STUDIES ON THE INTERACTION OF CHEMICAL CARCINOGENS WITH MACROMOLECULES

(INTERACTION OF STREPTOZOTOCIN AND METHYLGLYOXAL WITH DNA)

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CERTIFICATE

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INTRODUCTION

It is generally accepted that a high proportion of human cancers are attributable to environmental agents, mainly environmental chemicals. The distribution of potential carcinogens in the environment is essentially ubiquitous. The human diet contains a variety of naturally occuring mutagens and carcinogens (Ames, 1983). The predominance of certain foods in some countries has been related to the incidence of certain types of cancers in their populations. Therefore, dietary mutagens have attracted considerable interest in the last decade and a number of studies on dietary practices in relation to cancer have been undertaken. These studies suggest that a greater intake of fibre rich cereals, vegetables, and fruits and a lower consumption of fat rich products and excessive alcohol would be advisable (Doll and Peto,1981; Peto and Schneiderman,1981). Although quite a large number of dietary components have been evaluated in microbial and animal test systems, there is still a lack of definitive evidence about their carcinogenicity and mechanism of action. A majority of chemical carcinogens are known to form covalent adducts with DNA and there is a large body of evidence implicating DNA as a critical target in chemically induced cancer (Miller, 1978; O'Connor, 1981). In order to understand carcinogenesis at the molecular level, it is essential to determine the conformational changes in the target macromolecules and relate these findings to possible aberrations in the functioning of modified macromolecules. Of late, there has been

an increasing interest in oxygen radicals and lipid peroxidation as a source of damage to DNA and therefore as promoters of cancer (Harman, 1981; Genaler and Bernstein, 1981; Totter, 1980; Tappel, 1980). In addition, mammalian systems have evolved many defence mechanisms as protection against mutagens and carcinogens. The most important of such mechanisms may be those against oxygen radicals and lipid peroxidation. Therefore, some naturally occurring anticarcinogens are also included.

Mutagens and carcinogens in dietary plant material: It is obvious that food is a very complex substance to which humans are exposed. Most people perceive food substances of natural origin as free of risk. Such acceptance is largely based on faith because our objective knowledge on this topic is relatively poor. A large number of chemicals are synthesized by plants, presumably as a defence against a variety of invasive organisms, such as bacteria, fungi, and insects (Kapadia,1982; Clark, 1982; Pamukcu et al., 1980; Stich et al., 1981). The number of these toxic chemicals is extremely large and new plant chemicals are being continuously discovered (Jadhav et al., 1981; Griesebach and Ebel, 1978). It has been known for many years that plants contain carcinogens and a number of edible plants have demonstrated experimental carcinogenic activity for several species and various tissues. Widespread use of recently discovered short term

tests for detecting mutagens (Ames, 1979; Stich and San, 1981) and a number of animal cancer tests on plant substances have contributed to the identification of many natural mutagens and carcinogens in the human diet (Kapadia, 1982). Some examples of most frequently ingested compounds are discussed below.

Safrole and estragole are related compounds,which occur in certain spices and essential oils and are weak hepatocarcinogens (Penaroli, 1971; Guenther and Althausen, 1949). Recent studies have implicated 1'-hydroxysafrole and 1'-hydroxyestragole, respectively as proximate carcinogenic metabolites of safrole and estragole (Drinkwater et al., 1976; Borchert et al., 1973). Eugenol and anethole are structurally related to safrole and estragole,and are widely used as flavouring agents or as food additives. Black pepper contains small amounts of safrole and large amounts of a closely related compound piperine (Concon et al., 1979). Extracts of black pepper cause tumours in mice at a number of sites at a dose equivalent to 4 mg of dried pepper per day given for 3 months.

Ivie et al (1981) have reported that linear furocoumarins (psoralens),which are widespread in plants of the Umbelliferae family, are potent light activated carcinogens and mutagens. Three of the most common phototoxic furocoumarins are psoralen, xanthotoxin, and bergap-

ten. In addition to Umbelliferae, psoralen also occurs in plants from several other families (Ivie, 1978). Psoralens are potent photosensitizers and highly mutagenic in the presence of activating long wavelength UV light. They readily intercalate into duplex DNA, where they form light induced mono or di-adducts with pyrimidine bases. Psoralen, in the presence of light, is also effective in producing oxygen radicals (Ya et al., 1982).

Pyrrolizidine alkaloids are naturally occuring carcinogens and have been found in some fifty species of the families Compositae, Boraginaceae and Leguminosae (Schoental,1982), which are used as foods or herbal remedies. Several of these alkaloids are hepatotoxic and certain hepatotoxic pyrrolizidine alkaloids are also carcinogenic (Hirono et al., 1977; Schoental, 1976). Testing of pure pyrrolizidine alkaloids for carcinogenicity has not been extensive for reason of a limited supply of these chemicals. However, a number of these alkaloids have been reported to be mutagenic (Clark, 1960) in Drosophila and Aspergillus systems (Alderson and Clark, 1966). Recently, Mori et al. (1985) have used a hepatocyte primary culture-DNA repair test to screen seventeen pyrrolizidine alkaloids for their DNA damaging property. This test is highly responsive to carcinogenic pyrrolizidine alkaloids (Williams et al., 1980). Among the results obtained by these authors is the indication of a species difference in liver bioactiva-

tion of these alkaloids. This implies that there could be species difference in the carcinogenic potential of pyrrolizidine alkaloids.

In the last decade many studies on the mutagenicities of naturally occuring flavonoids have been reported. There are many flavonoids in plants and the mutagenicities of more than seventy naturally occuring flavonoids have been tested (Nagao et al., 1978; Sugimura et al., 1977). Of these, quercetin is the strongest mutagen, followed by kaempferol, rhamnetin, galangin, isorhamnetin, and fisptin. All these compounds, except quercetin, required metabolic activation by rat liver microsomal fraction and the mutagenicity of quercetin is further enhanced by rat liver enzymes. Quercetin is specially important since it occurs in conjugated or free form,sometimes in high concentrations, in many edible plant products including fruits, vegetables,and bracken fern. Evidence of the carcinogenicity of quercetin is conflicting. Whereas studies of Ambrose et al. (1952) report quercetin to be non-carcinogenic to rats fed 1 % quercetin for 410 days, more recent definitive studies of Pamukcu et al. (1980) demonstrate that quercetin was carcinogenic for the intestinal and bladder epithelium of the rat,when fed as a basic grain diet of 0.1 % quercetin (of purity $>$ 99 %) for 58 weeks. Although the mechanism of carcinogenicity of quercetin is not known, it has shown significant effects on DNA synthesis, lactate production, and cyclic adenosine 3',5'-monophosphate level in neopla-

stic cells (Podhajcer et al., 1980).

Edible mushrooms contain various hydrazine derivatives in relatively large amounts. Most hydrazines that have been tested have been found to be carcinogenic and mutagenic. The most common commerical mushroom, Agaricus bisporus, contains about 300 mg of agaritine, the γ-glutamyl derivative of the mutagen 4-hydroxymethylphenylhydrazine, per 100 g of mushrooms,as well as smaller amounts of the closely related carcinogen N-acetyl-4-hydroxymethylphenylhydrazine (Toth et al., 1982). Some agaritine is metabolized by the mushroom to a diazonium derivative, which is a potent carcinogen and is also present in the mushroom in smaller amounts. Many hydrazine carcinogens may act by producing oxygen radicals (Hochstein and Jain, 1981).

A number of 1,2-dicarbonyl compounds e.g., maltol, kojic acid, ethylmaltol, diacetyl, and glyoxal have been found to be mutagenic in the Salmonella/Microsome assay. Several compounds in this class are of toxicological interest because they occur in various foods. For example,maltol is a product of carbohydrate dehydration and is present in coffee, soyabeans,and baked cereals, such as bread. Kojic acid is a metabolite of many microorganisms including several fungi used in food production, while diacetyl is an aroma component of butter, beer, coffee, etc. (Fishbein, 1983).

Methylgloyxal (MG),also known as pyruvaldehyde or acetylformaldehyde, is a ketoaldehyde and may arise in the cell both enzymatically (Cooper and Andereon, 1970; Elliott, 1960; Sato et al., 1980) or nonenzymatically (Riddle and Lorenz, 1968) from free trioses. Besides, it has also been reported to be present in various foods, such as roasted coffee beans, tea, whisky, and soy sauce (Sugimura and Sato, 1983). Whether the enzymatic MG formation actually occurs in mammals has been controversial for many years (Meyer, 1953; Bonsignore et al., 1976; Salem, 1975; Van Eys et al., 1962; Riddle and Lorenz, 1968). The isolation of MG synthase from the enterobacteriaceae (Cooper, 1974; Yuan and Gracy,1977) and its presence in rat liver cells (Sato et al.,1980) confirmed that MG can be formed enzymatically from triose phosphates. Riddle and Lorenz (1968) observed that MG formation from both dihydroxyacetone phosphate (DHAP) and DL-glyceraldehyde is accelerated by polyvalent cations at physiological pH values.

Szent Györgyi (1967, 1977) proposed that MG interacts with the highly reactive sulfhydryl groups that may participate in the regulation of cell division in tissues and that this MG-SH complex can arrest cell division in rapidly dividing cells. MG and other similar aldehyde compounds exert significant effects on certain cancers by reducing their ascites fluid formation, prolonging the survival time of animals bearing those tumours and decreasing the mitotic index of normal and tumour cells (Jerzykowski et al., 1970; Fenselau and Long,

1976; Dianzani et al., 1978, 1980). The uncontrolled proliferation of tumour cells is supposed to be due to destruction of MG (Szent Gyorgyi, 1977) by two enzymes, namely, glyoxalase and α-ketoaldehyde dehydrogenase, which catalyze its oxidation to lactate, (Racker, 1951) and pyruvate (Monder, 1967), respectively.

In millimolar amounts, MG exerts several damaging effects on various biochemical parameters (Dianzani, 1979) and has been found to inhibit in vitro the growth of a variety of mammalian cell lines (Gregg, 1968; Klamerth, 1968; Scaife, 1969). Recently, in a screening performed to elucidate the DNA damaging activities of a series of biotic and xenobiotic aldehydes, Brambilla et al., (1984) found that exposure of cultured mammalian cells to non-toxic concentrations of MG resulted in the formation of macromolecular cross-links, mainly DNA protein type (Brambilla et al., 1985). Efforts have also been made to investigate the effect of MG on the microtubular system in order to understand the mechanisms of its antiproliferative activity (Gabriel et al., 1985).

A number of furans, such as 2-methylfuran, 2,5-dimethylfuran, furfural, 5-methylfurfural,and 2-furylmethylketone, are found in numerous food products including meat, milk products, various nuts, tea, and coffee (Maga, 1979). Stich et al. (1981) have reported that these

furans induced relatively high frequencies of chromatid breaks and chromatid exchanges,when they were exposed to cultured Chinese hamster ovary (CHO) cells in the absence of a liver microsomal preparation. The clastogenic doses of many of the furans were relatively high (100-3900 ppm), whereas the concentration in food products was relatively low. However, Stich et al. (1981) cautioned that the furans are not the only genotoxic chemicals in the complex mixture of heated, roasted, or boiled food products, and even if the furans do not pose a serious health hazard by themselves due to their small amounts in most food items, they may contribute significantly to the total genotoxicity of many consumable foods and beverages.

In addition to poyrrolizidine alkaloids, certain glycoalkaloids found in potato, such as solanine and chaconine, have been reported to be highly toxic as they are strong inhibitors of cholinestrase (Jadhav et al., 1981). Pyrrolizidine alkaloids and other glycoalkaloids can reach levels which can be lethal to humans in potatoes that are diseased or exposed to light (Katsui et al., 1982).

Cyclopropenoid fatty acids, present in cotton seed and other oils, have been reported to be carcinogenic, mitogenic having various toxic effects in farm animals. Among these, sterculic acid and malvalic acid are widespread in the human diet. They are also potentiators

of carcinogenicity of aflatoxins (Hendricks et al., 1980). Human exposure to these fatty acids results from the consumption of products of animals fed on cotton seed. Another major toxin in cotton seed is gossypol, which accounts for about 1 % of its dry weight. Gossypol causes male sterility through formation of abnormal sperm and is carcinogenic as well (Xue, 1980). It has been reported to be a potent initiator and also promoter of carcinogenesis in mouse skin (Haroz and Thomassan, 1980). Gossypol has been tested in China as a possible male contraceptive as it is inexpensive and causes sterility during use. Its mode of action as a spermicide is through the production of oxygen radicals.

A number of quinones and their phenolic precursors are found in the human diet and have been shown to be mutagens (Stich et al., 1981; Brown, 1980; Levin et al., 1982). Quinones are quite toxic as they can act as electrophiles or accept a single electron to yield the semiquinone radicals, which can react directly with DNA or generate superoxide radicals (Morimoto et al., 1983; Kappus and Sies, 1981). Many dietary phenols can autoxidize to quinones generating hydrogen peroxide at the same time. The amounts of these phenols in human diet are appreciable, for example, catechol, which appears to be mainly derived from metabolism of plant substances and is a potent promoter of carcinogenesis and an inducer of DNA damage (Carmella et al.,1982).

In addition, there are many other dietary compounds,which have been shown to be mutagenic and carcinogenic in various test systems. Allylisothiocyanate, a major flavour ingredient of mustard oil, is one of the main toxins of mustard seeds and has been shown to be a carcinogen in rats (Dunnick et al., 1982). Phorbol esters, present in plants of Euphorbiacea family, are potent promoters of carcinogenesis and cause nasopharyngeal and esophageal cancers (Hecker, 1981). A variety of carcinogens and mutagens are present in mold contaminated food grains, nuts, and fruits. Some of these, such as various aflatoxins, are among the most potent carcinogens and mutagens known (Hirono, 1981; Tazima, 1982). Nitrosoamines and other nitroso compounds formed from nitrate and nitrites in food have been directly related to the incidence of stomach and esophageal cancer. Nitrates are present in large amounts in spinach, radish, lettuce, and beans (Magee, 1982). Although alcohol is not a constituent of a normal human diet, in view of its widespread use by a large section of the human population, it would be relevant to mention its toxic role. Alcohol has long been associated with the cancer of mouth, pharynx, and liver (Tuyns et al., 1982). Alcohol metabolism generates acetaldehyde, which is a mutagen and possibly a carcinogen (Stich and Rosin, 1983; Campbell and Fantel, 1983). It also generates radicals that produce lipid hydroperoxides and other mutagens and carcinogens (Winston and Cederbaum, 1982; Videla et al., 1982).

Dietary fat- a possible source of carcinogens: Fat accounts for approximately 40 % of the calories in the human diet. There is epidemeological evidence relating high fat intake with colon and breast cancer. Animal studies have indicated that high dietary fat is a promoter and a presumptive carcinogens (Kinlen, 1983; Fink and Kritchevsky, 1981; Welsch and Aylsworh, 1983). Two plausible mechanisms, involving oxidative processes, have been considered to account for the relationship between high fat intake and the occurrence of cancer and heart diseases. According to the first mechanism, rancidity of fat yields a variety of mutagens and carcinogens,such as fatty acid hydroperoxides, cholesterol hydroperoxides, fatty acid epoxides, and aldehydes (Simic and Karel, 1980; Bischoff, 1969; Petrakis et al., 1981; Imai et al., 1980; Ferrali et al., 1980). Alkoxy and hydroperoxy radicals are also formed (Pryor, 1976-1982). Therefore, the colon and digestive tract are exposed to a variety of fat derived carcinogens. The second possible mechanism involves hydrogen peroxide, which is generated by the oxidation of dietary fatty acids by peroxisomes. Each oxidative removal of two carbon units generates one molecule of hydrogen peroxide, a known mutagen and carcinogen (Reddy et al., 1982; Plain, 1955). Some hydrogen peroxide may escape the catalase in the peroxisomes and thus contribute to the supply of oxygen radicals (Speit et al., 1982; Jones et al., 1981). Oxygen radicals in turn can damage DNA and start the rancidity chain raction, which leads to the

production of the mutagens and carcinogens, mentioned above (Pryor, 1976-1982).

Mutagens and carcinogens produced in cooking: Sugimura and his colleagues (1978,1979),as well as others (Pariza et al.,1983) have reported that the burnt and browned material from heating protein during cooking is highly mutagenic. Pyrolysis of protein produces strong frame-shift mutagnes that require metabolic activation by rat liver S-9 fraction (Nagao et al., 1977). Pyrolysates of amino acids also show various mutagenic activities (Matsumoto et al., 1977). Among the various amino acids, the pyrolysate of tryptophan has been found to be most mutagenic, followed by those of serine, glutamic acid, ornithine, and lysine.

Pyrolysates of various sugars, such as glucose, arabinose, fructose, and sorbitol, are all mutagenic in S. typhimurium system, without metabolic activation. Pyrolysate of glucose was found to contain acetaldehyde and glyoxal, which are mutagenic to S. typhimurium (Nagao et al., 1978). Caramel, which is sugar derived and widely used as a food colouring and flavouring agent is also mutagenic in Salmonella test systems, but had no carcinogenic effect, when fed to rats as 6 % of the diet for two years (Evans et al., 1977). Coffee contains a considerable amount of burnt material, including the muta-

genic pyrolysis product methylglyxal (Sugimura and Sato, 1983). One cup of coffee also contains about 250 mg of the natural mutagen chlorogenic acid (Stich et al., 1981) and about 100mg of caffeine which can cause birth defects at high levels in several experimental species (Fabro, 1982). There is inconclusive evidence to suggest that heavy coffee drinking is associated with cancer of the ovary, bladder, pancreas, and the large bowel (Trichopoulos et al., 1981). Rancidity reaction of cooking oils and animal fat is accelerated during cooking, thus increasing intake of mutagens and carcinogens (Simic and Karel, 1980).

Food additives: Sodium nitrite is extensively used as a preservative in meat, fish, and cheese. A possible formation of nitrosoamines from amines, present in or derived from the diet, occur by reaction with nitrous acid at acidic pH. In humans, gasteric juice attains a pH of nearly 1.0. Such high concentrations of hydrogen ions give rise to the nitrosyl cation No^{+} , which is a highly reactive nitrosylating agent. Nitrous acid itself is a known mutagen for various bacterial and fungal cells. Its mutagenicity is presumably related to the deamination of adenine and cytosine (Fishbein et al., 1970). Sodium bisulphite is used as a bacterial inhibitor in a variety of beverages and as a preservative in canned fruits and vegetables. The bisulphite anion reacts, rather specifically, with uracil and cytosine, within

single-stranded regions of DNA. It is also mutagenic to bacteria and bacteriophages (Singer, 1983). Ethylenediaminetetra-acetic acid (EDTA) and its alkali salts are widely used as sequestrants in various foods. They are useful as antioxidants due to their property of forming poorly dissociable chelate complexes with trace quantity of metal ions such as copper and iron in fats and oils. EDTA has been shown to induce chromosome aberrations and breakage in various plant species.

Saccharin was synthesized in the last century and since then it has been widely used as an artificial sweetener. Reports on the mutagenicity and carcinogenicity of saccharin are conflicting and there is some suggestion that these activities are thought to be due to impurities present in saccharin preparations (Kramers,1975). The possibility of an *in vivo* conversion of saccharin into a mutagenic metabolite has also been suggested (Batzinger *et al.*, 1970). Another artificial sweetener, which was widely used but is now banned in USA and many other countries,is cyclamate. Cyclamate induces chromosome breakage in cells of several plants and animal species. It is converted *in vivo* into cyclohexylamine, which is also an inducer of chromosome breaks (Fishbein *et al.*, 1970).

Oxygen radicals and cancer: One of the theories of etiology of cancer, which is being widely accepted, holds that the major cause is damage to DNA by oxygen radicals and lipid peroxidation (Ames, 1983, Totter, 1980). Several enzymes produce superoxide anion ($O_2^{\cdot -}$) during the oxidation of their substrates,for example, xanthine oxidase and peroxidase (Buettner et al., 1978; Duran et al., 1977). Numerous substances, such as reduced flavins and ascorbic acid, upon autoxidation produce superoxide anion. This radical may further accept an electron from a reducing agent, such as thiols, to yield peroxide (H_2O_2). There is in vitro evidence that H_2O_2 may then reacts with certain chelates of copper and iron to yield the highly reactive hydroxyl free radical ($^{\cdot}OH$) (Wolff et al., 1986). That the superoxide anion actually appears in metabolism is confirmed by the ubiquitous occurrence of superoxide dismutase. Indeed, certain white blood cells generate superoxide deliberately by means of a specialized membrane bound NADPH oxidase and this participates in the killing of microorganisms and tumour cells (Wolff et al., 1986).

It has been suggested that certain promoters of carcinogenesis act by generation of oxygen radicals, this being a common property of these substances. Fat and hydrogen peroxide are among the most potent promoters (Welsch and Aylsworth, 1983). Other well known cancer promoters are lead, calcium, phorbol esters, asbestos, and various quino-

nes. Inflammatory reactions lead to the production of oxygen radicals by phagocytes and this may be the basis of promotion by absestos (Hatch et al., 1980). Many carcinogens, which do not require the action of promoters and are by themselves able to induce carcinogenesis (complete carcinogens), also produce oxygen radicals (Demopoulos et al., 1980). These include nitroso compounds, hydrazines, quinones, and polycyclic hydrocarbons. Much of the toxic effect of ionizing radiation damage to DNA is also due to the formation of oxygen radicals (Totter, 1980). The mechanism of action of promoters may involve the expression of recessive genes and an increase in gene copy number through chromosome breaks and creation of hemizygosity (Kinsella,1982; Varshavsky, 1981). Promoters may also cause modification of prostaglandins, which are intimately involved in cell division, differentiation, and tumour growth (Fischer et al., 1982). Most data on radicals damage to biological macromolecules concern with the effects of radiation on nucleic acids because of the possible genetic effects. However, in view of the catalytic role of enzymes, damage to proteins is also considered important. It has been suggested that primary oxygen radicals, produced in cells and their secondary lipid radicals intermediates, modify and fragment proteins. The products are often more susceptible to enzymatic hydrolysis leading to accelerated proteolysis inside and outside the cells (Wolff et al., 1986).

Anticarcinogens: The protective defence mechanisms against mutagens and carcinogens include the shedding of surface layer of the skin, cornea, and the alimentary canal. If oxygen radicals play a major role in DNA damage, defence against these agents is obvioulsy of great importance (Totter, 1980). The major source of endogenous oxygen radicals are hydrogen peroxide and superoxide which, are generated as side products of metabolism (Pryor, 1976 - 1982). In addition, oxygen radicals also arise from phagocytosis after viral and bacterial infection or an inflammatory reaction (Tauber, 1982). The exogenous oxygen radical load is contributed by a variety of environmental agents as discussed in this dissertation and elsewhere (Pryor, 1976 - 1982). The enzymes that protect cells from oxidative damage are superoxide dismutase,glutathione peroxidase (Pryor,1976 - 1982). D.T. diaphorase (Lind et al.,1982) and glutathione transferases (Warholm et al.,1981). In addition to these enzymes, some small molecules in the human diet act as antioxidative agents and presumably, have an anticarcinogenic effect. Some of these compounds are discussed below.

Tocopherol (vitamin E) is an important trap of oxygen radicals in membranes (Pryor, 1976 - 1982) and has been shown to decrease the carcinogenic effect of quinones, adriamycin and daunomycin, which are toxic because of free radical generation (Ames, 1983). Protective effect of tocopherols against radiation induced DNA damage and dimeth-

ylhydrazine induced carcinogenesis have also been observed (Beckman, et al., 1982). β-carotene is a potent antioxidant present in the diet and is important in protecting lipid membranes against oxidation. Singlet oxygen is a highly reactive form of oxygen, which is mutagenic and is mainly generated by pigment mediated transfer of the energy of light to oxygen. Carotenoids are free radical traps and are remarkably efficient as quenchers of singlet oxygen (Packer et al., 1981). β-carotene and similar polyprenes are also the main defence in plants against singlet oxygen generated as a by product of the interaction of light and chlorophyll (Krinsky and Deneke, 1982). Carotenoids have been shown to be anticarcinogens in rats and mice and may also have a similar effect in humans (Mathews Roth,1982, Peto et al.,1981). Glutathione is present in food and is one of the major antioxidants and is antimutagenic in cells. Glutathione transferases are a major defence against oxidative and alkylating carcinogens (Warholm et al ., 1981). Dietary glutathione is effective anticarcinogens against aflatoxins (Novi, 1981). The cellular concentration of glutathione is influenced by dietary sulphur amino acids (Tateishi et al., 1981). Selenium, which is present in the active site of of glutathione peroxidase, is another important dietary anticarcinogen. Glutathione peroxidase is essential for destroying lipid hydroperoxides and endogenoous hydrogen peroxide and therefore, helps to prevent oxygen radical induced lipid peroxidation (Flohe, 1982). Several heavy metal toxins,

such as Cd^{2+} (a known carcinogen) and Hg^{2+} decrease glutathione peroxidase activity by interacting with selenium (Flohe, 1982). Some other dietary antioxidants include ascorbic acid and uric acid. The former has been shown to be anticarcinogenic in rodent treated with UV light and benzo(a)pyrene (Hartman, 1982). Uric acid is present in high concentrations in the blood of humans and is a strong antioxidant (Ames et al., 1981). A low uric acid level has been considered a risk factor in cigarette caused lung cancer; however, too high levels may cause gout.

In addition,edible plants contain a variety of substances, such as phenols that have been reported to inhibit or enhance carcinogenesis and mutagenesis in experimental animals (Ames, 1983). The inhibitory action of such compounds may be possibly due to the induction of cytochrome P-450 and other metabolic enzymes (Boyd et al., 1982). The optimum levels of dietary antioxidants have not been determined; however, there might be considerable variation among individuals. On the other hand, high doses of such compounds may lead to deleterious side effects. The differnces in cancer rates of various populations are generally considered to be due to environmental and life style factors such as smoking, dietary carcinogens and promoters. However, these differences may also be due, in good part, to insufficient amounts of anti- cartinogens and other protective factors in the diet (Maugh, 1979).

In the last two decades, there has been much emphasis on the induction of cancer by occupational and industrial pollution factors. There is growing recognition, however, that these may account for only a small fraction of human cancers. It is becoming increasingly clear from epidemeological and laboratory data that diet is an important factor in the etiology of certain human cancers. It has been suggested by Doll and Peto (1981) that in the United States diet accounts for 35 % of cancer deaths. According to these authors, there are five possible ways whereby diet may affect the incidence of cancer; (i) ingestion of powerful, direct acting carcinogens or their precursors; (ii) affecting the formation of carcinogens in the body; (iii) affecting transport, activation or deactivation of carcinogens; (iv) affecting 'promotion' of cells that are already initiated,and (v) overnutrition. Normal individual consumption of potentially mutagenic substances per day from foods and beverages is estimated to be between 1 to 2 gm. In addition, the endogenous conditions favour the formation of still more mutagens in vivo in humans (Oshshima and Bartsch, 1981).

Thus,it appears that no human diet is entirely free of mutagens and carcinogens and that such substances are perfectly natural and traditional in foods. However, it should be emphasized that most studies on the identification of dietary mutagens and carcinogens have been done in microbial and animal test syetems and therefore their

optimum toxic levels, which may vary among individuals, remain to be determined. Hence, the quantification of the approximate magnitude of the risk posed by the human diet remains a major challenge. Extrapolation of risk from experimental animals, such as rodents to humans is difficult for several reasons, including the longevity differences, antioxidant factors and presumably, the multicausal nature of most human cancers. Among the generally accepted risk factors for the induction of cancer, tobacco smoking is no doubt a major risk. It has been linked to both cancer and heart attacks. Diet, with its carcinogenic and anticarcinogenic components, is likely to be another major risk factor. Other risks include alcohol consumption, exposure to certain drugs, cosmetics, industrial pollution, and occupational exposure to dust and solvents.

Streptozotocin : Streptozotocin, an antibiotic produced by the strain Streptomyces achromogenes, is a 2-deoxy-D-glucose derivative of the carcinogen N-methyl-N-nitrosourea (Herr et al., 1967). In addition to its antibiotic properties, streptozotocin is widely used for induction of diabetes in experimental animals as it selectively destroys the pancreatic B-cells (Rerup, 1970). It has been shown to be a potent methylating agent and is used for the treatment of pancreatic neoplasms. The alkylation reaction was found to be similar to that of N-methyl-N-nitrosourea (MeNu), both in extent and the relative propor-

tion of methylation products formed in DNA. However, streptozotocin caused virtually no methylation in brain DNA, but both liver and kidney DNA were alkylated to a greater extent than that with MeNu (Bennett and Pegg, 1981). Nicotinamide has been reported to prevent the destruction of pancreatic B-cells but potentiates streptozotocin-mediated carcinogenesis in these cells (Dulin and Wyse, 1969; Kazumi et al., 1978; Rakieten et al., 1971). Neither the mechanism by which streptozotocin exerts the cytotoxic and carcinogenic effects nor that by which nicotinamide alters these effects is clearly understood. It is generally accepted that the alkylation of DNA by alkylating carcinogens, such as the alkylnitrosoureas, is a critical step in induction of tumours by these compounds (Singer ,1979). Rizvi and Hadi (1984) have reported N-ethyl-N-nitrosourea (EtNu) alkylated DNA to be more thermostable at neutral pH than native DNA. This was considered in view of the greater inhibitory effect of EtNu than several other alkylating agents on the template activity of DNA for RNA synthesis (Marushige and Marushige, 1983b). In this dissertation the effect of alkylation of DNA by streptozotocin on its secondary structure has been investigated. The influence of nicotinamide on streptozotocin-induced changes in native DNA has also been determined.

EXPERIMENTAL

MATERIALS

Chemicals used for the present studies were obtained from the sources given against their names.

Chemical	Source
Acetaldehyde	Fluka, Switzerland.
Benzoylated, naphthoylated diethylaminoethyl cellulose	Sigma Chemical Co., U.S.A.
Bovine serum albumin	Sigma Chemical Co., U.S.A.
Caffeine	Sigma Chemical Co., U.S.A.
Deoxyribonucleic acid (calf thymus)	Sigma Chemical Co., U.S.A.
Diphenylamine	B.D.H., India.
Ethylenediaminetetra-acetic acid	B.D.H., India.
Formaldehyde	Sarabhai M. Chemicals, India.
Formamide	Merck, India.
Glycerol	B.D.H., India.
Hydroxyapatite	Sigma Chemical Co., U.S.A.
Methylglyoxal	Sigma Chemical Co., U.S.A.
Nicotinamide	Sigma Chemical Co., U.S.A.
N-methyl-N-nitrosourea	Department of Chemistry, A.M.U., Aligarh.
Pea seed nuclease	Purified in our laboratory
Perchloric acid	Merck, India.
Potassium dihydrogen ortho-phosphate	B.D.H., India.

Di-potassium hydrogen phosphate 3-hydrate	Merck, Germany.
S_1 nuclease	Sigma Chemical Co., U.S.A.
Sodium acetate	Merck, India.
Sodium dodecyl sulphate	Sigma Chemical Co., U.S.A.
Streptozotocin	Sigma Chemical Co., U.S.A.
Tris (hydroxymethyl)-aminomethane	Fluka, Switzerland.

METHODS

Estimation of DNA and acid soluble nucleotides : DNA nucleotides, made acid soluble,were determined by the diphenylamine method of Schneider (1957) or spectrophotometrically by reading the absorbance at 260nm. To an 1.0ml aliquot, 2.0ml of diphenylamine reagent (freshly prepared by dissolving 1gm of recrystallized diphenylamine in 100ml of glacial acetic acid and 2.75ml of conc. H_2SO_4 was added. The tubes were heated in a boiling water bath for 20 minutes. The intensity of blue colour was read using Spectronic 20, Bausch and Lomb spectrophotometer. To determine the acid soluble material spectrophotometrically, an aliquot of the supernatant was suitably diluted and read at 260nm using Spectronic 21 UVD Bausch and Lomb spectrophotometer against a suitable blank. In some experiments, DNA was also determined by diphenylamine modified procedure of Burton (1956).This procedure considerably increases the sensitivity of the reaction. To an 1.0ml aliquot was added 1.0ml of 1N perchloric acid and the mixture heated at 70 $^{\circ}$C for 15 minutes. To this were added 0.1ml of 5.43mM acetaldehyde and 2.0ml of Burton's diphenylamine reagent (freshly prepared by dissolving 1.5gm of recrystallized diphenylamine in 100ml glacial acetic acid and 1.5ml of conc. H_2SO_4 . The blue colour, thus developed was read at 600nm.

Preparation of depurinated DNA from DNA treated with streptozotocin and MG: A 5.0ml solution of DNA (2mg/ml) in TNE (0.01M Tris-HCL, pH 7.5, 0.01M NaCl, and $1x10^{-4}$ M EDTA) was modified by adding sufficient streptozotocin and MG to obtain the desired DNA bp/streptozotocin and MG molar ratio. The mixture was incubated at 37 °C for 2 hours both with streptozotocin and MG. The pH of the mixture was noted at the end of incubation period and was found unchanged.Finally, the treated DNA was dialyzed againt 50 volumes of TNE (0.01M, pH 7.5) at 4 °C. Depurinated DNA was obtained by incubating the treated DNA at 50 °C for 6 hours to achieve the release of labile alkylated bases (Verly et al., 1973).

Alkaline hydrolysis of modified and depurinated DNA : Alkali labile acid soluble DNA nucleotides in both the treated and the depurinated DNA were determined by incubating the DNA with (a) 0.1M alkali for 30 minutes at room temperature or (b) 0.5M alkali for 1 hour at 37 °C. Under the former condition, alkali labile apurinic sites are hydrolysed in DNA,whereas the latter treatment also hydrolyses alkylphosphotriesters (Crathorn and Shooter, 1982).

Assay of S_1 nuclease : S_1 nuclease was assayed by estimating the acid soluble nucleotides,released from DNA as a result of enzymatic digestion. The reaction mixture, in a final volume of 1.0ml,contained 500 µg

of substrate (native, denatured or modified DNA), 0.1M acetate buffer, pH 4.5, 1mM zinc sulphate, water, and enzyme. The reaction mixture was incubated at 37°C (unless otherwise specified) for the desired period of time. At the end of the incubation period, the reaction was terminated by the addition of 0.2ml of 10 mg/ml bovine serum albumin (mixed thoroughly by shaking) and 1.0ml of ice cold 14 % perchloric acid. The tubes were immediately transferred to an ice bath and left at 4°C for at least 1 hour before centrifugation to remove precipitated protein and undigested DNA. The acid soluble DNA nucleotides were determined either by the diphenylamine method of Schneider (1957) or by reading the absorbance of an adequately diluted sample at 260nm against a suitable blank.

Preparation of denatured DNA: Denatured DNA was prepared by heating at 100°C for 7 minutes a 2 mg/ml solution of native calf thymus DNA in TNE (0.01M, pH 7.5) and cooling the solution rapidly in an ice bath (Verly and Lackroix, 1975).

Thermal denaturation of DNA as measured by the degree of S_1 nuclease digestion: Samples containing 300µg of native and modified DNA were heated to the desired temperature for 8 minutes and quickly quenched by the addition of 2.0 volumes of ice cold S_1 nuclease standard reaction buffer. The mixture was incubated with 600 units (unless other-

wise specified) of S_1 nuclease at 37°C for 3 hours. The reaction was stopped by the addition of 0.2ml of 10 mg/ml bovine serum albumin and 1.0ml of ice cold 14 % perchloric acid. The tubes were immediately transferred into an ice bath and kept at 4°C for at least 1 hour before centrifugation to remove precipitated protein and undigested DNA. The samples were processed as described above for the determination of acid soluble nucleotides.

Hydroxyapatite chromatography: Hydroxyapatite was suspended in phosphate buffer (0.01M, pH 7.0). The fine particles were removed and the slurry, mainly containing coarse particles, was poured into a column of 1cm cross-section. The stop cock was opened and sufficient amount of fluid was allowed to pass to obtain a 3cm bed. DNA samples (0.5ml containing 500 µg) were applied and the elution started with a stepwise gradient of phosphate buffer (pH 7.0). Generally, 3.0ml fractions were collected at the rate of 10 ml/hour. The DNA eluted in various fractions was determined by the diphenylamine reaction or spectrophotometrically by reading the absorbance at 260nm.

Purification of double-stranded DNA by BND-cellulose chromatography: Double-stranded DNA without single-strand breaks (SSB) was purified by BND-cellulose chromatography as described earlier(Peter Karran et al., 1977). Double-stranded DNA elutes from BND-cellulose with 1M NET (1M

NaCl, 10^{-4}M EDTA, 0.01M Tris- HCl, pH 7.5). DNA, with SSB or regions, requires 50 % formamide in 1M NET. The 1M NET eluate was dialysed against TNE (0.01M,pH 7.0) and used for incubations with MG.

Alkaline unwinding assay: Incubations with MG were carried out in volumes of 0.5 ml in sterile tubes. Each reaction mixture contained 75 µg DNA and MG in the desired DNA bp/MG molar ratio. Stoppered tubes were incubated at 37 °C for various time periods. Parallel controls, which did not contain MG, were also incubated. The pH of the reaction mixture was noted at the end of the incubation and was found unchanged. After incubation, the reaction mixtures were placed on ice and immediately subjected to alkaline unwinding by rapid addition of an equal volume of 0.06N NaOH in 0.01M Na_2HPO_4 (pH 12.5), followed by a brief vortexing. The tubes were immediately placed in a dark chamber at room temperature for the duration of the alkaline unwinding period (30 minutes). The tubes were gently removed and sufficient HCl (0.068N) was quickly added to bring the pH to neutrality, followed by gentle vortexing. 0.125ml of a 2% solution of SDS containing 0.025M EDTA was also added and the mixture vortexed thoroughly. The final volume at the end of alkaline unwinding operation ranged between 2.0-2.2 ml. The "unwound-neutralized" reaction mixtures were usually stored at 4 °C until they were analysed by hydroxyapatite chromatography. 0.5gm hydroxyapatite was brought to a boil in 5.0ml of

0.01M potassium phosphate buffer, pH 7.0, centrifuged and the supernatant discarded. The boiled hydroxyapatite was now suspended in 3.0ml of 0.01M phosphate buffer containing 10 % formamide and the tubes incubated at 60°C.Formamide to a final concentration of 10% was also added to the "unwound-neutralized" reaction mixtures before transfering them to the tubes containing boiled hydroxyapatite.The samples were incubated at 60°C for 2 hours with intermittent vortexing, followed by centrifugation. The supernatants were discarded and 3.0ml of 0.01M potassium phosphate buffer, pH 7.0, containing 20 % formamide was added. After thorough mixing,the tubes were again centrifuged and the supernatants discarded. The DNA was quantitatively adsorbed by hydroxyapatite as determined by the absence of UV absorbing material in the discarded supernatant. Single-stranded DNA was then selectively eluted from the gel by two successive 20 minute incubations (60°C) with 3.0ml, each of 0.125M potassium phosphate buffer, pH 7.0, containing 20 % formamide. The supernatants were collected after centrifugation. DNA in eluates was measured by absorption at 260nm or by diphenylamine method (Burton, 1956).

Calculations: According to Rydberg (1975), the relationship between strand separation of duplex DNA in alkali, where randomly distributed

breaks are introduced, is

$$\ln F = \frac{-K}{M_n} t^{\beta}$$

where F is the fraction of double-stranded DNA remaining after alkaline denaturation for time t, and K is an assumed constant for rotational and frictional forces. M_n is the number-average molecular weight between unwinding points and β is a constant less than 1.

From the above expression, Kanter and Schwartz (1979) have derived the following expression for calculating the number of unwinding points (P) per alkaline unwinding unit of DNA.

$$P = \frac{\ln F_x}{\ln F_o}$$

where F_x and F_o are the fractions of double-stranded DNA remaining after alkaline denaturation of treated and untreated samples, respectively. The number of breaks (n) per unit DNA is, therefore,

$$n = P - 1.$$

RESULTS AND DISCUSSION

(A) INTERACTION OF STREPTOZOTOCIN WITH DNA:

(a) Results:

S_1 nuclease and alkaline hydrolysis of streptozotocin alkylated DNA: DNA alkylated with increasing molar ratio of streptozotocin was subjected to hydrolysis by the single-strand specific nuclease S_1. The results given in Table I show that the production of acid soluble material increased with increasing streptozotocin concentration. Under the same conditions, control native and denatured DNA showed 13 and 62 % hydrolysis, respectively. Another native DNA control, which was incubated without the alkylating agent, showed 20 % hydrolysis. Thus, streptozotocin treatment transforms DNA into an effective substrate for S_1 nuclease and suggests a destabilization of the secondary structure. Similar findings with several other alkylating agents, where destabilization of secondary structure on alkylation was considered to be due to an accumulation of positive charges on opposite strands of native DNA, have earlier been reported from this laboratory (Wani et al.,1978;Rizvi et al.,1982;Rizvi and Hadi,1984).

In order to test whether apurinic or apyrimidinic sites are created or alkylphosphotriesters are formed on streptozotocin treatment (Wani et al., 1978; Crathorn and Shooter, 1982), the alkylated

Table-I

S_1 nuclease and alkaline hydrolysis of streptozotocin alkylated DNA:

Streptozotocin treatment of DNA was carried out as described in 'Methods' The S_1 nuclease reaction mixture in 1.0 ml contained native, denatured or treated DNA equivalent to 1.5 μ mole DNA nucleotides,100 mM acetate buffer, pH 4.5, 1 mM ZnSO4 and 100 units of S_1 nuclease. The incubation was at 40 °C for 2 hours and the reaction stopped by the addition of 0.2 ml of 10 mg/ml bovine serum albumin and 1.0 ml of ice cold 14% perchloric acid. Acid soluble nucleotides were measured by the diphenylamine procedure (Schneider,1957). Alkaline hydrolysis was carried out as described in 'Methods' using similar amounts of alkylated DNA.

DNA bp/streptozotocin molar ratio	S_1 nuclease hydrolysis		Alkaline hydrolysis			
			0.1M NaOH 22 °C		0.5M NaOH 37 °C	
	μ mole acid soluble DNA nucleotide	% DNA hydrolysed	μ mole acid soluble DNA nuleotide	% DNA hydrolysed	μ mole acid soluble DNA nucleotide	% DNA hydrolysed
Denatured DNA	0.94	62	-	-	-	-
Native DNA	0.20	13	-	-	-	-
No streptozotocin	0.30	20	0.05	3.2	0.05	3.2
1:1	0.42	28	0.13	8.6	0.15	9.9
1:2	0.54	36	0.15	9.9	0.18	11.9
1:4	0.71	47	0.20	13.2	0.25	16.5
1:8	-	-	0.29	19.2	0.34	22.5
1:16	-	-	0.34	22.5	0.39	25.8

DNA samples were subjeted to alkaline hydrolysis under two different conditions. Incubation with 0.1 M alkali causes the cleavage of apurinic sites by β-elimination (Tamm et al., 1953), whereas treatment with 0.5 M alkali at 37°C also leads to the hydrolysis of alkylphosphotriesters. As seen in Table I, hydrolysis with higher alkali concentration is higher than with 0.1 M NaOH at all the DNA bp/streptozotocin molar ratios tested. These results, therefore, indicate some alkylation of DNA phosphates, in addition to the alkylation of bases. Fig.1 shows the hydrolysis of DNA alkylated with streptozotocin alone and with nicotinamide in relation to native and denatured DNA with increasing S_1 nuclease concentration. With the maximum amount of enzyme used, 100 % of denatured DNA was made acid soluble compared with 15 % of native DNA. Alkylation of DNA in the presence of nicotinamide results in a greater maximum hydrolysis than in its absence.

Effect of NaCl on S_1 nuclease hydrolysis of streptozotocin and MeNu alkylated DNA: DNA was alkylated by streptozotocin or MeNu at a bp/alkylating agent molar ratio of 1:4 and subjected to hydrolysis by increasing S_1 nuclease concentration in the presence and absence of 0.1 M NaCl. It has earlier been shown that some hydrolysis of native DNA by S_1 nuclease at 40°C is always seen. However, this can bd suppressed to the extent of 90% in the presence of 0.1 M NaCl.

Fig.1. S_1 nuclease hydrolysis of DNA alkylated by streptozotocin in the presence and absence of nicotinamide:

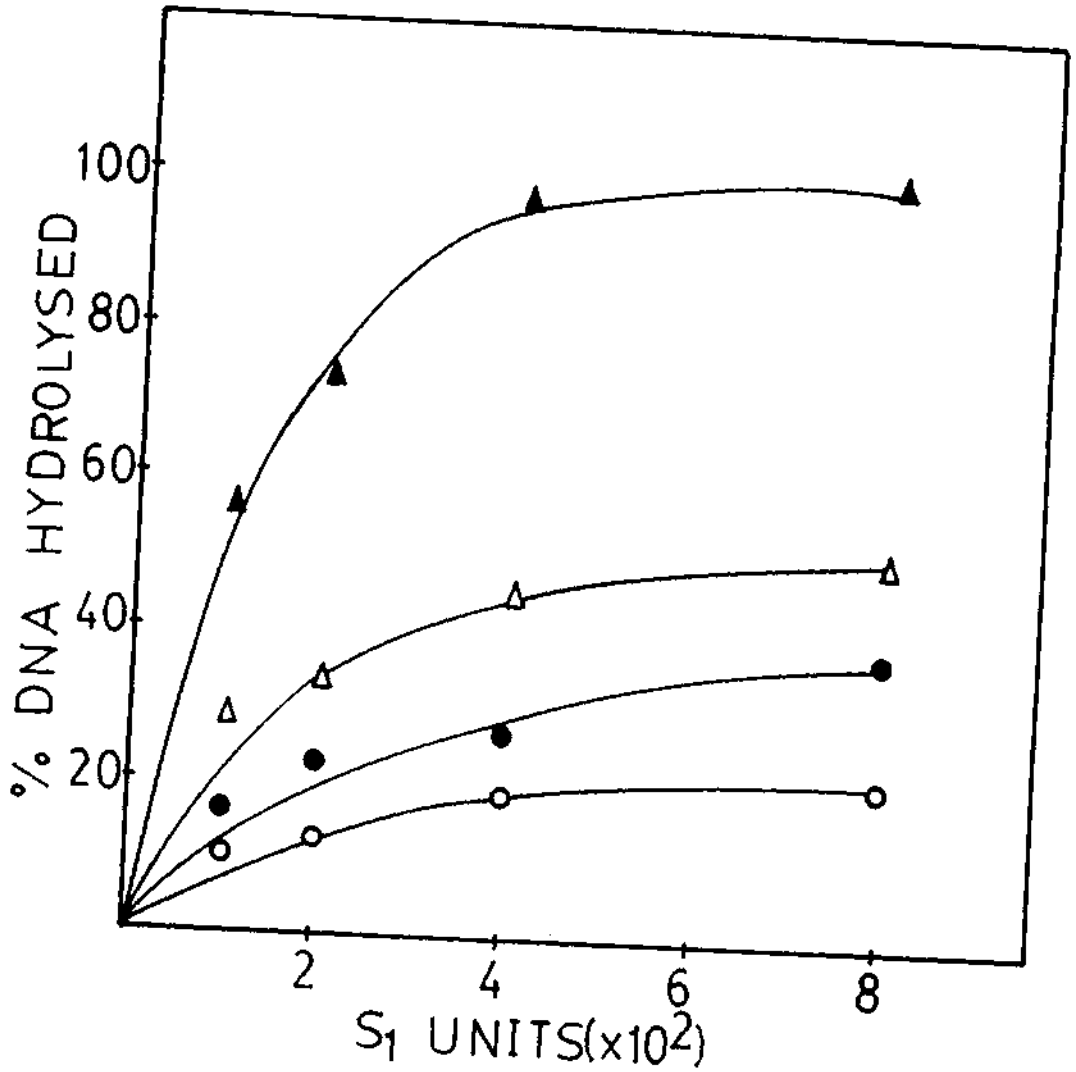
DNA was alkylated in the presence and absence of nicotinamide as described in 'Methods'at the DNA bp/streptozotocin molar ratio of 1:4. Nicotinamide, when present, was also added in the same ratio. The S_1 nuclease reaction was done as given in Table I.

Native DNA (O)

Denatured DNA (▲)

Streptozotocin alone (●)

Streptozotocin + nicotinamide (Δ)


% DNA HYDROLYSED
100
80
60
40
20
2
4
6
8
S1 UNITS(x10²)

On the other hand, denatured DNA hydrolysis is not affected by the presence of salt (Rizvi and Hadi, 1984). Fig.2A shows that NaCl has little effect on the degradation of DNA alkylated with streptozotocin alone. However, the hydrolysis of streptozotocin plus nicotinamide alkylated DNA is suppressed by salt to a considerably greater extent (Fig.2B),similar to the effect seen in Fig.2C with MeNu alkylated DNA. Also, the production of maximum acid soluble material in the absence of NaCl was found to be larger in the case of DNA alkylated in the presence of nicotinamide. High ionic strength has a stabilizing effect on the secondary structure of DNA phosphates (Von Hippel and Felsenfeld,1964). Alkylating agents,such as N-ethyl-N-nitrosourea, which primarily alkylate DNA phosphates, abolish the negative charges on DNA (Siebenlist and Gilbert, 1980). Therefore, on such DNA, NaCl would have little effect on localized melting in the duplex. Presumably, these results suggest a greater formation of alkylated bases in relation to alkylphosphotriesters, when alkylation is carried out by streptozotocin in the presence of nicotinamide, i.e. the phosphotriester formation is suppressed. The greater maximum hydrolysis in the absence of NaCl, seen in the case of DNA alkylated in the presence of nicotinamide, may be attributed to a greater destabilization of the secondary structure due to a larger accumulation of the positive charges of the quaternised alkylated bases. Finally, the results also indicate that the alkylated DNA

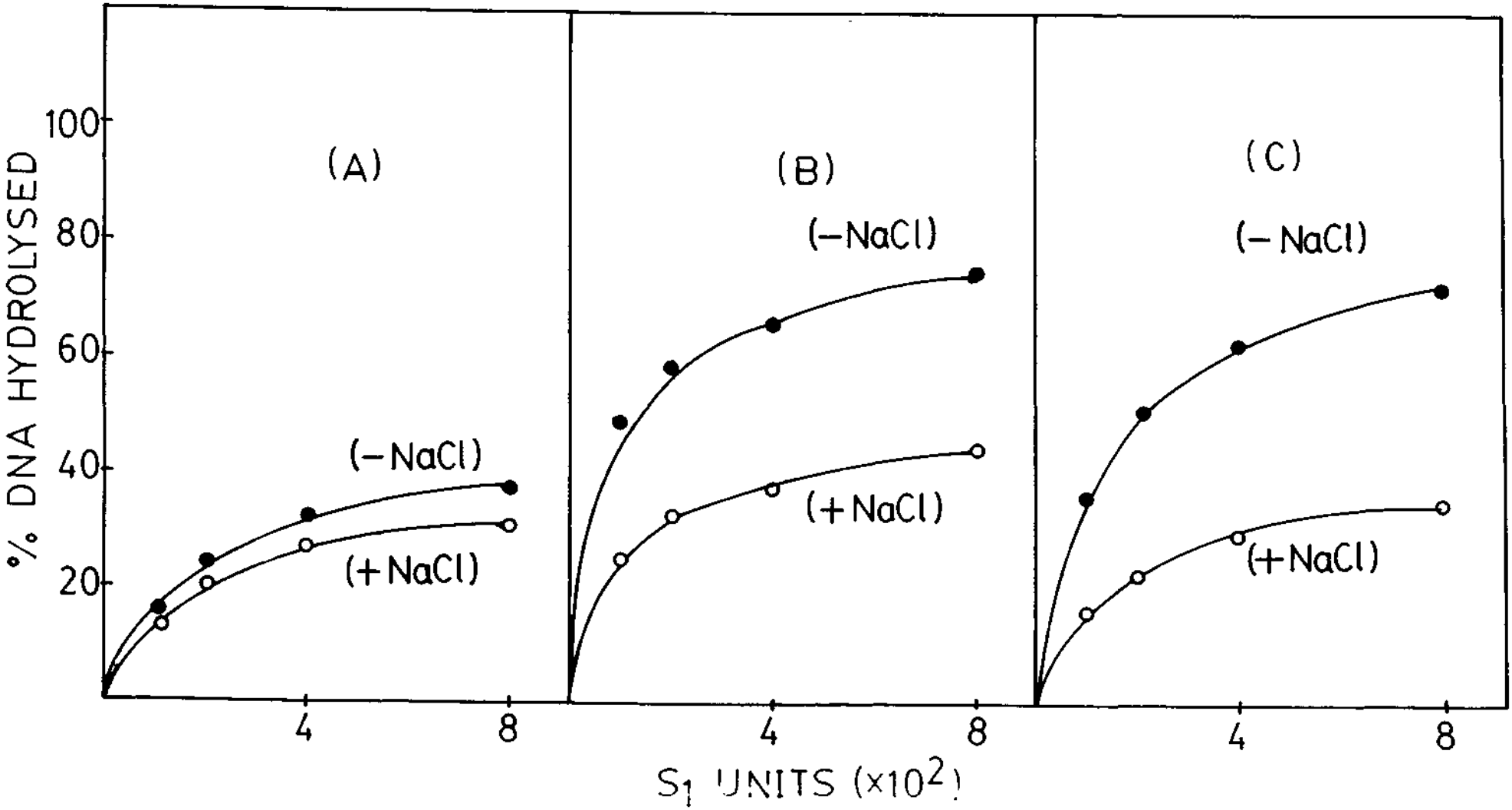
Fig.2. Degradation of streptozotocin alkylated DNA by S_1 nuclease in the presence and absence of NaCl:

Alkylation of DNA by streptozotocin and MeNu was done at the DNA bp/alkylating agent molar ratio of 1:4. Nicotinamide was also present in the same ratio. NaCl was added to the nuclease reaction mixture to a final concentration of 0.1M. The other reaction conditions were the same as given in Table I.

DNA alkylated with streptozotocin alone (A)

DNA alkylated with streptozotocin + nicotinamide (B)

DNA alkylated with streptozotocin + N-methyl-N-nitrosourea (C)


% DNA HYDROLYSED
100
80
60
40
20
(A)
(B)
(C)
(−NaCl)
(+NaCl)
(−NaCl)
(+NaCl)
(−NaCl)
(+NaCl)
4
8
4
8
4
8
S1 UNITS (×10²)

samples are essentially double-stranded, as complete hydrolysis of alkylated DNA by S_1 nuclease is not achieved. Heat-denatured alkylated DNA, on the other hand, is completely hydrolysed by S_1 nuclease under these conditions (results not shown; Rizvi and Hadi, 1984).

Thermal melting profiles of streptozotocin alkylated DNA: S_1 nuclease has been used to determine the thermal melting profiles of DNA through digestion of denatured strands (Case and Baker,1975). Fig.3A shows such an experiment with DNA alkylated by streptozotocin at DNA bp/alkylating agent molar ratios of 1:1, 1:2, and 1:4. The mid-range melting temperature (Tm) of control native DNA was determined to be 84 °C under the conditions employed. At the molar ratios of 1:1 and 1:2 there, is little change in the Tm, while at the ratio of 1:4, it is reduced to 78 °C. In Fig.3B a similar experiment is shown, in which the effect of nicotinamide on streptozotocin alkylation of DNA has been studied. The DNA used was alkylated at a bp/streptozotocin molar ratio of 1:4. Nicotinamide, when present, was also used at the same ratio. Similar to the previous experiment, DNA alkylated with streptozotocin alone shows a reduced Tm relative to native DNA. In the sample, where nicotinamide was also present during alkylation, a further reduction in Tm is observed, indicating an even greater destabilization of secondary structure. This is in agreement with our suggestion that in the presence of nicotinamide the formation of al-

Fig.3. Thermal denaturation of streptozotocin alkylated DNA as measured by the degree of S_1 nuclease digestion:

Samples containing 300 μg native or alkylated DNA were heated to the desired temperatures for 7 minutes and quickly quenched by the addition of 2.0 volumes of ice cold S_1 nuclease standard reaction buffer. The mixture was then incubated with 100 units of S_1 nuclease at 40 °C for 2 hours. The reaction was terminated and processed as described in Table I.

(A)
Native DNA (○)

DNA bp/streptozotocin molar ratio 1:1 (●)

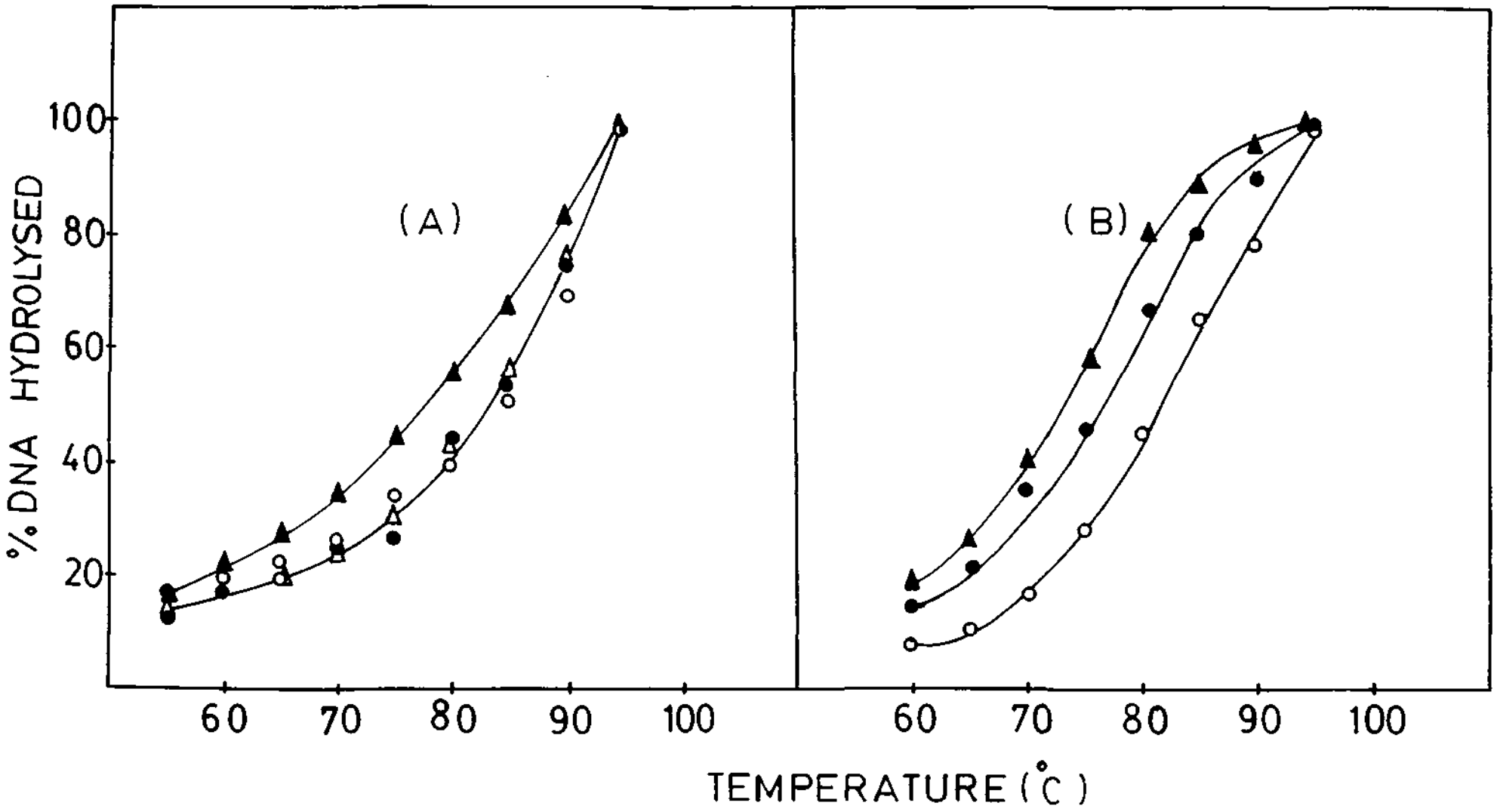
DNA bp/streptozotocin molar ratio 1:2 (Δ)

DNA bp/streptozotocin molar ratio 1:4 (▲)

(B)
Native DNA (○)

Streptozotocin alkylated DNA (●)

Streptozotocin + Nicotinamide alkylated DNA. (▲)


% DNA HYDROLYSED
100
80
60
40
20
(A)
(B)
60
70
80
90
100
TEMPERATURE (°C)

kylphosphotriesters is suppressed and a larger proportion of DNA bases are alkylated. Finally, the melting profiles also confirm the essential double-stranded nature of alkylated DNA, both in the absence and presence of nicotinamide.

In order to further substantiate the suggestion that in addition to bases, DNA phosphates are also alkylated by streptozotocin, hydroxyapatite chromatography of alkylated samples was carried out. The main factor involved in the adsorption of nucleic acids on hydroxyapatite is generally considered to be the interaction between phosphate groups and calcium ions on the surface of hydroxyapatite (Bernardi, 1971). It has earlier been shown that on extensive alkylation by EtNu, which mainly alkylates DNA phosphates and thereby abolishes the negative charges,duplex DNA behaves as single-stranded molecules on hydroxyapatite chromatography (Rizvi and Hadi, 1984). Goth and Rajewsky (1974) have reported a similar effect in their finding that on in vivo ethylation by EtNu a major part of radioactivity associated with nucleic acids did not bind to cation exchange columns and thus represented phosphotriesters. DNA was alkylated with DNA bp/streptozotocin molar ratios of 1:1, 1:2, and 1:4 and subjected to hydroxyapatite chromatography. Results given in Fig.4 show that at molar ratio of 1:1 the alkylated DNA eluted at the position of double-stranded DNA (0.25 M phosphate). As discussed

Fig.4. Hydroxyapatite chromatography of DNA alkylated with streptozotocin:

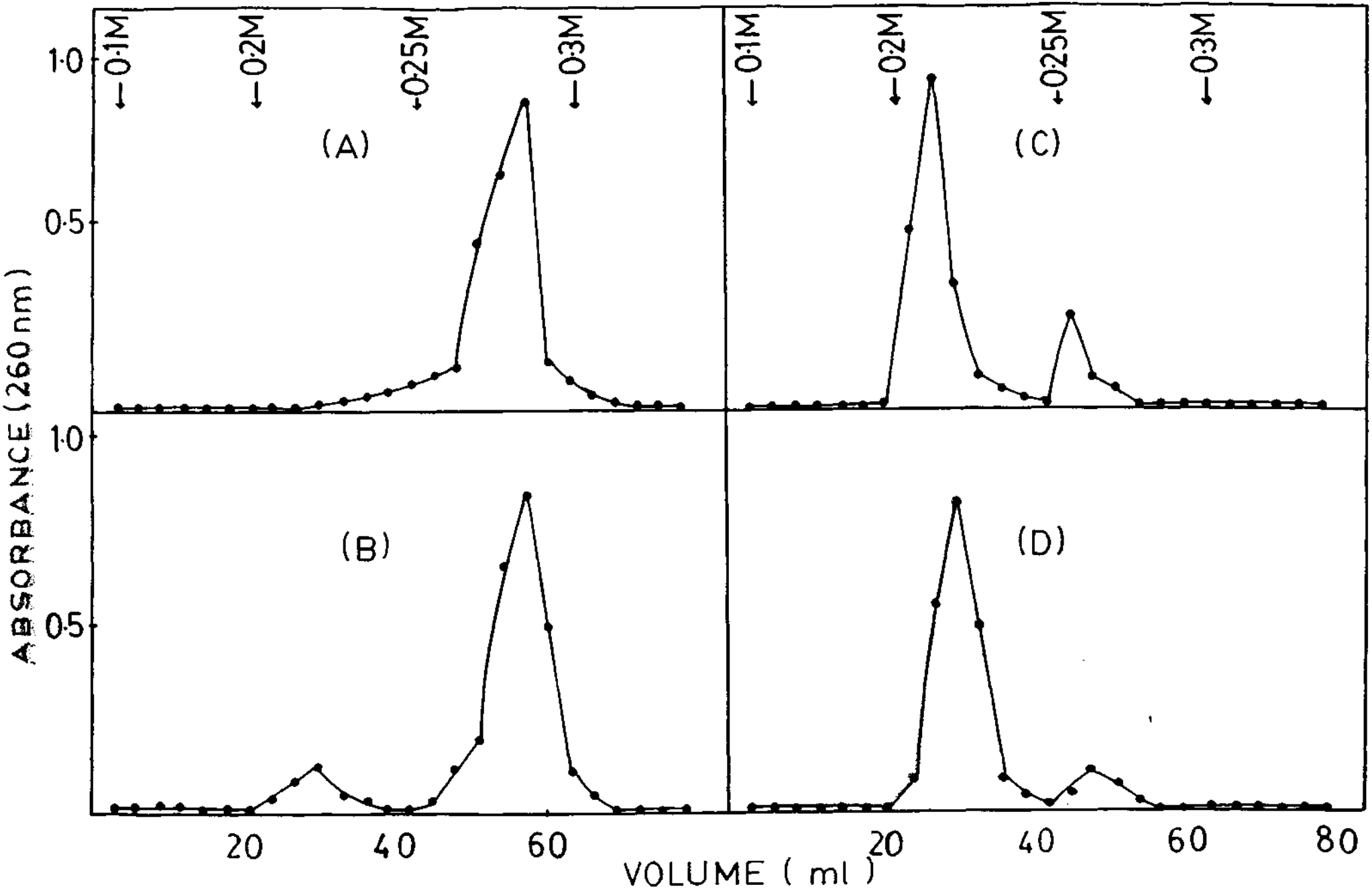
500 µg of alkylated DNA in 0.3ml of TNE was loaded on a 1x3cm column, previously equilibrated with 0.01M sodium phosphate buffer, pH 7.0. The column was washed with 20 ml of equilibrating buffer (pH 7.0) of the molarities indicated. 3.0 ml fractions were collected at the rate of 10ml/hour. Recoveries in all experiments were between 80 and 90 %.

Native DNA (A)

DNA bp/streptozotocin molar ratio 1:1 (B)

DNA bp/streptozotocin molar ratio 1:2 (C)

DNA bp/streptozotocin molar ratio 1:4 (D)


0·1M
0·2M
0·25M
0·3M
(A)
(C)
(B)
(D)
1·0
0·5
ABSORBANCE (260 nm)
20
40
60
80
VOLUME (ml)

above, this is presumably due to the absence of sufficient negative charges on DNA due to the formation of phosphotriesters.

(b) Discussion:

Monofunctional alkylating agents alkylate DNA at two sites; bases and phosphates, causing the formation of alkyl purines/pyrimidines and alkylphosphotriester . The major products of alkylation with streptozotocin are 7-methyl guanine, 7-and 3-methyl adenine and O^6 methyl guanine (Bennett and Pegg, 1981). However,no information has been available on the alkylation of DNA phosphates. We have presented indirect evidence to suggest that alkylation of DNA phosphates may also occur to a significant extent. It has previously been shown that alkylation of DNA may lead to the formation of partially denatured molecules, even in the absence of depurination of alkylated bases (Rizvi et al., 1982). Results presented in this dissertation on the S_1 nuclease hydrolysis and thermal melting profiles of streptozotocin alkylated DNA are in agreement with this conclusion. The increased stability of DNA on phosphate alkylation is related to neutralization of the negative charges on phosphate residues (Siebenlist and Gilbert, 1980). The greater stability of secondary structure may have important consequences in genetic expression, as the mechanism of action of many DNA binding proteins, such as RNA polymerase, involves partial

denaturation or destabilization of the secondary structure. It is conceivable that the differential cytotoxicity of streptozotocin in the absence and presence of nicotinamide is related to its differential effect on the secondary structure of DNA. It may be mentioned that N-methyl-N-nitrosourea, which primarily alkylates DNA at the N-7 position of guanine, and N-ethyl-N-nitrosourea, which mainly forms phosphotriesters,possess different carcinogenic potentials for various tissues (Marushige and Marushige, 1983a).

(B) INTERACTION OF METHYLGLYOXAL WITH DNA:

(a) Results:

S_1 nuclease and alkaline hydrolysis of MG treated DNA: In this laboratory the single-strand specific S_1 nuclease has earlier been used in studying the secondary structure of DNA, modified by various chemical mutagens, such as intercalating agents and DNA alkylating agents (Rizvi and Hadi, 1984; Alvi et al., 1985; Rizvi et al., 1986).

DNA treated with increasing molar ratio of MG was subjected to hydrolysis by S_1 nuclease. The results given in Table II show that the production of acid soluble material increased with increasing MG concentration. Under the same conditions, control native and denatured DNA showed 22 and 100 % hydrolysis, respectively. Thus, MG treatment transforms DNA into an effective substrate for S_1 nuclease and suggests a destabilization of its secondary structure. Similar findings with several other alkylating agents, where destabilization of secondary structure on alkylation was considered to be due to an accumulation of positive charges on opposite strands of native DNA, have earlier been reported from this laboratory (Wani et al., 1978; Rizvi et al., 1982; Rizvi and Hadi, 1984).

Table-II

S_1 nuclease and alkaline hydrolysis of MG treated and depurinated DNA :

MG treatment of DNA was carried out as described in 'Methods'. The S_1 nuclease reaction mixture in 1.0 ml contained native, denatured or treated DNA equivalent to 1.5 μ mole DNA nucleotides, 100 mM acetate buffer, pH 4.6, 1 mM $ZnSO_4$ and 20 units of S_1 nuclease. The incubation was at 37 °C for 2 hours and the reaction stopped by the addition of 0.2 ml of 10 mg/ml bovine serum albumin and 1.0 ml of ice cold 14% perchloric acid. Acid soluble nucleotides were measured by the diphenylamine procedure (Schneider, 1957) Alkaline hydrolysis was carried out as described in 'Methods' using similar amounts of treated and depurinated DNA.

DNA bp/MG molar ratio	S_1 nuclease hydrolysis		Alkaline hydrolysis							
			Treated DNA				Depurinated DNA			
			0.1M NaOH 18 °C		0.5M NaOH 37 °C		0.1 M NaOH 18 °C		0.5M NaOH 37 °C	
	μ mole acid soluble DNA nucleotide	%DNA hydrolysed	μ mole acid soluble DNA nucleotide	%DNA hydrolysed	μ mole acid soluble DNA nucleotide	% DNA hydrolysed	μ mole acid soluble DNA nucleotide	%DNA hydrolysed	μ mole acid soluble DNA nuleotide	%DNA hydrolysed
Denatured DNA	0.47	100	-	-	-	-	-	-	-	-
Native DNA	0.095	22	-	-	-	-	-	-	-	-
NO MG	0.095	22	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1:1	0.112	25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1:2	0.112	25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1:4	-	-	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1:8	0.190	36	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
1:16	0.271	43	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00

In order to test whether apurinic or apyrimidinic sites are created, or alkylphosphotriesters are formed on MG treatment (Wani et al., 1978; Crathorn and Shooter,1982), both treated and depurinated samples were subjected to alkaline hydrolysis under two different conditions. Incubation with 0.1M alkali causes the cleavage of apurinic sites by β-elimination (Tamm et al.,1953), whereas treatment with 0.5M alkali at 37 °C also leads to the hydrolysis of alkylphosphotriesters. As seen in Table II, no hydrolysis occurs under both the conditions at all the DNA bp/MG molar ratios tested. These results apparently rule out the possibility of alkylation of DNA phosphates or triesters formation.

Effect of NaCl on S_1 nuclease hydrolysis of MG treated DNA: DNA treated with MG at DNA bp/MG molar ratios of 1:8 and 1:16 was subjected to hydrolysis by increasing S_1 nuclease concentration in the presence and absence of 0.1M NaCl. It has earlier been shown that some hydrolysis of native DNA by S_1 nuclease at 40 °C is always seen. However, this can be suppressed to the extent of 90 % in the presence of 0.1M NaCl. On the other hand, denatured DNA hydrolysis is not affected by the presence of NaCl (Rizvi and Hadi, 1984). Fig.7 shows that NaCl had considerable effect on the degradation of DNA modified with MG. With the molar ratios of 1:8 and 1:16, the maximum hydrolysis was reduced to 3 % from the levels of 15 and 28 %, respectively. High ionic stre-

Fig.7. Degradation of MG treated DNA by S_1 nuclease in the presence and absence of NaCl:

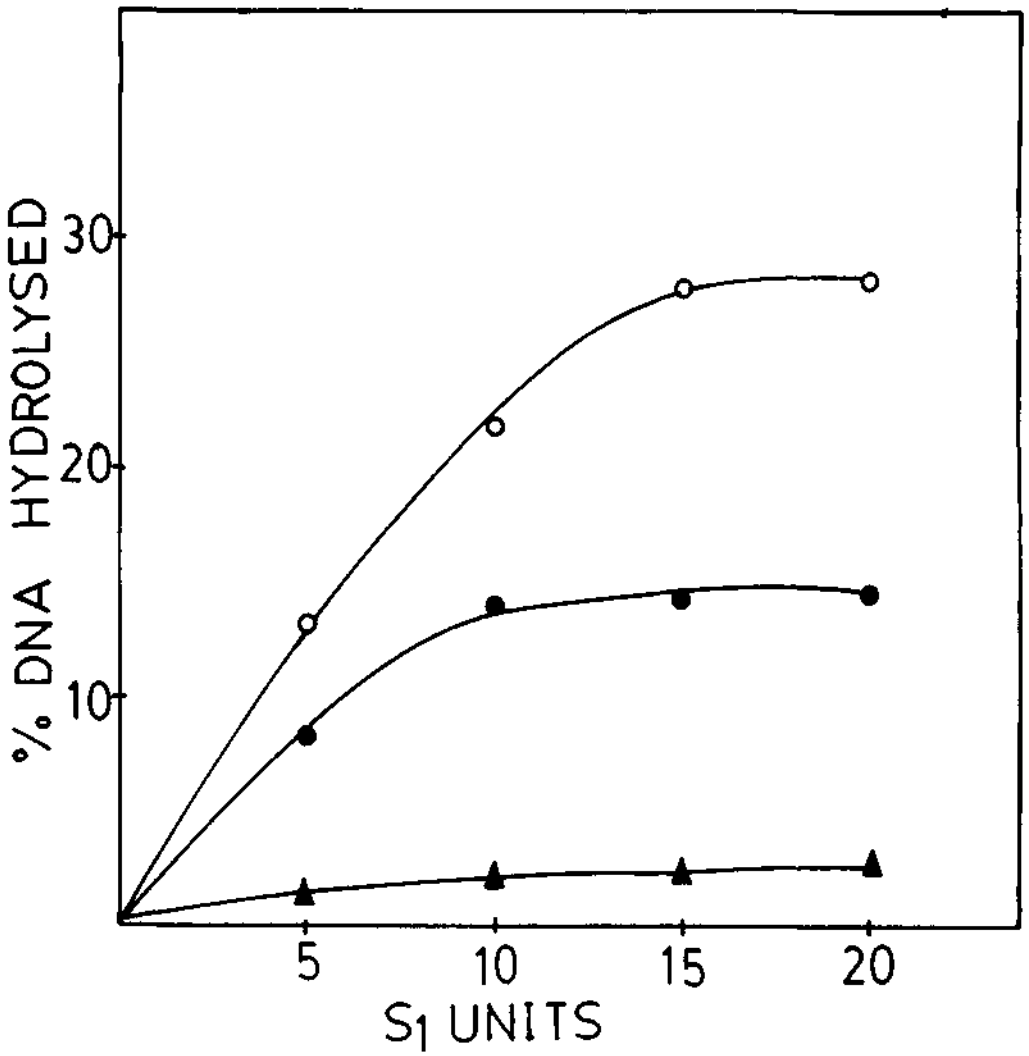
Treatment of DNA with MG was done at the DNA bp/MG molar ratios of 1:8 and 1:16. NaCl was added to the nuclease reaction mixture to a final concentration of 0.1M. The other reaction conditions were the same as given in Table II.

DNA bp/MG molar ratio 1:8 (●)

DNA bp/MG molar ratio 1:8 + NaCl (▲)

DNA bp/MG molar ratio 1:16 (○)

DNA bp/MG molar ratio 1:16 + NaCl (▲)


% DNA HYDROLYSED
30
20
10
5
10
15
20
S1 UNITS

ngth has a stabilizing effect on the secondary structure of DNA through the neutralizing effect of counter ions on the negative charges of DNA phosphates (Von Hippel ad Felsenfeld, 1964). On reaction with MG or on triester formation, these negative charges would be abolished and therefore the presence of NaCl should have little effect on S_1 nuclease hydrolysis. As the suppression of hydrolysis by NaCl is similar to that observed with native DNA, it may be suggested that reaction of MG with DNA does not involve DNA phosphates.

Thermal melting profiles of MG treated DNA: S_1 nuclease has been used to determine the thermal melting profiles of DNA through digestion of denatured strands (Case and Backer, 1975). Fig.5 shows such an experiment with DNA modified by MG at DNA bp/MG molar ratios of 1:2, 1:8, and 1:16. The mid-range melting temperature (Tm) of control native DNA was determined to be 75 $^{\circ}$C under the conditions employed. At the molar ratio of 1:2, the Tm was very similar to the control value. However, at the molar ratio of 1:8, a reduced Tm was observed (68 $^{\circ}$C). In addition, there was significant hydrolysis of DNA even after exposure to 60 $^{\circ}$C,suggesting the presence of some low melting regions in DNA. In contrast,at the higher molar ratio of 1:16, an increased value (80 $^{\circ}$C) was observed, indicating a stabilization of secondary structure relative to native DNA. These results can be accounted for by the possibility that at relatively lower concentrations, MG primarily causes the

Fig.5. Thermal denaturation of native and MG treated DNA as measured by the degree of S_1 nuclease digestion.

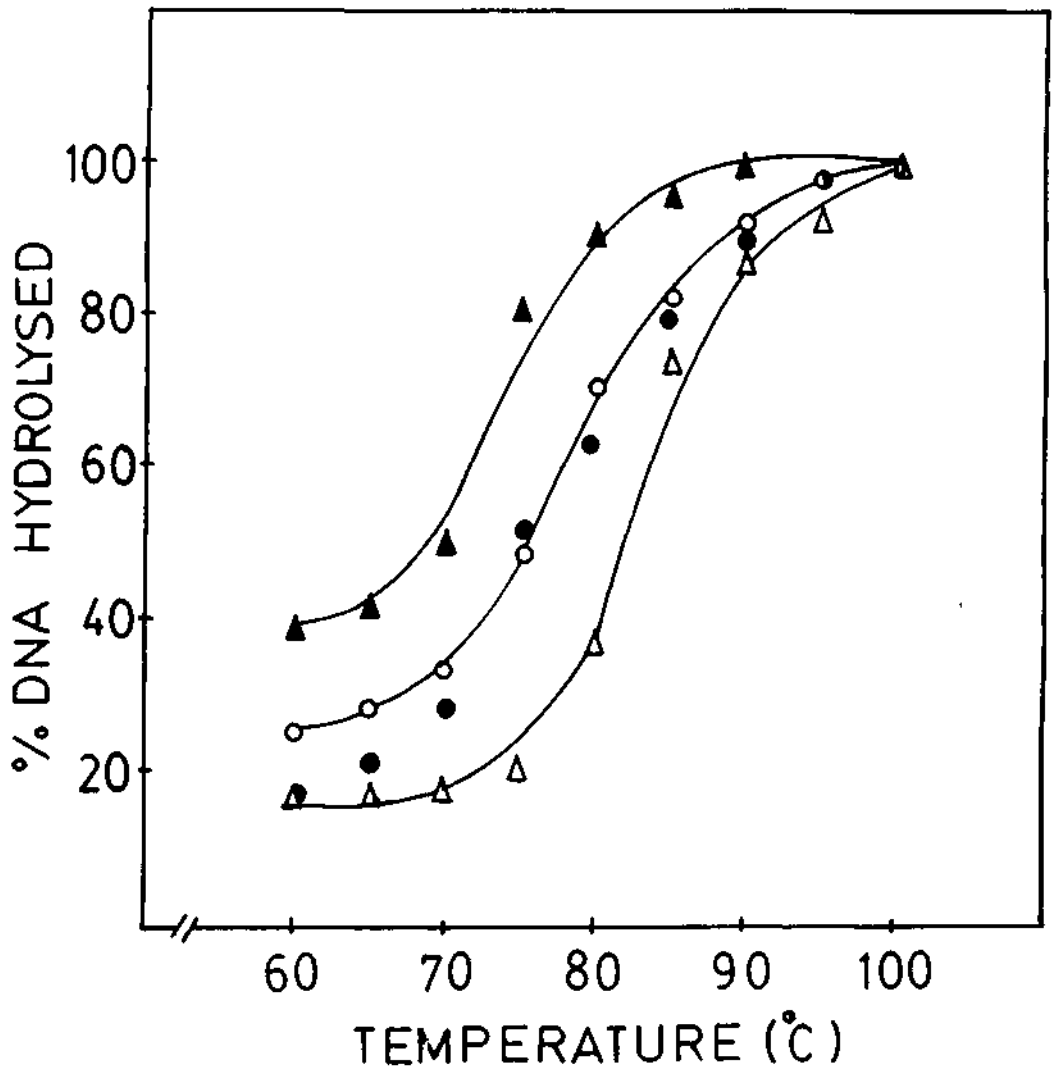
Samples containing 300 µg native or treated DNA were heated to the desired temperatures for 8 minutes and quickly quenched by the addition of 2.0 volumes of ice cold S_1 nuclease standard reaction buffer. The mixture was then incubated with 25 units of S_1 nuclease at 37 °C for 3 hours. The reaction was terminated and processed as described in Table I.

Native DNA (○)

DNA bp/MG molar ratio 1:2 (●)

DNA bp/MG molar ratio 1:8 (▲)

DNA bp/MG molar ratio 1:16 (Δ)


% DNA HYDROLYSED
100
80
60
40
20
60
70
80
90
100
TEMPERATURE (°C)

destabilization of the duplex DNA, presumably through the formation of SSB. At higher concentrations of MG, the formation of interstrand cross-links also occurs. With the technique used, such DNA would exhibit a higher T_m, although SSB are also present.

Degradation of MG treated DNA by S_1 nuclease: DNA treated at a bp/MG molar ratio of 1:16 for the indicated time at 37 °C was subjected to hydrolysis by S_1 nuclease (Fig.6). When untreated native DNA was incubated with S_1 nuclease, appreciable production of maximum acid soluble material was seen. Among the treated samples, 2 hour treated DNA was the most effective substrate, with the maximum production of acid soluble material of 42 %. Interestingly, however, the 8 hour treated DNA showed a reduced level of maximum hydrolysis (35 %). These results may be considered in support of the thermal melting profile pattern as the initial reaction of MG with DNA leads to the formation of SSB, giving rise to a higher rate of hydrolysis by S_1 nuclease. On introduction of an appreciable number of cross-links, the rate of hydrolysis is reduced.

Removal of A-T rich regions from native DNA by pea seed nuclease: Ames and co-workers (Levin et al., 1982) have suggested that various mono- and dialdehyde mutagens induce mutations at A-T base pairs. In order to test whether MG reacts preferentially with A-T rich regions in DNA,

Fig.6. Effect of increasing time of reaction of MG with DNA on S_1 nuclease hydrolysis:

Treatment of DNA with MG was carried out at DNA bp/MG molar ratio of 1:16 for the indicated time periods. 2 mg DNA, stock S_1 nuclease buffer and 100 units of S_1 nuclease were added and the volume made up to 5.0ml. Incubation was carried out at 40°C. Aliquots containing 400 µg DNA were removed at the indicated time intervals. The reaction was terminated and processed as described in Table I.

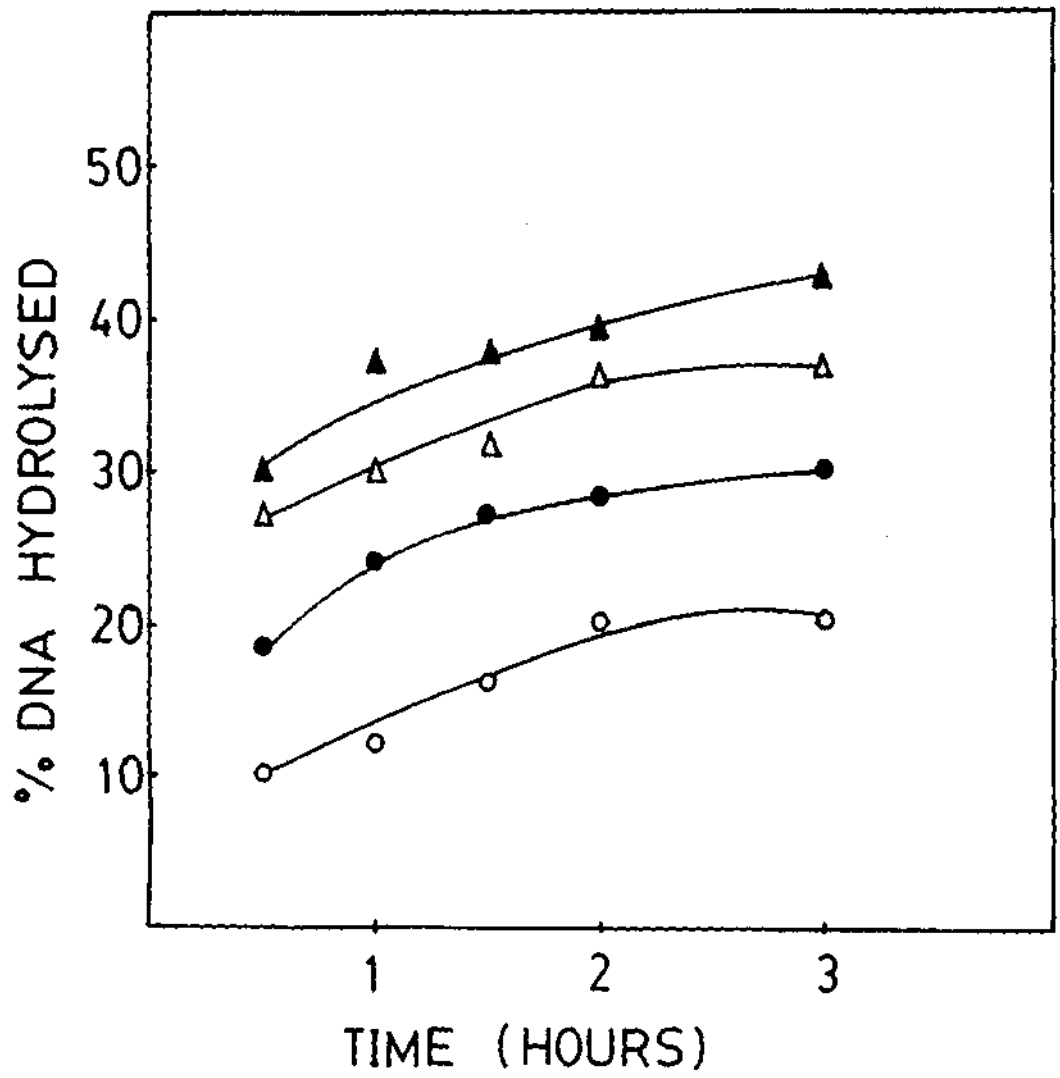
30 minute incubated native DNA (O)

8 hour incubated native DNA (O)

30 minute treated DNA (●)

2 hour treated DNA (▲)

8 hour treated DNA (Δ)


% DNA HYDROLYSED
50
40
30
20
10
1
2
3
TIME (HOURS)

the following protocol was followed. The predominantly A-T rich areas were removed by hydrolysis with pea seed nuclease before the reaction of DNA with MG. The single-strand specific nuclease from pea seed has been purified and characterized in this laboratory (Wani and Hadi, 1979). This enzyme was subsequently shown to preferentially hydrolyse partially denatured A-T rich areas in native DNA (Wani and Hart, 1981). The hydrolysis of double-stranded DNA by pea seed nuclease was carried out at 48 °C in order to expose the relatively less stable A-T rich regions to nucleolytic action. As shown in Fig. 10 and as expected, A-T base pair depleted DNA showed a higher melting temperature as compared to double-stranded DNA (81 vs 86 °C).

Assuming the GC percentage of 42 for calf thymus DNA, the same value for A-T base pair depleted DNA was calculated to be 54 % (Mandel and Marmur, 1968).

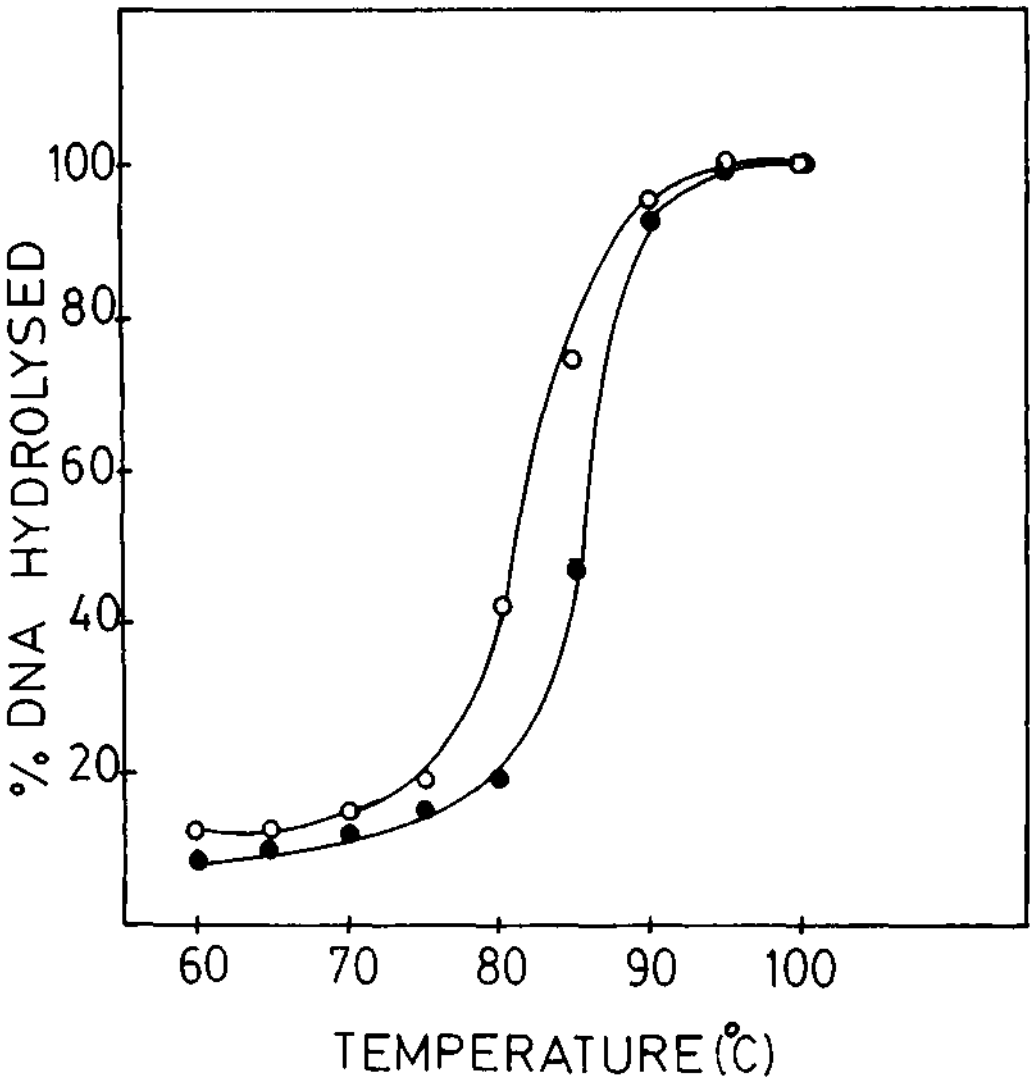
Quantitation of DNA strand breaks by alkaline unwinding assay: The fidelity of hydroxyapatite in a batch assay to fractionate the DNA in the neutralized alkaline unwinding mixture has been well established by Kanter and Schwartz (1979). However, these and other authors have used the alkaline unwinding assay for measurement of DNA damage in whole mammalian cells. We have adapted this procedure for the determi-

Fig.10. Thermal melting profiles of pea seed nuclease treated and native DNA:

10 mg of calf thymus DNA was incubated with 3 units of single-strand specific pea seed nuclease for 1 hour at 48 °C in a standard reaction mixture (Wani and Hadi, 1979). The undigested DNA was precipitated with 3.0 volumes of ethanol in the presence of 0.1M sodium acetate. The precipitated DNA was washed once, dried in air and suspended in 2.0 ml of TNE (0.01M, pH 7.5). The amount of recovered DNA was 7.2mg. Thermal melting profiles were determined as described in 'Methods' using S_1 nuclease.

Native DNA (○)

Pea seed nuclease treated DNA (●)


100
80
60
40
20
% DNA HYDROLYSED
60
70
80
90
100
TEMPERATURE (°C)

nation of strand breaks in DNA after in vitro treatment with various direct acting dietary mutagens and carcinogens. The details are given in 'Methods'.

Fig.8 illustrates an experiment, where BND-cellulose purified DNA has been treated with increasing DNA bp/MG molar ratio. The alkaline unwinding of treated DNA was carried out for 30 minutes before neutralization and fractionation of double and single-stranded DNA. It may be seen that the fraction of duplex DNA remaining decreases in almost a linear fashion with increasing concentration of MG. These results have been used to determine the number of strand breaks formed per unit of DNA by the procedure given in 'Methods' and the results are given in Table III.

In all the experiments described above, the MG treatment of DNA was carried out for 2 hours at 37 C. Fig.9 represents the results of an experiment, in which BND-cellulose purified DNA was subjected to treatment with a fixed molar ratio of MG (1:1) for various time periods. Alkaline unwinding of the treated sample was carried out and the fraction of duplex DNA measured. As seen in Fig., the duplex DNA decreases upto 2 hours but shows a gradual increase after this period and reaches a value almost equal to untreated DNA at the end of 5 hours. These results are in support of our earlier assertion that the initial

Fig.8. Fraction of duplex DNA recovered after reaction with increasing MG molar ratio:

75 µg of BND-cellulose purified calf thymus DNA was incubated with the indicated molar ratios of MG for 2 hours at 37 °C. The alkaline unwinding of the treated samples was immediately started as described in 'Methods'.

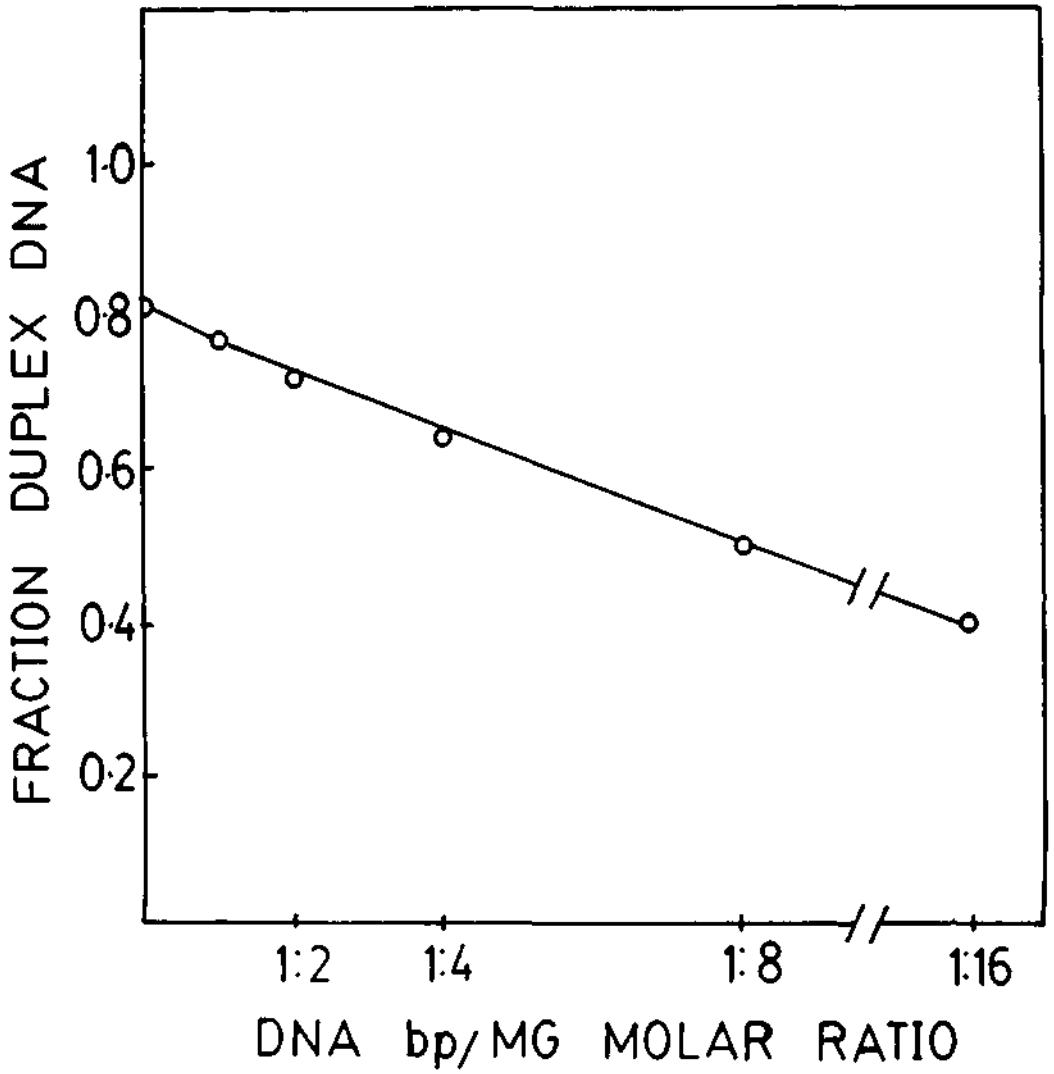

FRACTION DUPLEX DNA
1·0
0·8
0·6
0·4
0·2
1:2
1:4
1:8
1:16
DNA bp/MG MOLAR RATIO

Table - III

DNA strand breaks induced by MG:

DNA bp/MG molar ratio	Fraction of duplex DNA	No. of breaks per unit DNA
No MG	0.82	-
1:1	0.77	0.3
1:2	0.73	0.6
1:4	0.64	1.2
1:8	0.50	2.5
1:16	0.40	3.6

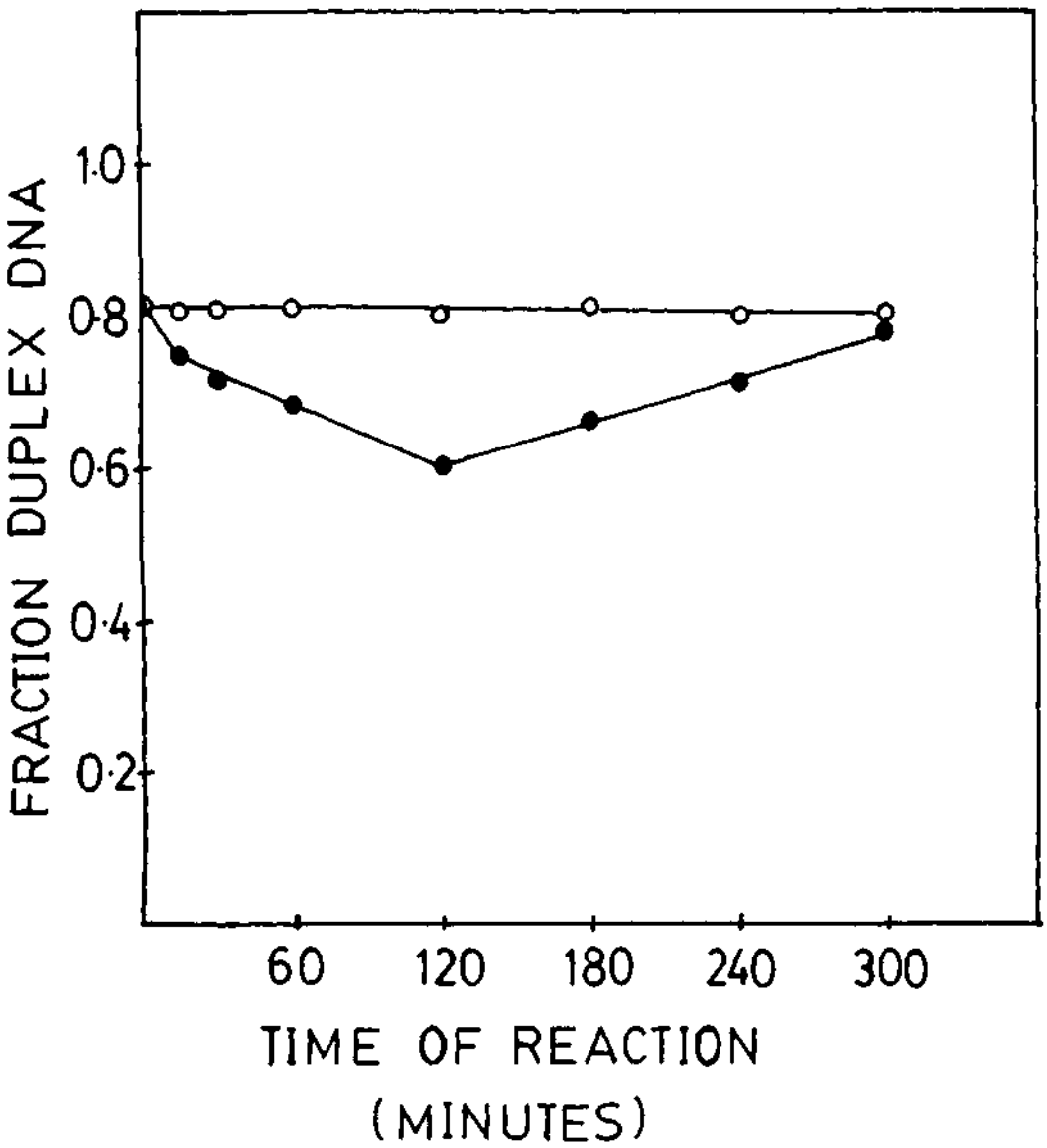
Data given have been calculated from the values obtained in Fig.8.

Fig.9. Effect of time of reaction of MG with DNA on the fraction of duplex DNA recovered:

DNA bp/MG molar ratio used for treatment was 1:1 and incubation at 37 °C was carried out for the indicated time periods. The alkaline unwinding of the treated samples was done as described in 'Methods'.

Native DNA (O)

DNA bp/MG molar ratio 1:1 (●)


FRACTION DUPLEX DNA
1·0
0·8
0·6
0·4
0·2
60
120
180
240
300
TIME OF REACTION
(MINUTES)

interaction of MG with DNA leads to the formation of SSB, and interstrand cross-links are introduced only at a later stage. The results obtained in this experiment can be explained by the fact that the alkaline unwinding assay would be unable to detect strand breaks in a DNA molecule, which also contains interstrand cross-links (Table IV).

S_1 nuclease hydrolysis of A-T base pairs depleted MG treated DNA: The pea seed nuclease bydrolysed A-T base pairs depleted DNA was purified by ethanol precipitation and reacted with MG at a bp/MG molar ratio of 1:16. The treated samples (both A-T base pairs depleted and native DNA) were subjected to hydrolysis by S_1 nuclease. The results obtained in Fig.11 show that the maximum extent of hydrolysis achieved with native DNA was more than double of that with A-T base pairs depleted DNA. However, in the absence of MG treatment, both types of samples gave significantly lower but similar extent of hydrolysis. These results suggest that the removal of A-T rich areas from native DNA reduces the number of reaction sites for MG and indicates that the mutagen preferentially reacts with A-T rich regions.

Alkaline unwinding of A-T base pairs depleted MG treated DNA: In order to further substantiate the above result,a similar experiment was also performed using the alkaline unwinding assay. The pea seed nuclease hydrolysed A-T base pairs depleted DNA was further purified by

Table - IV

Effect of increasing time of reaction of MG with DNA on strand breaks:

Reaction time of MG with DNA (Minutes)	Fraction of duplex DNA in control samples	Fraction of duplex DNA in treated samples	No. of breaks per unit DNA
15	0.81	0.74	0.4
30	0.81	0.71	0.6
60	0.81	0.68	0.8
120	0.80	0.60	1.3
180	0.81	0.66	1.0
240	0.80	0.71	0.5
300	0.80	0.78	0.1

Data given have been calculated from the values obtained in Fig.9.

Fig.11. S_1 nuclease hydrolysis of native and A-T base pairs depleted DNA:

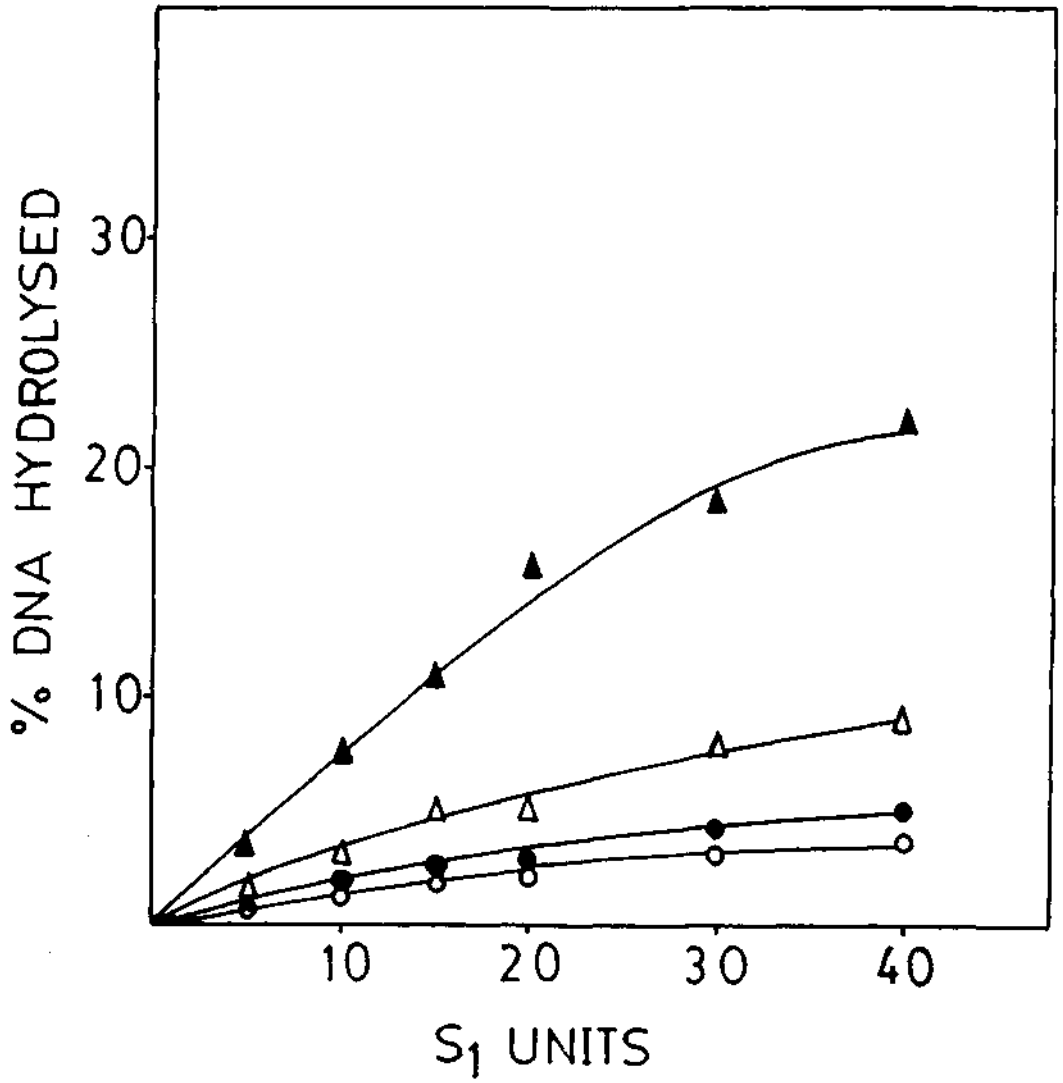
Equivalent amounts (300 µg) of native and A-T base pairs depleted DNA were treated at the DNA bp/MG molar ratio of 1:16 and subjected to hydrolysis by increasing S_1 nuclese concentration in the standard S_1 nuclease reaction mixture. The other reaction conditions were the same as given in Table II.

Native DNA (Δ)

Native DNA + MG (▲)

A-T base pairs depleted DNA (O)

A-T base pairs depleted DNA + MG (●)


% DNA HYDROLYSED
30
20
10
10
20
30
40
S1 UNITS

BND-cellulose chromatography, reacted with MG and subjected to alkaline unwinding. The results are given in Table V. Whereas with native DNA the number of SSB produced increased with increasing molar ratio of MG, the A-T base pairs depleted DNA did not show the formation of any SSB. The same results have been plotted in Fig.12 and show no change in the fraction of duplex DNA remaining in the case of A-T base pairs depleted DNA. These results strongly support the previous observation that MG preferentially reacts with A-T rich regions in native DNA.

Effect of ionic strength and temperature on the reaction of MG with DNA: In Table VI and VII is given the effect of increasing ionic strength and temperature on the formation of SSB in DNA by MG. DNA bp/MG molar ratio in both the experiments was 1:4. Table VI shows that the presence of salt has a stimulatory effect on the number of breaks formed per unit DNA. Almost a 3-fold increase in the number of breaks formed is recorded. Increasing temperature of reaction with MG, on the other hand, shows no significant effect. However, elevated temperatures (48 and 55 ^{o}C) appear to reduce the number of breaks formed per unit DNA.

Presently, no satisfactory explanation for the above results can be offered. However, one explanation could be that MG preferenti-

Fig.12. Fraction of duplex DNA recovered in MG treated native and A-T base pairs depleted DNA:

Treatment of equivalent amounts of BND-cellulose purified native and A-T base pairs depleted DNA was carried out as described in Fig.8. The alkaline unwinding of the treated samples was done as described in 'Methods'.

Native DNA + MG (O)

A-T base pairs depleted DNA + MG (●)

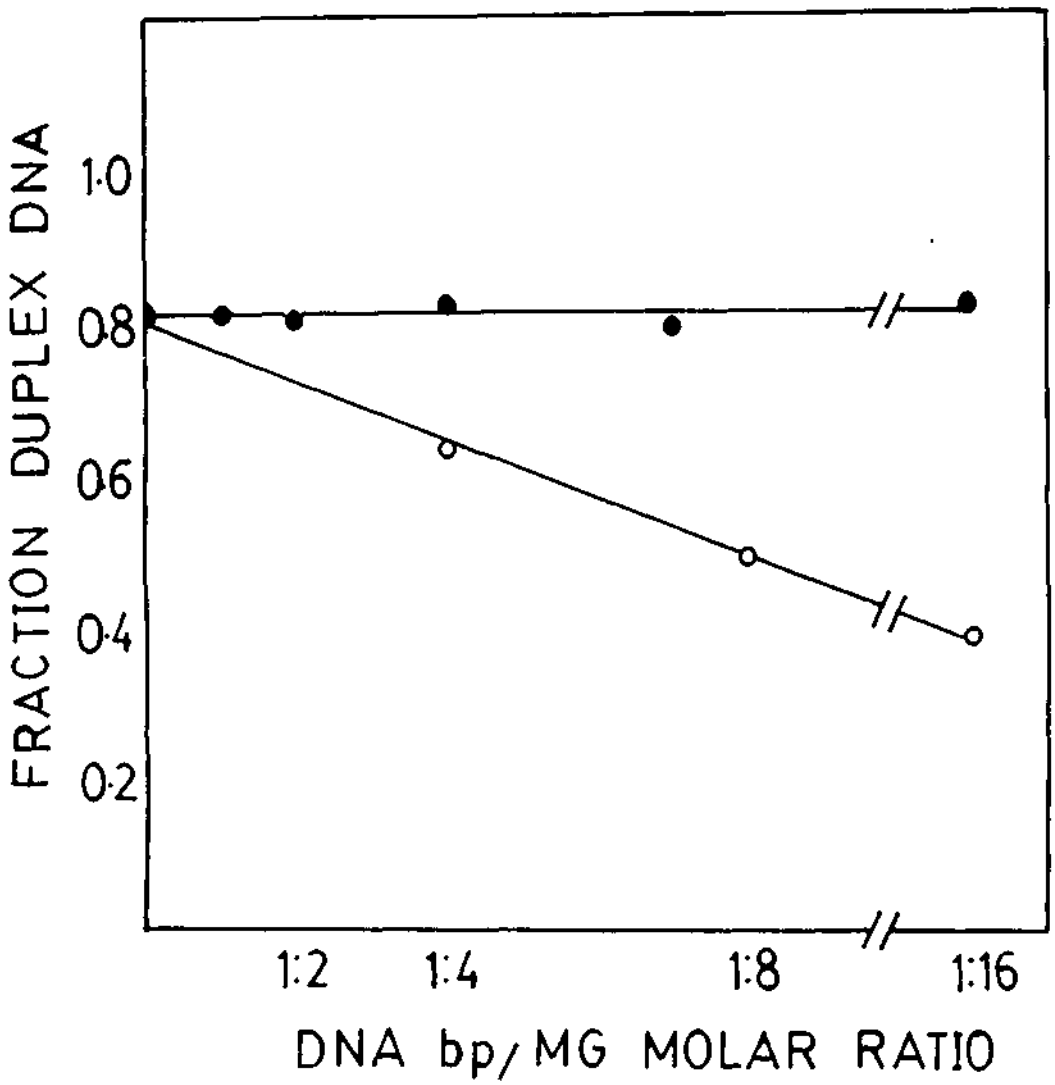

FRACTION DUPLEX DNA
1·0
0·8
0·6
0·4
0·2
1:2
1:4
1:8
1:16
DNA bp/ MG MOLAR RATIO

Table - V

Strand breaks induced by MG in native and A-T base pairs depleted DNA:

Native DNA:

DNA bp/MG molar ratio	Fraction of duplex DNA	No. of breaks per unit DNA
No MG	0.80	-
1:4	0.63	1.1
1:8	0.49	2.2
1:16	0.37	3.5

A-T base pairs depleted DNA:

DNA bp/MG molar ratio	Fraction of duplex DNA	No. of breaks per unit DNA
No MG	0.81	-
1:1	0.81	0.00
1:2	0.80	0.05
1:4	0.81	0.00
1:8	0.79	0.10
1:16	0.80	0.05

Data given have been calculated from the values obtained in Fig.12.

Table - VI

Effect of NaCl on MG induced strand breaks in DNA: BND-cellulose purified DNA was treated with MG at a DNA bp/MG molar ratio of 1:4 for 2 hours at 37°C. The incubation mixture also contained NaCl in the final concentrations indicated.

Concentration of NaCl (Molarity)	Fraction of duplex DNA in control samples	Fraction of duplex DNA in treated samples	No. of breaks per unit DNA
0.0	0.81	0.62	1.3
0.1	0.82	0.57	1.8
0.2	0.81	0.54	1.9
0.3	0.81	0.52	2.1
0.4	0.82	0.52	2.3
0.5	0.81	0.43	3.0

Table - VII

Effect of temperature on MG induced strand breaks in DNA: BND-cellulose purified DNA was treated with MG at the DNA bp/MG molar ratio of 1:4 at the indicated temperatures for 2 hours.

Temperature (°C)	Fraction of duplex DNA in control samples	Fraction of duplex DNA in treated samples	No. of breaks per unit DNA
20	0.81	0.73	0.5
37	0.84	0.66	1.4
48	0.78	0.68	0.6
55	0.78	0.69	0.5

ally reacts with double-stranded DNA. Since high salt concentration has a stabilizing effect on the secondary structure of DNA, the extent of reaction is enhanced under these conditions. On the other hand, elevated temperature below T_m causes partial denaturation, especially in A-T rich regions, the reaction with MG is, therefore, inhibited.

An alternative but more likely explanation could be that high ionic strength inhibits the interstrand cross-linking, but does not affect the formation of strand breaks to the same extent. Therefore, with high salt concentration, a larger number of strand breaks are detected. On the other hand, the extent of reaction of MG with DNA at higher temperatures may be greater but larger number of strand breaks are not detected due to simultaneous formation of interstrand cross-links. Further experiments need to be carried out to understand these observations.

(b) Discussion:

As noted in 'Introduction', MG is a ketoaldehyde, which may arise in the cell both by enzymatic and nonenzymatic mechanisms. In addition, it is also present in various foods and is found in cigarette smoke (Johnstone and Plimmer, 1959). In view of its genotoxic and anticancer properties (see Introduction), there has been some interest in its mechanism of action. Recently, Brambilla et al. (1985) have demonstrated the formation of DNA-protein cross-links by MG in cultured mammalian cells. The results presented in this dissertation demonstrate that MG causes a destabilization/disruption in the secondary structure of DNA as a consequence of the formation of SSB. This is evidenced by an increased susceptibility to S_1 nuclease, and a decreased melting temperature at certain concentrations of MG. Experiment on alkaline hydrolysis of treated DNA indicated that depurination of DNA does not occur on reaction with MG.

An interesting finding of these results is that at DNA bp/MG molar ratio higher than 1:8, the melting profiles gave greater values of Tm than control native DNA. This implied a stabilization of the secondary structure of DNA. This result is further strengthened by the experiment shown in Fig.6, where an extended reaction time with MG gives a reduced rate of hydrolysis with S_1 nuclease. As discussed

below, these observations can be explained by an interstrand cross-linking of DNA at relatively higher concentrations of MG. The results given in Fig.9 are also explained by the same mechaniams. In this experiment,a relatively lower concentration of MG has been used (DNA bp/ MG molar ratio 1:1). On alkaline unwinding, the initial recovery of duplex DNA decreased, but returned to almost its original value after an extended period of treatment with MG. Presumably, the earlier formation of SSB allows the DNA to unwind at various breaks. However, in later stages when interestrand cross-links also appear, the unwinding at strand breaks is not possible. A similar observation has been made by Brambilla et al. (1985), who have reported that MG treatment hides the expression of strand breaks, induced by MMS in cultured Chinese hamster ovary cells.

Some of the cytotoxic properties of MG have been considered to be similar to those of formaldehyde (Brambilla et al., 1985). Similar to MG, formaldehyde has been shown to cause the formation of cross-links between DNA and proteins. In addition, it also gives rise to SSB produced by ionizing radiation (Grafstrom et al., 1983). Various mono-and dialdehydes are generally considered to react with $-NH_2$ group of macromolecules. Amino groups in native DNA involved in hydrogen bonding do not react with formaldehyde, implying that no in vitro reaction with double-stranded DNA occurs (Haselkorn and Doty, 1961).Also,

the reaction with denatured DNA appears to be reversible (Grossman et al., 1961). Ames and co-workers (Levin et al., 1982) have recently reported that various mono-and dialdehydes cause mutations in A-T base pairs in a Salmonella mutagenesis tester strain with A-T base pairs at the site of mutation. As described in the results (Table IV, Fig.7 and 8), our findings strongly suggest that MG preferentially reacts with A-T rich sequences in the native DNA. This can be accounted for by the fact that A-T base pairs possess a partially single-stranded character at temperature significantly lower than Tm , thereby facilitating their reaction with aldehydes. On single stranding, the $-NH_2$ groups of A-T base pairs would be free of hydrogen bonding and therefore would be available for reaction with MG.

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