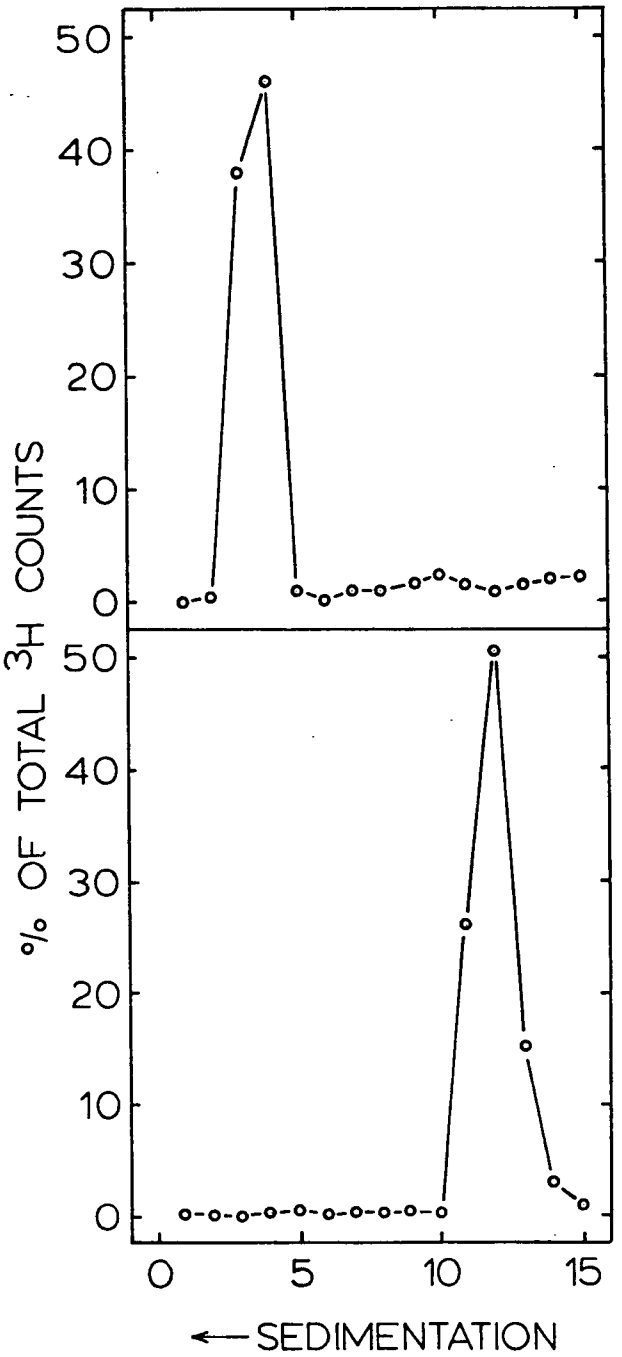

50
40
30
20
10
0
50
40
30
20
10
0
% OF TOTAL ^{3}H COUNTS
0
5
10
15
← SEDIMENTATION

Figure 23A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed $CuSO_4 \cdot 5H_2O$ (5×10^{-5} M) plus glycine (5×10^{-4} M)(○), or 0.5 ml of sodium ascorbate (0.15 M)(●) at 0 hr and sampled at 4 hr.

Figure 23B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed $CuSO_4 \cdot 5H_2O$ (5×10^{-5} M) plus glycine (5×10^{-5} M) plus sodium ascorbate (0.15 M) in a total volume of 0.5 ml at 0 hr and sampled at 4 hr (○) or 48 hr (●).

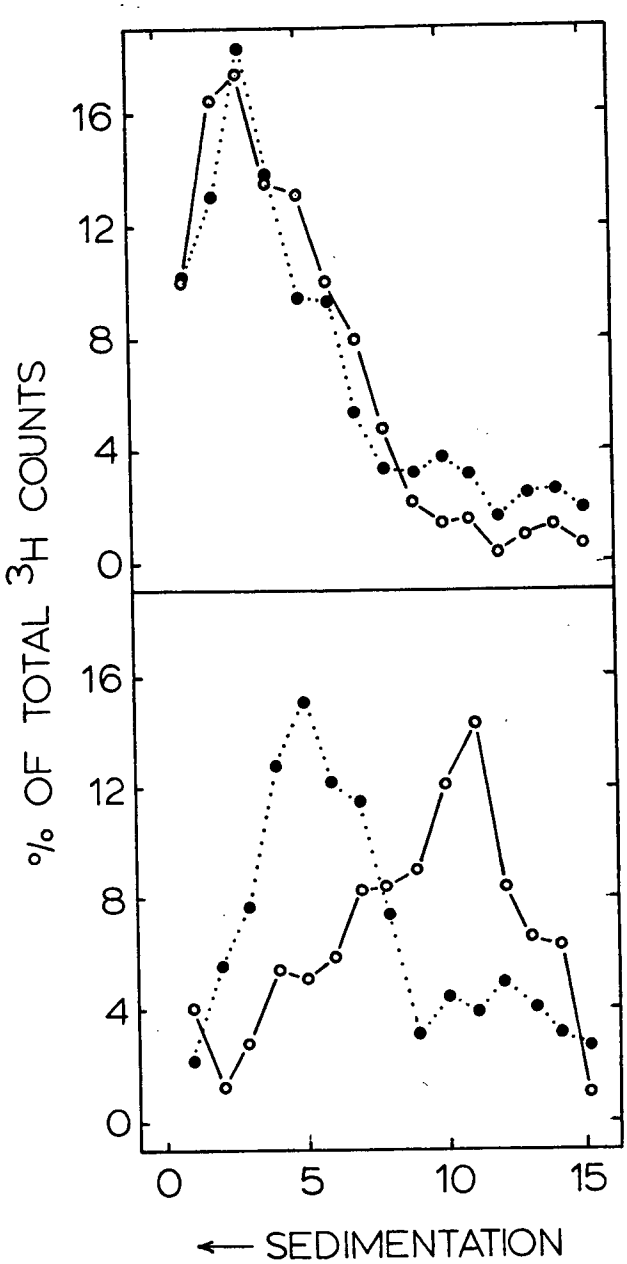

% OF TOTAL ^{3}H COUNTS
16
12
8
4
0
16
12
8
4
0
0
5
10
15
← SEDIMENTATION

Figure 24

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to dimethylnitrosamine (5×10^{-3} M) without S9 activation system for 0.5 hr.

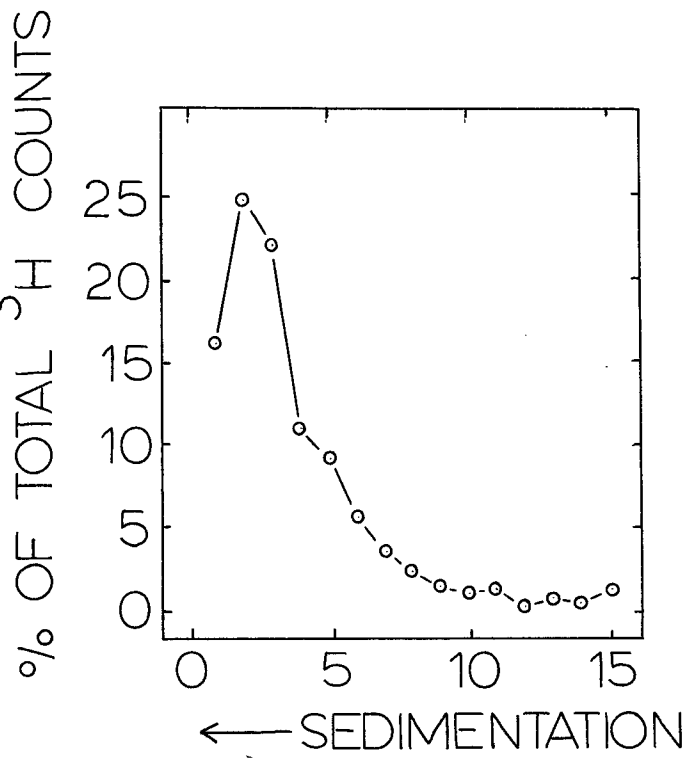
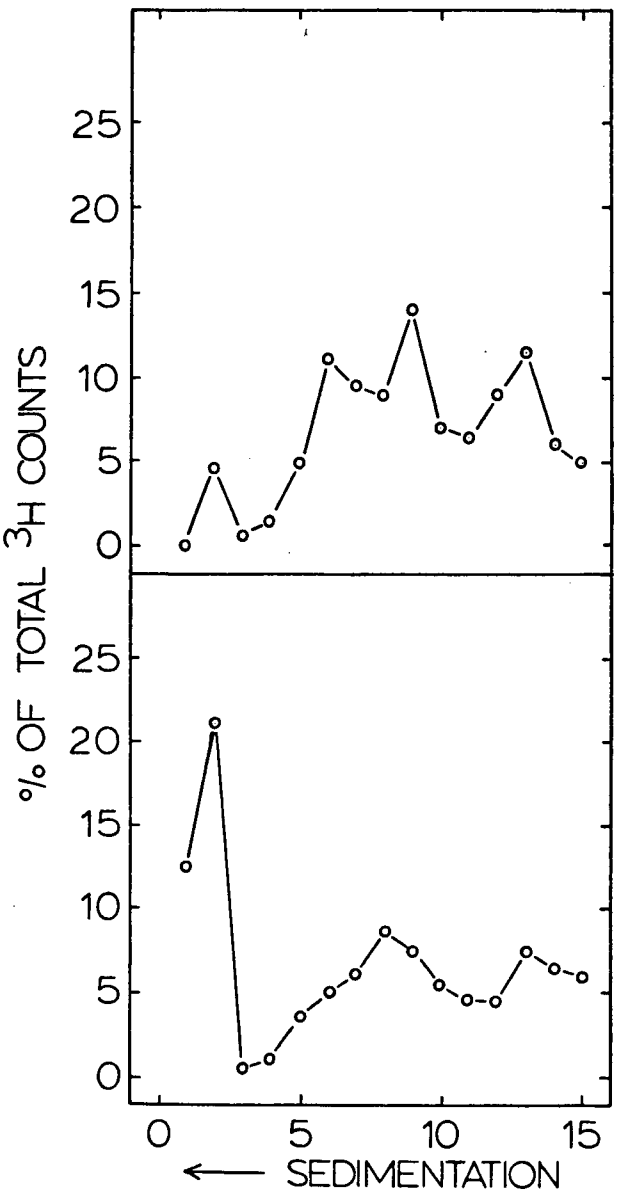

% OF TOTAL ^{3}H COUNTS
25
20
15
10
5
0
0
5
10
15
SEDIMENTATION

Figure 25A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to dimethylnitrosamine (5×10^{-3} M) in the presence of an S9 activation system for 0.5 hr.

Figure 25B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to dimethylnitrosamine (5×10^{-3} M) in the presence of an S9 activation system and sodium ascorbate (1×10^{-2} M) for 0.5 hr.


% OF TOTAL ^{3}H COUNTS
25
20
15
10
5
0
25
20
15
10
5
0
0
5
10
15
SEDIMENTATION

activation system, since the precarcinogen sterigmatocystin was not inhibited in its ability to induce DNA repair in the presence of 1 X 10^{-2} M sodium ascorbate and S9 activation system (Lo and Stich, 1978). Sterigmatocystin requires epoxidation to form carcinogenic species, in a manner analogous to BP (Wogan, et al., 1979). Therefore, inhibition of activated DMN is implicated (although some cellular interaction of sodium ascorbate cannot be ruled out in this case. S9 activation system alone exerts no fragmenting effect on cultured human fibroblast DNA (Laishes, 1974).

3) While sodium ascorbate may inhibit the action of activated carcinogens, it may also inhibit the non-enzymatic activation of those carcinogens. When cultured human fibroblasts were incubated in the presence of the nitrosation products of methylguanidine for 1 hr, DNA fragmentation resulted (Fig. 26A). However, when 1.5 X 10^{-3} M sodium ascorbate was added to the reaction vessel where nitrous acid was used to nitrosate the methylguanidine, the resulting nitrosation products were unable to exert as great a fragmentation effect (Fig. 26B). The implication is that the formation of nitrosation products was inhibited by the presence of sodium ascorbate.

4) As demonstrated above, sodium ascorbate may inhibit the action of activation of precarcinogens. In addition, direct-acting, ultimate carcinogens may be affected as well. When equal volumes of MEM containing sodium ascorbate and MNNG were added to cell cultures, in that order, such that the final concentrations are 1 X 10^{-3} and 1 X 10^{-5} M, respectively, considerable fragmentation of DNA resulted (Fig. 27A). However, when the sodium ascorbate and MNNG were incubated for 30 min at 37^{o} C in a closed vessel prior to addition to cells, much less fragmentation occurred (Fig. 27B). The same situation exists in vivo. When MNNG (20 mg per kg body weight) and sodium

Figure 26A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to the nitrosation products of methylguanidine (starting concentration of methylguanidine was 5 X 10^{-4} M) for 1 hr.

Figure 26B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to the nitrosation products of methylguanidine (starting concentration of methylguanidine was 5 X 10^{-4} M) where nitrosation had taken place in the presence of sodium ascorbate (1.5 X 10^{-3} M). Exposure was for 1 hr.

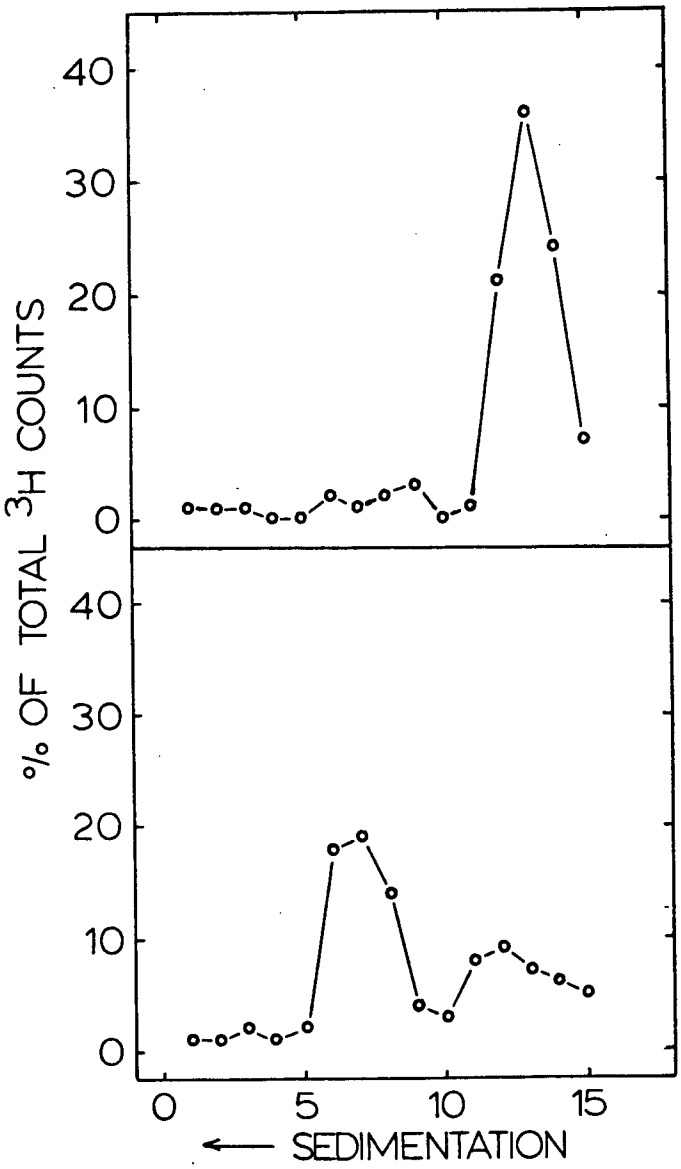
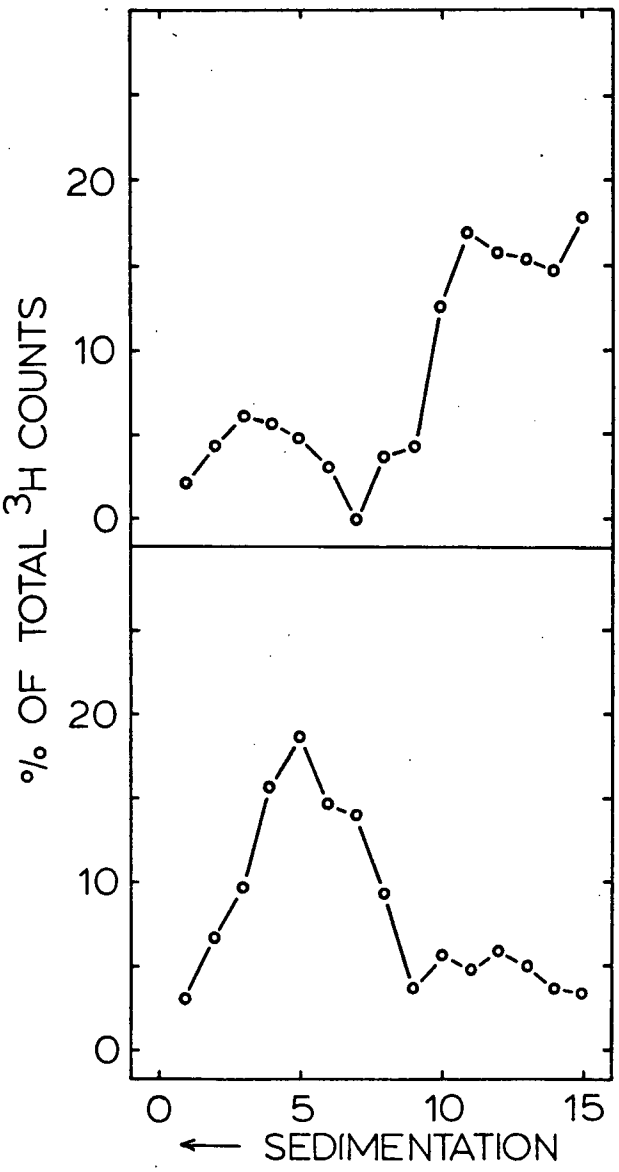

% OF TOTAL ^{3}H COUNTS
40
30
20
10
0
40
30
20
10
0
0
5
10
15
SEDIMENTATION

Figure 27A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to MNNG (1×10^{-5} M) in the presence of sodium ascorbate (1×10^{-3} M) for 0.5 hr.

Figure 27B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to MNNG (1×10^{-5} M) in the presence of sodium ascorbate (1×10^{-3} M) that was incubated at 37^{o} C for 0.5 hr prior to addition to cells. Exposure was for 0.5 hr.


% OF TOTAL ^{3}H COUNTS
20
10
0
20
10
0
0
5
10
15
← SEDIMENTATION

ascorbate (100 mg per kg body weight) were mixed and immediately (within 30 sec) force-fed to mice in a volume of 0.5 ml, damage to DNA results (Fig. 28A). On the other hand, incubation of the mixture for 0.5 hr at 37° C before force-feeding resulted in decreased DNA fragmentation of mouse gastric mucosal cells (Fig. 28B). The concentrations used here (100 mg per kg body weight) were higher than those used to inhibit DNA repair (cf. Fig. 10,11)(40 mg per kg body weight). The low ascorbate concentrations were used previously to minimize long-term toxicity to cells being tested for their ability to repair damage over 72 hr, while the high concentrations (Fig. 27) were used to maximize the observed inhibition of damage over short-term 4 hr treatments.

5) Although incubation of sodium ascorbate with some carcinogens (e.g., DMN) decreased their DNA-fragmenting ability, sodium ascorbate may also enhance the DNA-fragmenting ability of others. MNNG (1×10^{-5} M), when administered to cultured human fibroblasts, fragmented DNA (Fig. 29A). When equal volumes of MEM containing sodium ascorbate and MNNG were added to cell cultures, in that order, such that the final concentrations were 1×10^{-3} M and 1×10^{-5} M, respectively, considerably more fragmentation of DNA resulted (Fig. 29B). In vivo, when MNNG (40 mg per kg body weight) was force-fed to mice, fragmentation resulted (Fig. 30A). When MNNG (40 mg per kg body weight) was mixed with sodium ascorbate (100 mg per kg body weight) and immediately (within 10 sec) force-fed, increased fragmentation of gastric mucosal cells resulted (Fig. 30B).

The increased damage to DNA was not due to the independent action of sodium ascorbate, since sodium ascorbate alone did not fragment DNA in vivo (Fig. 31A) or in vitro (Fig. 31B).

Figure 28A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed a mixture of MNNG (20 mg per kg body weight) and sodium ascorbate (100 mg per kg body weight) immediately after mixing. Samples were taken 4 hr after force-feeding.

Figure 28B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed a mixture of MNNG (20 mg per kg body weight) and sodium ascorbate (100 mg per kg body weight) after a 0.5 hr incubation of the mixture at 37^{o}. Samples were taken 4 hr after force-feeding.

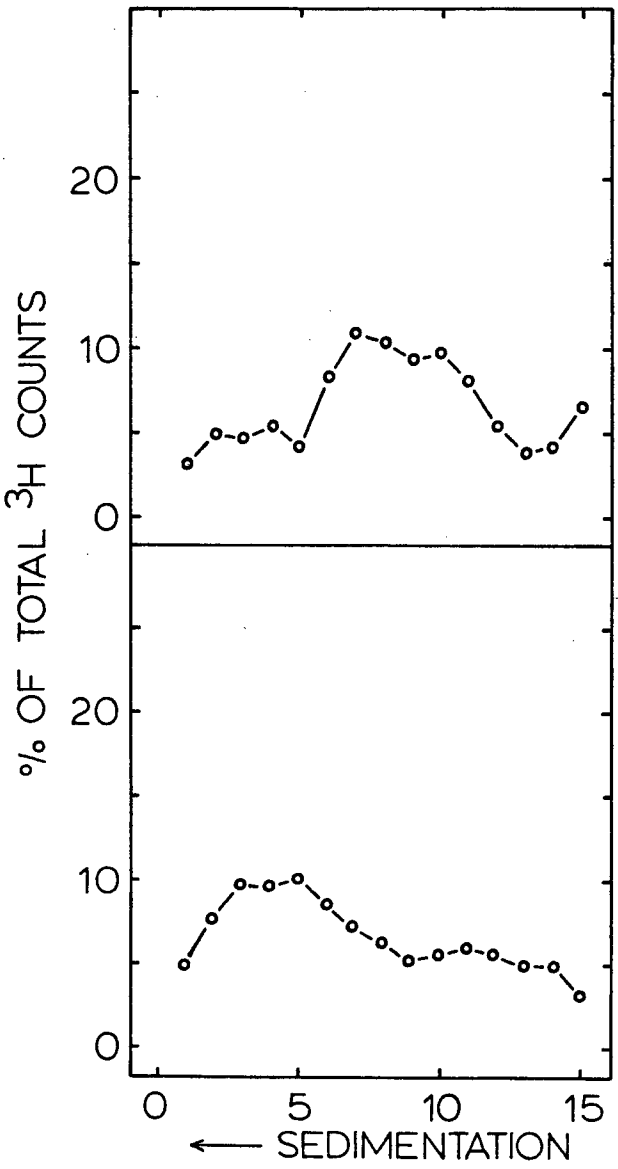

% OF TOTAL ^{3}H COUNTS
20
10
0
20
10
0
0
5
10
15
SEDIMENTATION

Figure 29A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to MNNG (1×10^{-5} M) for 0.5 hr.

Figure 29B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to MNNG (1×10^{-5} M) in the presence of sodium ascorbate (1×10^{-3} M) for 0.5 hr.

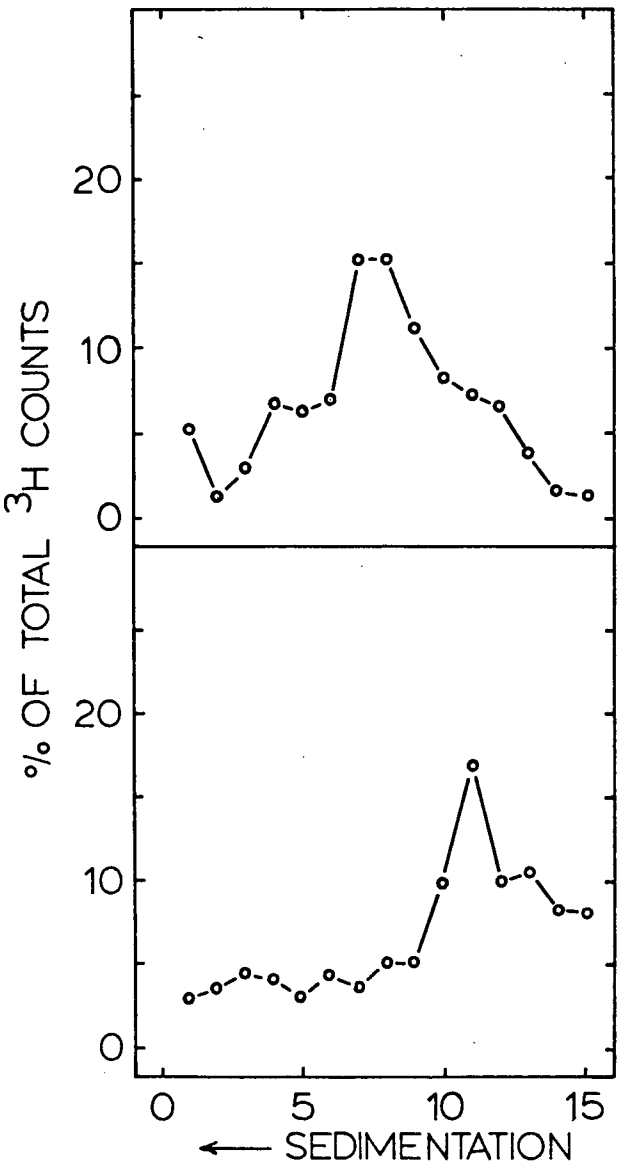

% OF TOTAL ^{3}H COUNTS
20
10
0
20
10
0
0
5
10
15
SEDIMENTATION

Figure 30A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed MNNG (40 mg per kg body weight) at 0 hr and sampled at 4 hr.

Figure 30B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed MNNG (40 mg per kg body weight) in the presence of sodium ascorbate (100 mg per kg body weight) at 0 hr and sampled at 4 hr.

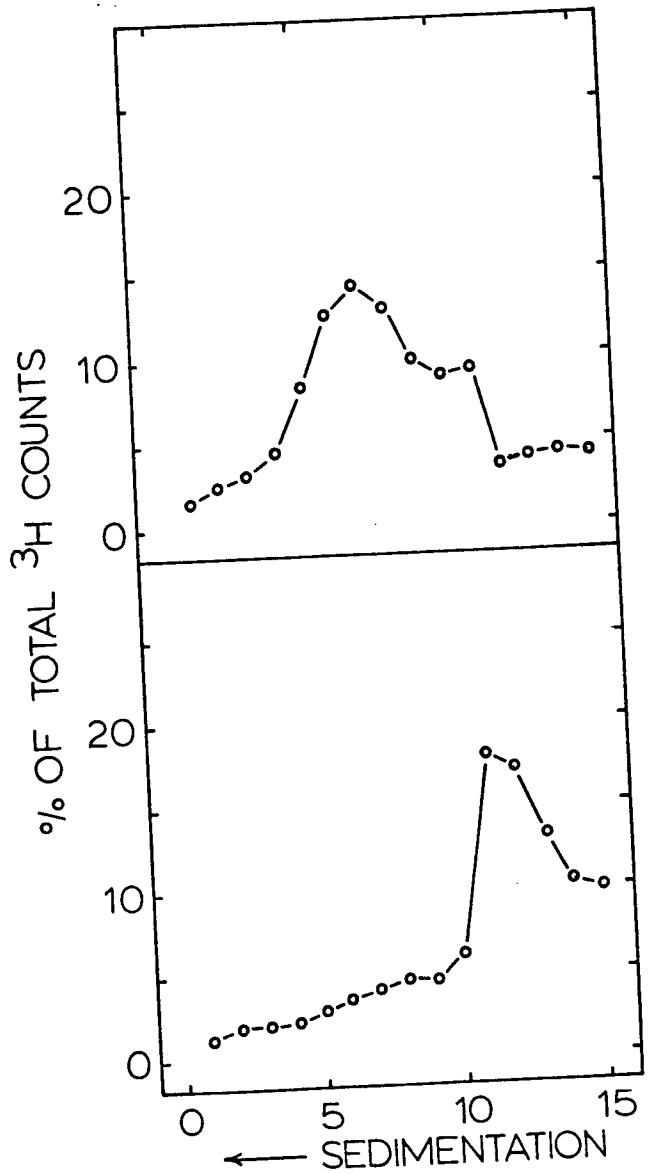
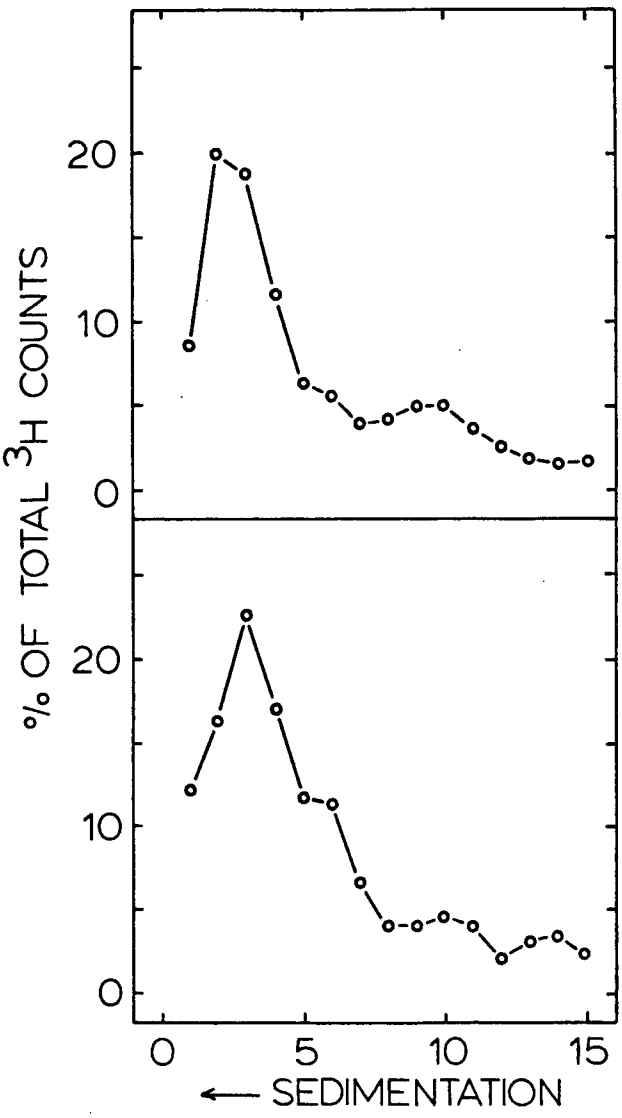

20
10
0
% OF TOTAL ^{3}H COUNTS
20
10
0
0
5
10
15
SEDIMENTATION

Figure 31A

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from gastric mucosal cells of mice force-fed sodium ascorbate (100 mg per kg body weight) at 0 hr and sampled at 4 hr.

Figure 31B

Alkaline sucrose gradient sedimentation profile of ^{3}H-DNA from cultured human fibroblasts exposed to sodium ascorbate (5×10^{-3} M) for 0.5 hr.


% OF TOTAL ^{3}H COUNTS
20
10
0
20
10
0
0
5
10
15
← SEDIMENTATION

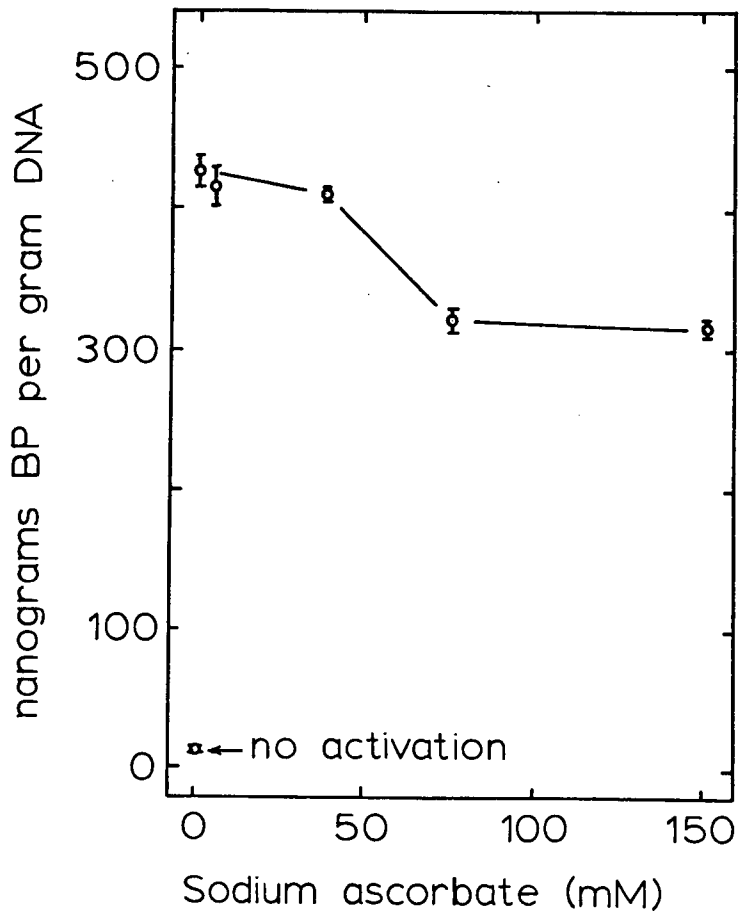
6) It might be argued that the decrease in fragmenting ability of MNNG observed after incubation with sodium ascorbate might have been due to loss of sodium ascorbate from the system by oxidation, binding to protein in solution, etc., during the incubation period. By this argument, sodium ascorbate would have only the ability to enhance fragmentation in this system, and the decrease in fragmentation seen after incubation would be due to loss of enhancement only. However, when sodium ascorbate was incubated for 0.5 hr with MNNG and force-fed to mice (Fig. 28B) fragmentation of DNA was less than that observed when MNNG alone was force-fed to mice, even when the MNNG force-fed alone was at a dose 25% higher than that force-fed with sodium ascorbate (Fig. 30A). Thus, the decrease in DNA-damaging ability of MNNG after incubation with sodium ascorbate was likely due to the action of acorbate or dehydroascorbate on MNNG, rather than merely the loss of sodium ascorbate from the system.

Effect of sodium ascorbate and other agents on binding of BP to DNA:

1) Since sodium ascorbate may inhibit damage to DNA by scavenging electrophilic carcinogenic and mutagenic species, the ability of sodium ascorbate to prevent binding of BP to DNA in vitro and in vivo was investigated. When sodium ascorbate was incubated with BP and S9 activation mixture over CHO cells, increasing concentrations decreased the amount of ^{3}H-BP label associated with purified DNA (Fig. 32). When BP (500 ng per mouse) was mixed with sodium ascorbate of various concentrations and force-fed to mice, ^{3}H-BP label associated with DNA decreased with increasing sodium ascorbate concentration. However, a low concentration of sodium ascorbate (15 mM) produced an increased

Figure 32

Binding of ^{3}H-BP to DNA from cultured CHO cells exposed to ^{3}H-BP (2×10^{-7} M) and various concentrations of sodium ascorbate in the presence of an S9 activation system for 2 hr. Plates were rinsed with PBS and DNA isolated immediately. The mean of 5 samples is plotted for each concentration, ± standard error.


500
300
100
0
nanograms BP per gram DNA
no activation
0
50
100
150
Sodium ascorbate (mM)

level of bound BP (Fig. 33) in two separate experiments.

2) Other reducing agents besides sodium ascorbate may affect BP binding to DNA. When the reducing agent propyl gallate was incubated with BP and S9 activation system over CHO cells, reduced binding of BP resulted (Fig. 34) in a manner similar to that observed with sodium ascorbate. However, when propyl gallate was mixed with BP and force-fed to mice, binding to gastric cell DNA increased with increasing propyl gallate concentration (Fig. 35). Due to the limited solubility of propyl gallate, only about half the molar concentration of sodium ascorbate used to exert effect in the case of propyl gallate.

Sulfhydryl as well as hydroxyl reducing agents may exert an effect on binding of BP to DNA. The sulfhydryl reducing agent glutathione, when incubated with BP and S9 activation system over CHO cells, increased the binding of BP to DNA by over 3 fold (Fig. 36). When glutathione was mixed with BP and force-fed to mice, the same increase in binding of BP to DNA occurred (Fig. 37). In this case, the extreme acidity of glutathione solutions allowed molar concentrations of only about one-sixth that of sodium ascorbate to be used. These solutions were neutralized by the addition of concentrated 10 M NaOH.

3) Since alteration in binding of BP by reducing agents may be due to differential inhibition of activating and deactivating enzyme systems, the effect of two compounds known to exert this effect - harman and norharman - was investigated in this system. Incubation of harman with BP and S9 activation system over CHO cells exerted no effect on BP binding over the concentration range studied (Fig. 38). The same lack of effect was observed with norharman (Fig. 39).

Figure 33

Binding of ^{3}H-BP to DNA from gastric cells of mice force-fed ^{3}H-BP (500 ng per mouse) in the presence of various concentrations of sodium ascorbate. A total volume of 0.5 ml was administered to each mouse. Samples were taken at 12 hr after force-feeding. The mean of values from 10 mice is plotted for each concentration, ± standard error.

The concentrations indicated are those of the force-fed solutions, not stomach contents after force-feeding. See discussion for further consideration.

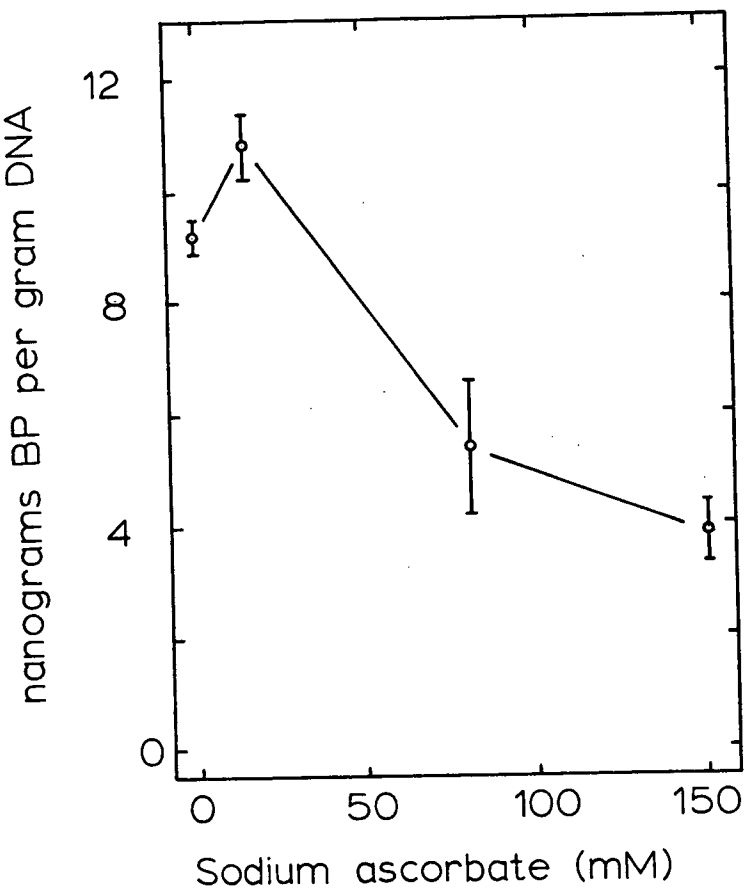

nanograms BP per gram DNA
12
8
4
0
0
50
100
150
Sodium ascorbate (mM)

Figure 34

Binding of ^{3}H-BP to DNA from cultured CHO cells exposed to ^{3}H-BP (2×10^{-7} M) and various concentrations of propyl gallate in the presence of an S9 activation system for 2 hr. Plates were rinsed with PBS and DNA isolated immediately. The mean of 10 samples is plotted for each concentration, ± standard error.

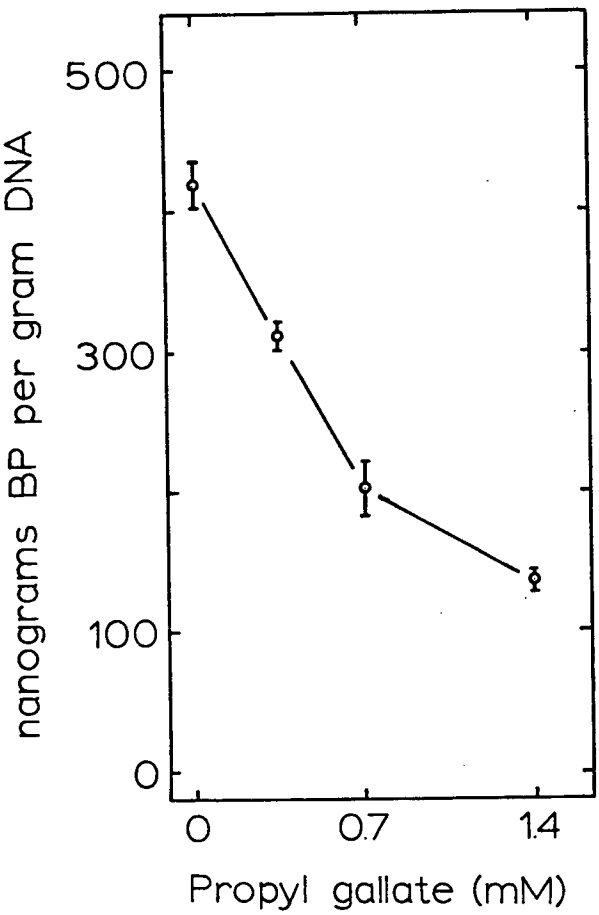

500
300
100
0
nanograms BP per gram DNA
0
0.7
1.4
Propyl gallate (mM)

Figure 35

Binding of ^{3}H-BP to DNA from gastric cells of mice force-fed ^{3}H-BP (500 ng per mouse) in the presence of various concentrations of propyl gallate. A total volume of 0.5 ml was administered to each mouse. Samples were taken 12 hr after force-feeding. The mean of values from 10 mice is plotted for each concentration, ± standard error.

The concentrations indicated are those of force-fed solutions, not stomach contents after force-feeding. See discussion for further consideration.

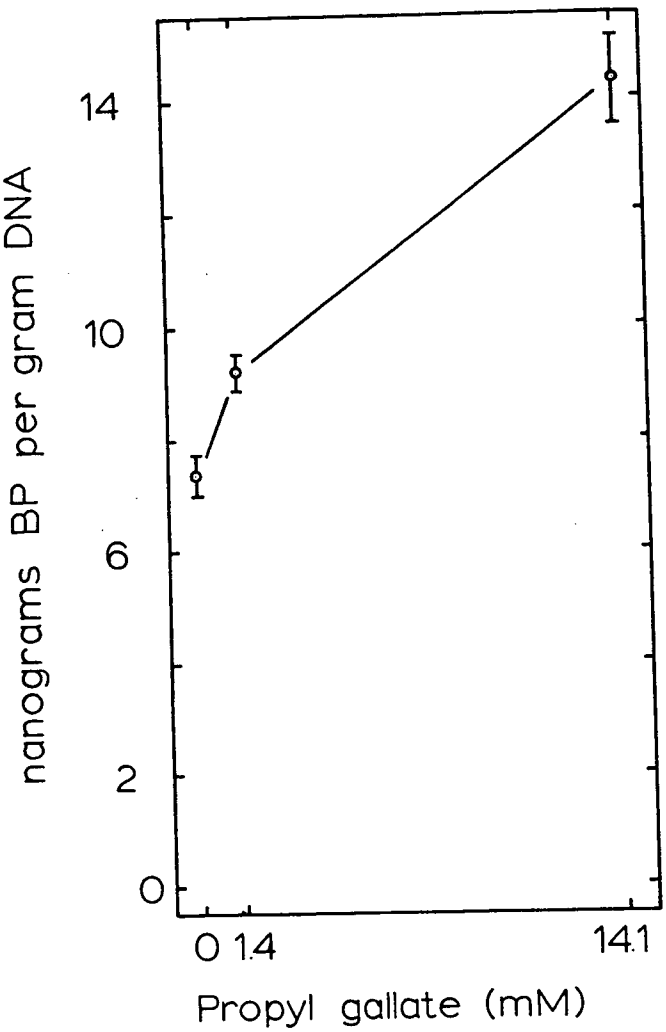

14
10
6
2
0
nanograms BP per gram DNA
0 1.4
14.1
Propyl gallate (mM)

Figure 36

Binding of ^{3}H-BP to DNA from cultured CHO cells exposed to ^{3}H-BP (2×10^{-7} M) and various concentrations of reduced glutathione in the presence of an S9 activation system for 2 hr. Plates were rinsed with PBS and DNA isolated immediately. Results from duplicate plates at each concentration are plotted.

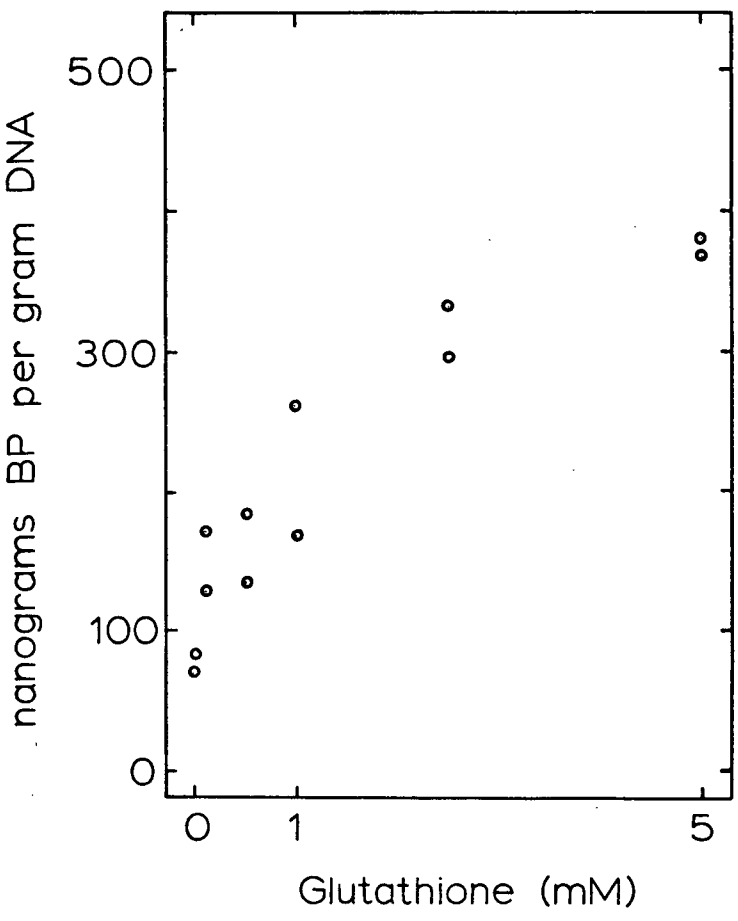

500
300
100
0
nanograms BP per gram DNA
0
1
5
Glutathione (mM)

Figure 37

Binding of ^{3}H-BP to DNA from gastric cells of mice force-fed ^{3}H-BP (500 ng per mouse) in the presence of various concentrations of reduced glutathione. A total volume of 0.5 ml was administered to each mouse. Samples were taken at 12 hr after force-feeding. Results from duplicate samples at each concentration are plotted

The concentrations indicated are those of the force-fed solutions, not stomach contents after force-feeding. See discussion for further consideration.

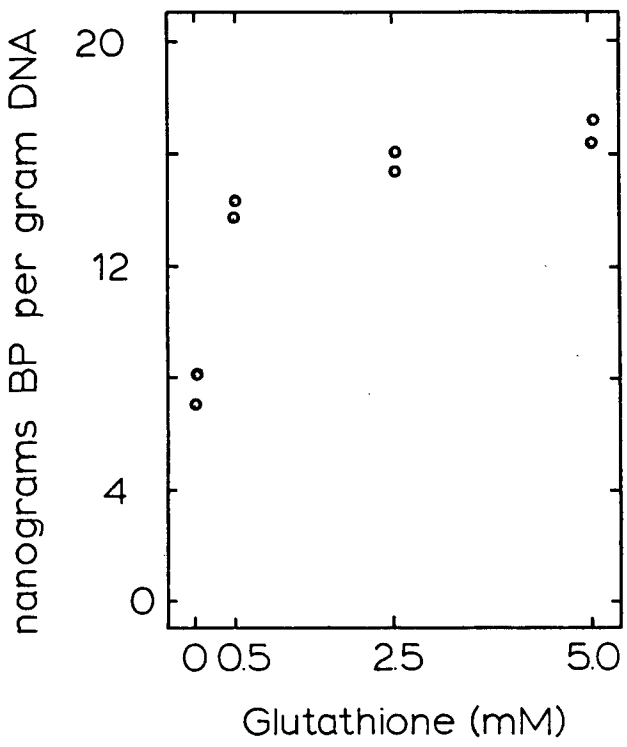

nanograms BP per gram DNA
20
12
4
0
0 0.5
2.5
5.0
Glutathione (mM)

Figure 38

Binding of ^{3}H-BP to DNA from cultured CHO cells exposed to ^{3}H-BP (2×10^{-7} M) and various concentrations of harman in the presence of an S9 activation system for 2 hr. Plates were rinsed and DNA isolated immediately. Results from triplicate samples for each concentration are plotted.

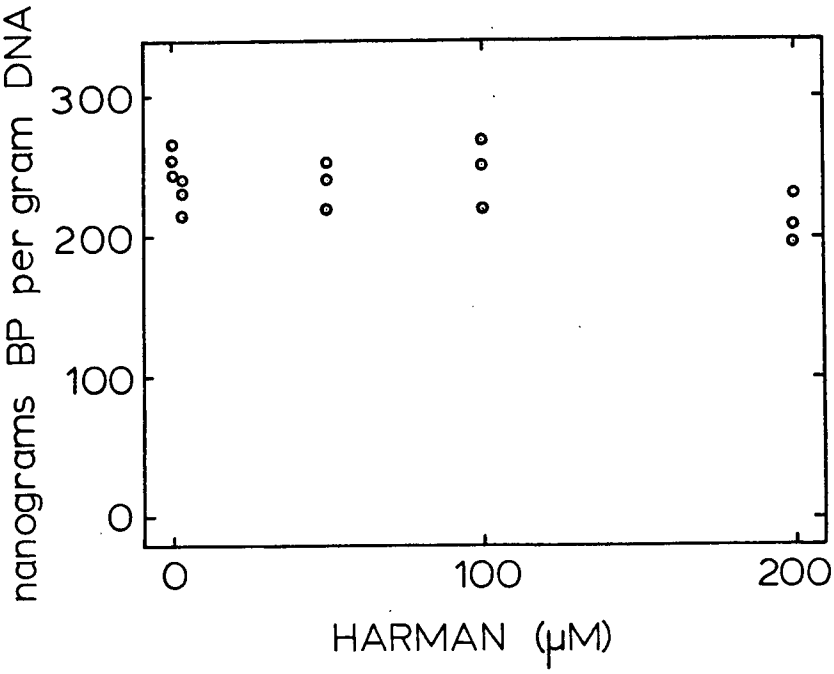
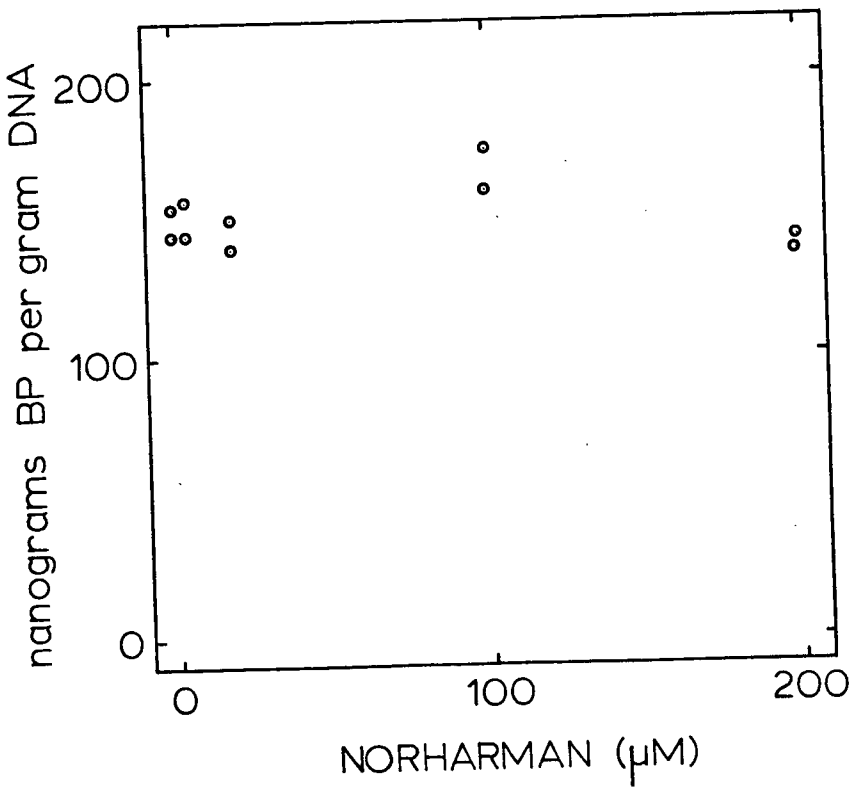

nanograms BP per gram DNA
300
200
100
0
0
100
200
HARMAN (μM)

Figure 39

Binding of ^{3}H-BP to DNA from cultured CHO cells exposed to ^{3}H-BP (2×10^{-7} M) and various concentrations of norharman in the presence of an S9 activation system for 2 hr. Plates were rinsed and DNA isolated immediately. Results from duplicate samples for each concentration are plotted.


nanograms BP per gram DNA
200
100
0
0
100
200
NORHARMAN (μM)

DISCUSSION

Studies aimed at identification of chemical carcinogens have historically focussed on two aspects of the problem. First, the investigation of substances that are chemically constructed for their ability to induce cancer, and which are virtually absent from the environment, have been useful because of the information they have provided on the process of cancer initiation (e.g., 7,12-dimethyl benz(a)anthracene and 4-nitroquinoline-1-oxide). Secondly, those relatively exotic chemicals made by man for some industrial, medical or domestic purpose have proved a fruitful source of carcinogenic substances. Their reactivity (the source of their usefulness in the above situations) makes them good candidates for the potential electrophilic reactants that have been implicated as ultimate carcinogens and mutagens. Only recently have the great mass of ubiquitous, naturally-occurring substances come under the eye of investigators. Realization of the ultimate electrophilic nature of carcinogens has made it possible to "screen" the previously intimidating number of compounds so that particularly promising candidates are recognizable by their potential carcinogenic nature (capable of being realized by cell-mediated or non-cellular chemical modification) rather than by their initial, unmetabolized reactivity.

In order to recognize all possible carcinogens in the environment, a variety of screening tests have been proposed to identify them. These range from actual induction of tumours in rodents and other mammals to the detection of short-term cellular and tissue changes that have been causally or empirically associated with induction of cancer. This second group includes short-term tests of three types: 1) those that assay

for changes induced by chemicals in isolated DNA or chromatin (e.g., unwinding of bacteriophage DNA cleaved by chemicals (Kuhnlein, 1980), 2) those that assay for chemical or morphological changes in cultured mammalian or bacterial cells (induction of DNA repair, chromosome aberrations, DNA alterations, germ cell anomalies, DNA-carcinogen adducts, etc. (reviewed by Stich and San, 1980)), and 3) those that assay for chemical or morphological changes in mammalian cells in vivo. These include many of the tests available for cultured cells, as well as those possible only in systems containing organs capable of exhibiting development of preneoplastic tissue (Solt and Farber, 1977) or specific locus mutations (Maier and Zbinden, 1980).

This array of tests is a useful one for detection of chemicals which may induce cancer, but it cannot be used to detect chemicals which may modify the induction of cancer by chemical or other agents. It would be highly desirable to have an assay system that could be used for the detection of such modifying chemicals, for use both in vitro and in vivo, and one of the aims of this work was to evaluate the usefulness of the alkaline sucrose gradient and BP-DNA adduct tests for DNA damage and repair as suitable candidates for such an application.

As an application of these tests, both were used to assay the ability of sodium ascorbate to modify the capacity of both cultured cells and mammalian epithelial cells in situ to repair damage to DNA caused by direct-acting carcinogen (MNNG) or a precarcinogen (BP) applied in the presence of an appropriate activating system.

It was found that sodium ascorbate, supplied to cells after the application of MNNG to fragment DNA, inhibited their ability to restore the capacity of DNA to sediment quickly in alkaline sucrose gradients. This restoration normally takes place by 30 hours after

carcinogen treatment (Koropatnick, 1978). Inhibition of repair took place for the period that unoxidized sodium ascorbate was present, and repair capacity was restored, both to cultured cells in vitro and gastric cells in vivo, when sodium ascorbate treatments were stopped. Sodium ascorbate treatment alone had no DNA-fragmenting effect on DNA. In addition, sodium ascorbate supplied to cells after the appoication of BP to form covalent DNA adducts inhibited their ability to excise those adducts. It was found that, in the absence of sodium ascorbate, cultured CHO cells rapidly lost approximately 60% of bound ^{3}H-BP during the first 12 hours after BP treatment, and thereafter lost only very small amounts of ^{3}H-BP label up to 72 hours post-treatment. This is similar to results abtained when rats were injected with ^{3}H-BP and the extent of specific aryl substitution at the 0-6 site of guanine in liver was determined (P. Kleihues, personal communication). The presence of sodium ascorbate in the medium present during the repair period strongly inhibited the excision during the first 12 hours, but, at low concentrations of sodium ascorbate (1×10^{-3} M), ^{3}H-BP binding returned to near-control levels by 24 hours after BP treatment. At high concentration (5×10^{-3} M), control levels were not reached until 36-48 hours post-treatment.

The same situation was observed in vivo in mouse gastric cells. Bound BP reached a peak in these cells 12 hours after force-feeding ^{3}H-BP. ^{3}H-binding fell to 50% of this high level in the first 36 hours after peak binding, and then did not significantly change up to 48 hours after peak binding. In the presence of sodium ascorbate, force-fed after the peak of ^{3}H-binding was reached, virtually no increase in bound ^{3}H-BP levels occurred. Within four hours after

the end of sodium ascorbate treatments, bound ^{3}H-BP levels fell rapidly, and normal binding levels were reached between 12 and 36 hours after cessation of sodium ascorbate treatment.

One of the questions raised by the observed inhibition of restoration of normal DNA is: at which stage of restoration of DNA does sodium ascorbate exert its effect? Since sodium ascorbate inhibited the excision of BP adducts and restoration of fast sedimentation of DNA, it appears that the inhibitory effect is exerted at a very early point in repair, at or before the excision stage of damaged regions of DNA (assuming a common repair mechanism for BP and MNNG-induced DNA repair). Since MNNG may alkylate DNA to produce apurinic sites that are converted to the single-strand breaks that are observed on alkaline sucrose gradients, such excision of repairable regions of DNA is a necessary prerequisite in the repair of MNNG-induced damage, as well as repair of BP-adducts. In addition, restoration of fast sedimentation to DNA on alkaline sucrose gradients is dependent upon ligation of single-strand breaks, as well as excision of damaged regions. The inhibition of repair of DNA observed in the presence of sodium ascorbate might therefore be due to inhibition of DNA ligation or inhibition of any of the processes leading up to it. Thus, sodium ascorbate may inhibit repair at one or more steps.

A second question thay may be asked is: does the sodium ascorbate alone inhibit excision of BP adducts and restoration of fast sedimentation of DNA, or are the oxidative or other metabolic products of sodium ascorbate responsible for this effect? Sodium ascorbate is oxidized *in vivo* to dehydroascorbate by a reversible process, and dehydroascorbate is irreversibly oxidized to diketogulonate and oxalate. The initial oxidation step may be catalyzed by sodium ascorbate itself,

especially if metal ions are added (Halliwell and Foyer, 1976; Michelson, 1973). Thus, because of the irreversible nature of later oxidation steps, sodium ascorbate concentration in a solution will quickly decline in favour of increasing concentration of its oxidation products. If these oxidation products were responsible for repair inhibition, then older sodium ascorbate solutions would be more efficient repair inhibitors than fresh solutions. However, when a single solution of sodium ascorbate was added to cultured human fibroblasts recovering, over a period of 30 hours, from damage inflicted by MNNG, repair was less inhibited than when fresh solutions of sodium ascorbate were added every 4 hours up to 20 hours. Thus, either sodium ascorbate, or some substance produced by it in the initial oxidation step, are necessary for inhibition of DNA repair.

Third, the question of relevance of the concentrations used to retard DNA in these experiments must be raised. The concentrations of sodium ascorbate used to inhibit repair in cultured cells ranged from 1×10^{-3} M to inhibit restoration of fast sedimentation to DNA from cultured human fibroblasts to 5×10^{-3} M to inhibit excision of BP adducts from cultured CHO cells (although 1×10^{-3} M sodium ascorbate could be used to inhibit excision of BP adducts in the first 12 hours after administration of BP). Calculation of the effective concentration of sodium ascorbate in experiments involving force-feeding, however, are more difficult, although empirical comparisons to human consumption are easier. There are two ways to consider the effective sodium ascorbate concentration on gastric mucosal cells in mice force-fed the sodium ascorbate: 1) by assuming the sodium ascorbate is diluted equally throughout the whole body volume, and 2) by assuming that sodium ascorbate is diluted only to the

volume of the stomach. Because complete dissemination of chemicals in the body requires a lengthy time period, the first method is better suited to observation of long-term effects of administered chemicals. Since restoration of fast-sedimentation of DNA from cultured human fibroblasts on alkaline sucrose gradients occurs between 12 and 18 hours after MNNG administration (Koropatnick, 1978) and prerequisite metabolic steps (e.g., excision of DNA adducts) occur at their greatest rate in the first 24 hours after carcinogen administration, long-term incubation is not suitable here. Also, increase or decrease of concentration of chemical by excretion, detoxification, or chemical instability, are not taken into account by the whole-body dilution system.

The second method - calculating effective concentration by stomach volume - seems more appropriate here. The test cells of the gastric mucosa line the stomach, and so are subjected to bathing chemicals before they are absorbed into the blood stream. Concentrations of sodium ascorbate differ here from those administered to cultured cells. Because of food present in the stomach, non-specific binding or oxidation of ascorbic acid may decrease the effective concentration. Variation in mouse stomach volume (estimated to be 0.002 to 0.005 litres) may also affect concentration. Also, mice do not require sodium ascorbate as a dietary supplement, and produce their own supply by biosynthesis from glucuronic acid. Unlike humans, they also possess an ascorbic acid reductase enzyme that has the ability to metabolize ascorbic acid and remove it from the system (Brown and Reynolds, 1963). If this enzyme is available in sufficient levels in gastric mucosal cells, rapid metabolism could reduce the length of time over which the sodium ascorbate is effective. Finally, movement of stomach contents into the small intestine will also reduce the effective time of action of sodium ascorbate.

With this in mind, the concentration of sodium ascorbate to which gastric mucosal cells were exposed, based solely on the volume of mouse stomach, was 1.4×10^{-2} M to 3.5×10^{-2} M in the case of alkaline sucrose gradient analysis of DNA repair and 3.5×10^{-2} M to 8.75×10^{-2} M in the case of BP-DNA adduct analysis of DNA repair. For reasons outlined above, these may be considered the upper limits of ascorbate concentrations actually applied to the cells.

In humans, stomach volume varies roughly between 0.5 and 2.0 litres. In order to achieve sodium ascorbate concentrations in the range effective in reducing repair in mice, doses of approximately 1-35 grams per treatment, administered periodically over the period of repair of mucosal cell DNA, would have to be given. This is a high level in comparison with that required for normal health (30-40 mg per day)(Pett, 1955). However, some recommended levels of sodium ascorbate administration for the prevention of colds (Pauling, 1976; Stone, 1972) and treatment of cancers (Cameron, et al., 1975; Cameron and Pauling, 1978) are within this range.

While sodium ascorbate was able to inhibit repair as measured by alkaline sucrose gradients and excision of BP adducts to DNA in vivo and in vitro, removal of sodium ascorbate (in the case of alkaline sucrose gradient analysis of repair in cultured human fibroblasts) and non-renewal of sodium ascorbate treatments (in the case of ASG analysis of repair in mouse gastric mucosal cells, and BP adduct analysis of repair in both cultured CHO cells and mouse gastric mucosal cells) resulted in the eventual return of the DNA of the cells in question to a state of repair that was close to that observed in cells untreated with sodium ascorbate. This is an indication that a) the inhibitory effect is reversible, and b) that sodium ascorbate is rapidly decomposed in the

system to remove its effect, or the cell becomes acclimatized to the presence of the ascorbate. Since mice force-fed ascorbate returned to normal levels of excision of BP adducts from DNA more quickly than cultured cells after cessation of sodium ascorbate treatments, and fresh treatments of sodium ascorbate increased the length of time over which repair inhibition took place in cultured human fibroblasts treated with MNNG, it seems likely that rapid decomposition of sodium ascorbate and its removal from the system is responsible for its eventual loss of effect, rather than adaptation of cellular repair systems to the presence of ascorbate.

While the observations made in both systems appear to favour the suggestion that sodium ascorbate inhibits repair, other explanations can be invoked to explain the observed effects. One suggestion is that sodium ascorbate may potentiate damage caused by carcinogens such that, when carcinogen is removed from cell systems and sodium ascorbate added, the observed retention of damage is not due to lack of repair, but rather to promotion of damage by residual carcinogen left behind by incomplete removal and rinsing. It was observed that sodium ascorbate may enhance the fragmentation of DNA in cultured human fibroblasts *in vitro* and mouse gastric mucosal cells *in vivo*, caused by MNNG. Sodium ascorbate in the presence of glycine-complexed copper could be used to fragment the DNA of cultured human fibroblasts *in vitro* and mouse gastric mucosal cells *in vivo*. Because of the possibility that enhancement of DNA damage was the *cause* rather than the *effect* of the observed retention of damage, the effect of sodium ascorbate on initial damage to DNA was measured.

When sodium ascorbate and BP were administered to mice by force-feeding, an initial *increase* in binding of BP was observed at low sodium ascorbate concentrations, followed by a decrease in BP binding with

increasing sodium ascorbate concentration. This small but statistically significant and reproducible increase in binding was observed at close to the same concentration (100 mg sodium ascorbate per kg body weight) that was used to cause the maintenance of high levels of BP adducts and slow-sedimenting DNA characteristics (40 mg per kg body weight) of mouse gastric mucosal cells treated in vivo. However, sodium ascorbate administered to cultured cells in combination with BP did not induce any increase in BP binding to DNA at ascorbate concentrations as high or higher than those used to inhibit repair of DNA fragmentation or BP adducts in cultured CHO cells or human fibroblasts.

Thus, even when BP is administered with sodium ascorbate in concentrations much higher than those likely to be left behind by incomplete rinsing, only a very small amount of increased binding is observed, and then only in vivo. This small increase does not appear to be enough to account for the retention of high levels of BP adducts in DNA observed in the presence of sodium ascorbate in the bathing medium of repairing cells. Therefore, it seems likely that the retention of MNNG and BP-induced DNA modification seen in the presence of sodium ascorbate is due to an effect on repair of DNA rather than on the initial DNA-damaging events.

While sodium ascorbate could be used to inhibit the repair of DNA fragmentation and BP adducts, the "scavenging" ability of sodium ascorbate may also be used to inhibit the action of several carcinogens. The DNA fragmenting property of S9-activated DMN was inhibited by the presence of sodium ascorbate, presumably by inhibition of action of the carcinogen rather than by inhibition of the activation system (Lo and Stich, 1978). Sodium ascorbate inhibited the non-enzymatic formation of nitrosation products of methylguanidine, as shown by the decreased DNA-fragmenting

ability of methylguanidine reacted in the presence of ascorbate. Sodium ascorbate, when incubated with MNNG for 30 minutes prior to treatment if cultured cells or mouse gastric mucosal cells, inhibited the DNA-fragmenting action of the MNNG. This was in contrast to the enhancement of MNNG fragmentation seen when mixed MNNG and sodium ascorbate were applied in vitro or in vivo without the 30 minute incubation. This enhancement was due, presumably, to inhibition of repair of DNA. This, the reducing potential of sodium ascorbate could be employed to "scavenge" electrophiles and prevent their action.

However, sodium ascorbate could be used to fragment DNA when co-administered with copper, both in vitro and in vivo. This fragmentation was repairable, since mouse gastric mucosal cells treated in vivo were able to restore near-control sedimentation properties to their DNA by 48 hours after treatment. This fragmenting ability was due, presumably, to DNA damage caused by hydrogen peroxide produced by autoxidation of sodium ascorbate in the presence of transition metals (Stich, et al., 1979).

Since the biological reducing agent sodium ascorbate had an effect on scavenging reactive electrophiles, an attempt was made to measure the scavenging ability of two other reducing agents - propyl gallate and glutathione. Propyl gallate, a food additive used to prevent oxidation (as is sodium ascorbate), was found to inhibit the binding of activated BP in cultured CHO cells, as expected from data gathered using sodium ascorbate. However, when propyl gallate was force-fed to mice in the presence of S9 activation system, an increase in bound BP in relation to propyl gallate concentration was found. In addition, reduced glutathione was found to increase binding of BP, both in vitro and in vivo. Because of the limited solubility of propyl gallate, only half the molar concentration of sodium ascorbate

required to alter BP-adduct formation was used. In addition, only relatively low concentrations of glutathione (approximately one-sixth of the molar concentration of sodium ascorbate required for effect) were used. In view of this disparity, the difference in effect might be due to concentration rather than qualitative effects. In fact, the initial increase in BP-binding to DNA *in vivo* observed in the presence of sodium ascorbate occurred at close to the same molar concentration of propyl gallate that caused a two-fold increase in binding *in vivo*. This may indicate that, at higher propyl gallate and glutathione concentrations *in vivo*, the same inhibition of BP-binding to DNA as that observed in the presence of sodium ascorbate might be seen.

Because of the differential effect of propyl gallate (inhibiting BP binding *in vitro* but enhancing BP binding *in vivo*), the tryptophan pyrolysis products harman and norharman (already shown to either enhance or inhibit BP mutagenicity in bacteria, depending on concentration) were administered to CHO cells in the presence of BP to observe whether such an effect was observable in this system. No significant inhibition or enhancement of BP binding was observed over the range of concentrations where inhibition or enhancement of BP mutagenicity in bacteria was seen (Fujino, *et al.*, 1978). Therefore, it seems unlikely that such a differential effect, due to differences in degree of inhibition of activating and inactivating enzyme systems associated with BP, is at work here. While the reducing properties of propyl gallate (as the driving force behing a "scavenging" effect on reactive electrophiles) may be invoked to explain decreased BP binding, it is intriguing to speculate that the *increase* in bound BP observed may be due to inhibition of repair mechanisms. However, it must be kept in mind that differential enzyme inhibition may still be a factor

in vivo, as difficulties in determination of propyl gallate concentration and time of exposure in the stomach, as outlined above, exist. Also, the enzymes responsible for BP metabolism in gastric mucosal cells may differ from the liver enzymes used in the S9 activation system. On the other hand, glutathione will increase binding of BP in vitro with controlled glutathione exposure and S9 activation, as well as in vivo. Thus, inhibition of repair may well be a reasonable mechanism to explain increased binding of BP in the presence of glutathione.

Summary

In general, sodium ascorbate was shown to inhibit repair of lesions introduced into DNA by MNNG or BP. Inhibition of both the early lesion excision steps and late DNA ligation and chromatin reorganization steps was observed in cultured CHO cells or cultured human fibroblasts, respectively, as well as in gastric cells of mice treated with carcinogen and sodium ascorbate in vivo.

While sodium ascorbate was demonstrated to inhibit DNA damage by "scavenging" reactive electrophiles, and could also fragment DNA in vivo and in vitro when co-administered with copper, these effects do not appear to be responsible for the observed retardation of excision of BP adducts from DNA or the restoration of fast-sedimenting ability of DNA on alkaline sucrose gradients.

In addition, the reducing agents propyl gallate and sodium ascorbate were shown to inhibit BP binding in vitro, but propyl gallate enhanced BP-binding in vivo. The reducing agent glutathione also enhanced BP binding, both in vivo and in vitro. It is possible that inhibition of DNA repair may be responsible for the observed

increase in DNA lesions.

Perspectives

While the reducing agent sodium ascorbate has been implicated in reducing the extent of DNA repair of damage caused by chemical carcinogens, it remains to be seen what relevance this observation has to the effect of vitamin C levels in human health. The best link of reduced repair capacity and induction of cancers comes from data gleaned from studies of human patients. Sufferers of xeroderma pigmentosum, Louis-Bar syndrome, Fanconi's anaemia, familial rectal polyposis, and Cockayne's syndrome (German, 1977; Paterson, 1977; German, 1978) all show predilection for cancer in association with reduced excision repair capacity. However, there are some individuals suffering from these diseases that exhibit normal repair capacity. Therefore, the possibility exists that enhanced susceptibility to cancer depends upon the quality of repair of damage, and not only on reduced quantity of repair. It seems reasonable to suppose that a decrease in DNA repair may lead to increased cell death rather than initiation of tumours (Kihlman, 1977). Sodium ascorbate has been shown to potentiate the toxicity of nitro-aromatic compounds (Koch, et al., 1979) and ascorbic acid has been shown to inhibit the expression of cultured mouse cell foci transformed by 3-methyl cholanthrene (Benedict, et al., 1980). Sodium ascorbate has also been shown to promote mitotic inhibition in cells treated with carcinogenic chemicals (Stich, et al., 1979). In the light of this, inhibition of the extent of DNA repair after carcinogen treatment may prevent tumour initiation in mammals by promoting the death of affected

cells. It is desirable that *in vivo* experiments be done in which sodium ascorbate is applied to animal test subjects after carcinogen administration to inhibit the short term repair that occurs in hours or days. The effect on the production of tumours or preneoplastic tissue would then be observed. In this way, the electrophile-scavenging effect and anti-cancer cell properties implicated for vitamin C may be excluded, and only the effect of inhibition of DNA repair events observed. Also, the effect of sodium ascorbate on repair of UV-induced lesions in DNA would be desirable. This would exclude the effect of electrophile-scavenging by sodium ascorbate, as well as avoid the difficulties associated with the presence of residual carcinogen after treatment of cells.

In the case of propyl gallate and glutathione, the ultimate effect of increased DNA damage by carcinogens in their presence remains unknown. It has been observed that, while sodium ascorbate enhances cytotoxicity of nitro-aromatic compounds, sulphydryl reducing agents such as glutathione, cysteine, cysteamine and mercaptoethanol inhibit cytotoxicity of these compounds (Koch, *et al.*, 1979). While the mechanism of these phenomena remains unknown, further investigation is certainly warranted. Experiments using combinations of glutathione and sodium ascorbate *in vitro*, to determine whether there is a chemical competition between the effects, would be a fruitful avenue of investigation.

As general methods for observing the effect of compounds that may modify the short-term effects of carcinogenic compounds, BP adduct and ASG sedimentation analysis of DNA appear to be well-suited. The most widely used short-term assay for DNA repair employs unscheduled incorporation of ^{3}H-TdR (Stich and San, 1980). This

assay cannot be used to differentiate between decreased damage and decreased repair, so that inhibition of incorporation of ^{3}H-TdR observed in the presence of some modifying compound could be due to either effect. Also, repair *in vivo* is technically difficult to observe using this method. The BP adduct assay may be used *in vitro* or *in vivo*. In addition, the ASG sedimentation assay may also be used to distinguish between both DNA damage and DNA repair, both *in vivo* and *in vitro*. It may also be used to measure the late-occurring repair events, up to and including chromatin reorganization, that are not addressed by measurement of unscheduled ^{3}H-TdR uptake or BP adduct excision from DNA.

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