INTERACTION OF ANTIOXIDANTS WITH DNA

(Studies on the interaction with DNA of the dietary antioxidant and drug curcumin)

DISSERTATION

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HASEEB AHSAN

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FACULTY OF LIFE SCIENCES
ALIGARH MUSLIM UNIVERSITY
ALIGARH (INDIA)

TELEPHONE : 400741
TELEX : 564—230 AMU IN

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF LIFE SCIENCES
ALIGARH MUSLIM UNIVERSITY
ALIGARH—202002 (INDIA)

Ref. No._____ Dated 6.4.96

CERTIFICATE

This is to certify that the work embodied in this dissertation has been carried out by **Mr. Haseeb Ahsan** under my supervision and is suitable for the award of M.Phil degree in Biochemistry.

(S.M. HADI)
Professor of Biochemistry

AS A TOKEN OF LOVE
AND DEEPEST AFFECTION
TO MY PARENTS

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(HASEEB AHSAN)

CONTENTS

LIST OF ABBREVIATIONS

EDTA	-	Ethylenediaminetetraacetic acid
µg	-	microgram
µM	-	micromolar
µmol	-	micromoles
mM	-	millimolar
M	-	Molar
nmol	-	nanomoles
Tris-HCl	-	Tris(hydroxymethyl)aminomethane hydrochloride
v/v	-	volume/volume
w/v	-	weight/volume

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INTRODUCTION

INTRODUCTION

The predominance of certain foods in some countries has been related to the incidence of certain types of cancers in their populations. The human diet contains a variety of naturally occurring mutagens and carcinogens (Ames, 1983). Therefore, dietary mutagens have attracted considerable interest in the last decades and a number of studies on dietary practices in relation to cancer have been undertaken. 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 (O'Connor, 1981; Miller, 1978). 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 also 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; Gensler 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.

Mutagens and carcinogens in dietary plant material

A large number of toxic 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; Stich et al, 1981a; Jadhav et al, 1981; Pamukcu et al, 1980; Griesebach and Ebel, 1978). It has been known for many years that plants contain carcinogens, and a number of edible plants have shown experimental carcinogenic activity for several species and various tissues. Wide use of short term tests for detecting mutagens (Stich and San, 1981; Ames, 1979) and a number of animal cancer tests on plant substances have contributed to the identification of many mutagens and carcinogens in the human diet (Kapadia, 1982).

Safrole and estragole are related compounds, which occur in certain spices and essential oils and are weak hepatocarcinogens (Fenaroli, 1971). Studies have implicated

1'-hydroxysafrole and 1'-hydroxyestragole as proximate carcinogenic metabolites of safrole and estragole, respectively (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 closely related compound, piperine (Concon et al, 1979). Extracts of black pepper cause tumors in mice at a number of sites.

Ivie et al, (1981) have reported that linear furocoumarins (psoralens), are potent light activated carcinogens and mutagens. Some of the most common phototoxic furocoumarins are psoralen, xanthotoxin and bergapten. 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 diadducts with pyrimidine bases. Psoralen in the presence of light is also effective in producing oxygen radicals (Ya et al., 1982).

Pyrrolizidine alkaloids are naturally occurring carcinogens and are found in some fifty species of plants, 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). A number of these alkaloids have been reported to be mutagenic in Drosophila and Aspergillus systems. Mori et al., (1985) have used a hepatocyte primary culture DNA repair test to screen a number of 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 the carcinogenic potential of pyrrolizidine alkaloids. In addition to pyrrolizidine alkaloids, certain glycoalkaloids found in potato, such as solanine and chaconine, have been reported to be highly toxic as they are strong inhibitors of cholinesterase (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).

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

common commercial mushroom, Agaricus bisporus contains about 300 mg of agaritine, the -glutamyl derivative of the mutagen, 4-hydroxymethylphenylhydrazine, per 100 gm of mushroom (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., maltole, kojic acid, ethylmaltole, 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, maltole is a product of carbohydrate dehydration and is present in coffee, soyabeans and baked cereals. 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).

A number of furans, such as 2-methylfuran, dimethylfuran, furfural, 5-methylfurfural and 2-furylmethylketone are found in numerous food products including meat, milk products, tea, coffee (Maga, 1979).

Stich et al., (1981b) 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, they also 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 do contribute significantly to the total genotoxicity of many consumable foods and beverages.

Cyclopropenoid fatty acids present in cotton seed and other oils, have been reported to be carcinogenic and mitogenic having various toxic effects in farm animals. Among these, sterculic acid and malvalic acid are widespread in the human diet. They are also potentiaters of carcinogenicity of aflatoxins (Hendricks et al., 1980). Another major toxin in cotton seed is gossypol. Gossypol causes male sterility through formation of abnormal sperm. It is a potent initiator and also promoter of carcinogenesis

in mouse skin (Haroz and Thomassan, 1980) and is carcinogenic as well (Xue, 1980). Gossypol has been tested as a possible male contraceptive, as it is inexpensive and causes sterility during use. Its mode of action as a spermicide is presumably 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 (Levin et al, 1982; Stich et al, 1981b; Brown, 1980). 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 amount of these phenols in human diet are appreciable). Catechol which is mainly derived from metabolism of plant substances 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. Allyloisothiocyanate, 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 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 (Tazima, 1982; Hirono, 1981). Nitrosoamines and other nitrosocompounds 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).

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 as a possible source of carcinogens

There is epidemiological evidence relating high fat

intake with colon and breast cancer. Animal studies have indicated that high dietary fat is a promoter and a presumptive carcinogen (Kinlen, 1983; Welsch and Aylsworth, 1983; Fink and Kritcheusky, 1981). Two plausible mechanisms involving oxidative processes, have been considered to account for the relationship between high fat intake and the occurrence of heart diseases and cancer. 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 (Petrakis et al, 1981; Simic and Karel, 1980; Imai et al, 1980; Ferrali et al, 1980) and alkoxy and hydroperoxy radicals (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 unit generates one molecule of hydrogen peroxide, a known mutagen and carcinogen (Reddy et al., 1982). 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 reaction, which leads to

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

Mutagens and carcinogens produced in cooking

Pariza and colleagues (1983) as well as Sugimura and Nagao (1979) have reported that the burnt and browned materials from heating proteins during cooking is highly mutagenic. Pyrolysis of proteins produces strong frameshift mutagens that require metabolic activation by rat liver S9 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 is widely used as a food colouring and flavouring agent, is also mutagenic in Salmonella test systems. Coffee contains a considerable amount of burnt material including the mutagenic pyrolysis

product methylglyoxal (Sugimura and Sato, 1983). A cup of coffee also contains the natural mutagen chlorogenic acid (Stich et al., 1981a) and caffeine which can cause birth defects at high levels in several experimental species (Fabro, 1982). 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 used as a preservative in meat, fish and cheese. A possible formation of nitrosoamines from amines, present in or derived from the diet, occurs by reaction with nitrous acid at acidic pH. A high concentration of hydrogen ions in the human stomach (gastric juice has a pH of around 1.0) gives 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 and RNA. It is also mutagenic to

bacteria and bacteriophages (Singer, 1983). Alkali salts of EDTA are widely used as sequesterants 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 drosophila and various plant species.

An artificial sweetner, which was widely used but is now banned in many countries, is cyclamate. Cyclamate induces chromosome breakage in cells of several plant 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 ($O^{\bullet}_2{}^{-}$) during the oxidation of their substrates, for example, xanthine oxidase and peroxidase (Buttner *et al*, 1978; Duran *et al*, 1977). Numerous substances such as reduced flavins and ascorbic acid upon autoxidation produce

superoxide anion. This radical further accepts 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 react with certain chelates of copper and iron to yield the highly reactive hydroxyl free radical ($OH^{\bullet}$). That the superoxide anion actually appears in metabolism is confirmed by the ubiquitous presence 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 quinones. Inflammatory reactions lead to the production of oxygen radicals by phagocytes and this is the basis of promotion by asbestos (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 involves the expression of recessive genes and an increase in gene copy number through chromosome breaks and creation of hemizygosity (Kinsella, 1982; Varshavsky, 1981). Promoters also cause modification of prostaglandins which are intimately involved in cell division, differentiation and tumour growth (Fischer et al., 1982). Most data on radical 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 radical 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 alimentary canal. If oxygen radicals play a major role in damage to DNA, defence against these agents is obviously 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. 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. The enzymes that protect cells from oxidative damage are superoxide dismutase, glutathione peroxidase (Pryor, 1976-1982), 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.

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 dimethylhydrazine induced carcinogenesis have also been observed (Beckman et al., 1982). ß-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 generated by the pigment mediated transfer of light energy to oxygen. Carotenoids are free radical traps and are remarkably efficient as quenchers of singlet oxygen (Packer et al., 1981). ß-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 anticarcinogenic in rodents 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 an effective anticarcinogen against aflatoxins (Novi, 1981). Selenium, which is present in the active site of glutathione peroxidase, is another important dietary anticarcinogen. Glutathione peroxidase is essential for destroying lipid

hydroperoxides and endogenous hydrogen peroxide and therefore, helps to prevent oxygen radical induced lipid peroxidation. Several heavy metal toxins, such as Cd^{2+} 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 rodents treated with UV light and benzo(α)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 due to the induction of cytochrome P-450 and other metabolic enzymes (Boyd et al., 1982). A high dose of such compounds may even lead to deleterious side effects. The differences 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 to insufficient amounts of anticarcinogens and other protective factors in the diet (Maugh, 1979).

In the past 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 epidemiological and laboratory data that diet is an important factor in the etiology of certain human cancers. According to Doll and Peto (1981), 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 about 1-2 gm. In addition, the endogenous conditions favour the formation of still more mutagens <u>in vivo</u> in humans (Oshshima and Bartsch, 1981).

Antioxidant role of curcumin

An antioxidant is any substance that when present at low concentrations compared to that of an oxidizable substrate, significantly inhibits or delays oxidation of that substrate (Halliwell and Gutteridge, 1989). Oxygen is not totally innocuous and it has long been known to be toxic to many animals including humans. The deleterious effects of oxygen are known to result from its metabolic reduction to highly reactive and toxic species, known as reactive oxygen species (ROS) (Buechter, 1988). These oxygen free radicals in living organisms which include hydroxyl radical ($OH^{\bullet}$), superoxide anion ($O^{\bullet}{}_2^-$), hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), etc; can cause tissue damage by reacting with lipids in cellular membranes, nucleotides in DNA and sulphydryl groups in proteins. Free radicals can originate endogenously from metabolic processes or exogenously as components of tobacco smoke and air pollutants apart from other sources.

Antioxidant defence against free radical damage includes vitamin E, vitamin C, ß-carotene, glutathione, uric acid, bilirubin and several metalloenzymes such as superoxide dismutase (SOD), catalase, glutathione peroxidase and proteins such as ceruloplasmin. Several dietary

micronutrients also contribute greatly to the protective system. Many of these protective antioxidants are essential nutrients. Many enzymes and secondary compounds of higher plants have been demonstrated to protect against oxidative damage by inhibiting or quenching free radicals and reactive oxygen species (Machlin and Bendich, 1987).

The free radical oxidation of lipids in foods is a matter of concern for food manufacturers as the degradation of fats gives rise to unpleasant end products. The use of antioxidants in food packaging minimizes such deterioration. Natural foods of plant origin contain components that may have antioxidative properties. Thus, there is increasing interest in the use of natural antioxidants from plants in the preservation of food (Aruoma, 1993).

An important constituent of an Indian diet is turmeric, rhizome of the plant Curcuma longa, commonly used as a spice in cooking and also as a food preservative and colouring agent. Curcumin (diferuloyl methane), a yellow orange compound which is the active constituent or the major pigment of this rhizome has been identified as a natural antioxidant. Curcumin is used to colour cheese and butter, in cosmetic formulations and in some pharmaceutical

preparations (Govindarajan, 1980). It has been approved for use as a colourant and preservative in food processing (WHO Food Additives, 1975) and is also used as a colouring principle in drugs (Auslander et al., 1977). Toxicologically, curcumin is relatively inert. It does not appear to be toxic to animals or humans even at high dosage (Shankar et al, 1980; Deodhar et al, 1980; Abraham et al, 1976). The pharmacological safety of curcumin can be assessed by its consumption for centuries by people upto 100 mg/day in some countries (Ammon and Wahl, 1991).

Curcumin exhibits a variety of toxicological, pharmacological and photochemical activities, including phototoxicity to bacteria, especially the Gram positive species (Dahl et al., 1989) and to rat basophilic leukemia cells in culture (Dahl et al., 1994). The anticarcinogenic properties of curcumin in animals has been demonstrated by its inhibition of tumor initiation induced by benzo(a) pyrene and 7,12-dimethylbenz(a)anthracene (DMBA) and tumor promotion induced by phorbol esters in mouse skin (Huang et al., 1992a). It is believed that it possibly inhibits tumorigenesis by modulating arachidomic acid metabolism (Huang et al., 1992b, 1991). A recent study demonstrated

that it inhibits azoxymethane-induced colon carcinogenesis (Rao et al., 1995). Recently, it has also been shown that curcumin inhibits TPA-induced expression of c-fos, c-jun and c-myc proto-oncogene mRNAs (Kakar and Roy, 1994). Curcumin has also been found to inhibit the formation of the potentially mutagenic DNA-adduct of 8-hydroxydeoxyguanosine in mouse skin (Shih and Lin, 1993). Curcumin is being developed in the treatment of arthritic patients and such studies are in phase II of clinical trials (Srimal, 1993; Asthana, 1992-93). It also possesses antiinflammatory (Rao, 1982) and antioxidant properties (Toda et al., 1988, 1985).

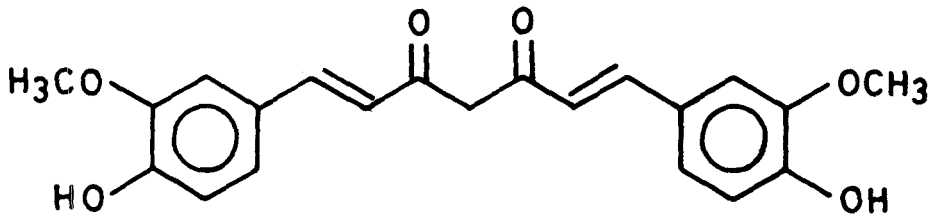
Curcumin has a dual effect in oxygen radical reactions. It can act as a scavanger of hydroxyl radicals or catalyse their formation from hydrogen peroxide in vitro, depending upon the experimental conditions (Tonnensen and Greenhill, 1992; Kunchandy and Rao, 1990; Tonnensen, 1989). Curcumin is capable of photoproducing active oxygen species - $^1O_2$ and $O^{\bullet}{}_2^-$ (upon irradiation with visible light). Illuminated curcumin initiates multiple photochemical pathways, including photogeneration of $^1O_2$, photoreduction of O_2 to $O_2^{\bullet -}$ and H_2O_2 and production of carbon centered radicals that may subsequently react with O_2 (Dahl et al., 1994). Studies have shown that it is a good scavanger of hydroxyl

radicals at high concentration, but at low concentration it activated the Fenton system to generate an increased amount of hydroxyl radicals. Curcumin is also a potent scavanger of superoxide radical and this property may be responsible for its good antiinflammatory activity (Kunchandy and Rao, 1990). Curcumin may inhibit the promotion of tumors by functioning as an $OH^{\bullet}$ scavanger (Shih and Lin, 1993).

Anticarcinogenic and other therapeutic activities of natural compounds are correlated with their ability to protect biomolecules against prooxygens (Foote et al., 1970). Curcumin has been shown to be responsible for the protection of DNA from free radicals induced damage (Donatus et al., 1990), hemoglobin from nitrite induced oxidation (Unnikrishnan and Rao, 1992), RBC from primaquine induced oxidative damage (Tonnensen et al., 1994), hepatocytes from various toxins (Shalini and Srinivas, 1990). It also inhibits lipid peroxidation. Curcumin enclosed in liposomes can be used as a drug delivery system (Tonnensen et al., 1993).

Thus, various studies support the role of curcumin as an effective radical quencher and antioxidant which helps in controlling oxygen radical mediated pathologies.

Figure 1 : Structure of curcumin.



Diferuloylmethane (curcumin, Mr = 368)

SCOPE OF THE WORK

A common feature of many DNA-binding drugs (including a number of anti-tumor agents) is their capacity to provoke breakage of DNA stands *in vivo*. For two group of antibiotics (i.e., streptomycin and bleomycin) this seems to be the basis of their effect on cells. Several anti-cancer drugs such as bleomycin, adriamycin, phenanthroline cleave DNA in the presence of a transition metal ion [Cu(II), Fe(III), Fe(II)] and a reducing agent (Eliot *et al*, 1984; Sigman *et al*, 1979; Sausville *et al*, 1976). The generation of oxygen radicals in the proximity of DNA is well established as a cause of strand scission by various drugs (Wong *et al*., 1984). Copper and iron are essential trace elements that are ubiquitous in many proteins and often participate in the catalytic function of enzymes (Beinert, 1991). However, upon their leakage out of stores and their release out of macromolecular structures, they could become involved in deleterious processes to tissues (Lesnefsky, 1992; Edwards *et al*, 1991; Aruoma *et al*, 1991). These deleterious processes are catalysed by the fraction of free copper and iron that is redox active. As these metal ions are sparingly

soluble under physiological conditions, they must attach and form complexes with biomolecules and serve as redox active centres for repeated production of free radicals leading to damage at or near the metal binding site.

Certain antioxidants such as naturally occuring flavonoids eg., quercetin, function as scavangers of ROS like singlet O_2, superoxide anion and H_2O_2. Previous studies from this laboratory, have demonstrated that flavonoids cause strand scission in DNA in vitro in presence of Cu(II) and molecular oxygen (Rahman et al., 1992). In addition, the natural physiological antioxidant, uric acid has also been shown to cause strand breakage in DNA (Shamsi and Hadi, 1995).

Oxygen radicals have been suggested to be involved in the action of a number of DNA damaging drugs. Curcumin has significant abilities to protect DNA against singlet oxygen ($^1O_2$), a reactive oxygen species with potentially known genotoxic/mutagenic properties. The protective ability of curcumin is reported to be more than that of well known biological antioxidants, eg ; lipoate, α-tocopherol, ß-carotene (Subramanian et al., 1994).

In view of the previous findings, which suggest that several of the biological antioxidants are themselves capable of DNA damage in the presence of transitional metals, I have investigated the effect of curcumin on DNA in the presence of Cu(II).

EXPERIMENTAL

MATERIALS

Chemicals used for the present study were obtained from the following sources :

Chemicals	Source
Agarose	Bio-Rad Laboratories, USA
Bathocuproinedisulfonic acid (disodium salt)	Sigma Chem. Co., USA
Bovine serum albumin (BSA)	Sigma Chem. Co., USA
Calf Thymus DNA (type I, sodium salt)	Sigma Chem. Co., USA
Catalase	Sigma Chem. Co., USA
Cupric chloride (Cu^{++})	S.D. Fine Chem. Ltd., India
Curcumin	Aldrich Chem. Co. Ltd., UK
Diphenylamine (DPA)	E.Merck (India) Ltd., Bombay
Ethidium bromide	Sigma Chem. Co., USA
EDTA (disodium salt)	Qualigens Fine Chem., Bombay
L-Histidine	E.Merck (India) Ltd., Bombay
Mannitol	CDH Lab. Reagents, Delhi
Neocuproine hydrochloride	Sisco Research Lab., Bombay
Nitroblue tetrazolium chloride (Nitro BT)	Sisco Research Lab., Bombay
Nuclease S_1 (*A.oryzae*)	Sigma Chem. Co., USA
Potassium iodide	Merck, Germany
Sodium acetate	Qualigens Fine Chem., Bombay

Sodium azide	Merck, Germany
Sodium benzoate	Merck, Germany
Supercoiled plasmid DNA (pBR322)	prepared in the lab. according to the method of Sambrook, et al (1989)
Superoxide dismutase (SOD), Bovine Erythrocyte	Sigma Chem. Co., USA
Tris(hydroxymethyl)-amino methane	E. Merck (India) Ltd., Bombay
Triton X-100	Loba Chemie, Bombay

All the other chemicals were of analytical grade.

METHODS

Assay of S_1 nuclease hydrolysis

The enzyme assay was done by estimating the acid soluble nucleotides released from DNA as a result of enzymatic digestion. The reaction mixture (0.5 ml) contained 10 mM Tris-HCl (pH 7.5) and 500 µg of calf thymus DNA (native, denatured and treated). The reaction was started by the addition of Cu(II). All solutions were autoclaved before use.

Nuclease S_1 digestion of the mixture was carried out in a total volume of 1 ml by adding sodium acetate buffer (0.1 M, pH 4.5), 1 mM $ZnSO_4$ and 20-30 units of S_1 enzyme. The reaction mixtures were incubated for 2 hours at 37°C. The reaction was stopped by adding 0.2 ml of bovine serum albumin (10 mg/ml) and 1 ml of 14% perchloric acid (cold). The tubes were transferred to 4°C for at least an hour before centrifugation, to remove the undigested DNA. Nucleotides were determined in the supernatant using the method of Schneider (1957). To a 1 ml aliquot, 2 ml diphenylamine reagent (freshly prepared by dissolving 1 gm of diphenylamine in 100 ml glacial acetic acid and 2.75 ml of conc. H_2SO_4) was added. The tubes were heated in a

boiling water bath for 20 min. The intensity of blue colour was read at 600 nm, after cooling.

Treatment of supercoiled plasmid pBR322 DNA with curcumin and Cu(II)

Reaction mixtures (30 µl) contained 10 mM Tris-HCl (pH 7.5) and other components as described in the 'legends'. All the solutions were autoclaved before use.

After incubation, 10 µl of a solution containing 40 mM EDTA, 0.05% bromophenol blue tracking dye and 50% glycerol (v/v) was added to stop the reaction. The reaction mixtures were subjected to electrophoresis in Tris-acetate EDTA (TAE) buffer (pH 8.0) in a 1-1.4% submarine agarose gel. The gel was stained with ethidium bromide (0.5-1 µg/ml), viewed and photographed on a transilluminator.

Detection of superoxide anion ($O^{\bullet}_2{}^-$)

Superoxide anion was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described by Nakayama, et al (1983). A typical assay mixture contains 50 mM sodium phosphate buffer (pH 8.0), 70 µM NBT, 0.1 mM EDTA and 0.06% triton X-100 in a total volume of 3 ml. The reaction was started by the addition of NBT. Immediately after mixing, the absorbance was read at 560 nm against a

blank which did not contain curcumin. To confirm the formation of $O^{\bullet}_2^-$, SOD was also added to the reaction mixture before adding curcumin.

Estimation of hydroxyl radical ($OH^{\bullet}$)

Hydroxyl radical ($OH^{\bullet}$) production was determined by the aromatic hydroxylation method. This assay is based on the ability of $OH^{\bullet}$ radical to hydroxylate aromatic rings at almost diffusion controlled rates and the measurement of hydroxylated products by simple colourimetric method using salicylate (2-hydroxybenzoate) as a detector molecule (Richmond, et al., 1981). The reaction mixture (2 ml) contains 2 mM salicylate, 0.1 mM EDTA, 0.1 mM Cu(II) and 50 mM KOH-KH_2PO_4 buffer (pH 8.0). The reaction was started by the addition of appropriate amounts of curcumin and incubated at room temperature for half an hour. The reaction was stopped by adding 80 µl of 11.6 M HCl, 0.5 gm NaCl and 4 ml of chilled diethyl ether. The contents were mixed by vortexing for a minute. Next, 3 ml of the upper ether layer was extracted and evaporated to dryness at around 50°C. The dried residue was dissolved in 0.25 ml of cold distilled water and the following added in sequence - 0.125 ml 10% (w/v) TCA (in 0.5 M HCl), 0.25 ml of 10% (w/v) sodium tungstate (in water) and 0.25 ml of 0.5% (w/v) $NaNO_2$ (sodium

nitrite, freshly prepared). After standing for 5 min, 0.5 M KOH was added and the absorbance read after a minute at 510 nm.

Assay of hydrogen peroxide (H_2O_2) production

The production of H_2O_2 was assayed by the method of Nakayama, et al., (1983). Titanium sulphate solution was prepared from titanium dioxide and concentrated sulphuric acid (Snell and Snell, 1949).

A 2 ml sample containing different amounts of curcumin was mixed with 2 ml sodium phosphate buffer (50 mM, pH 7.2) and incubated at 37°C for one hour. An aliquot (2 ml) of the mixture was added to 2 ml of $Ti(SO_4)$ solution. Absorbance was measured at 410 nm against a blank which did not contain $Ti(SO_4)$ but contains 2 ml sulphuric acid. In order to confirm that the colour change was due to the generation of H_2O_2, 0.4 ml catalase (1 mg/ml) was added in a separate reaction before incubation at 37°C.

Spectroscopy

The absorption spectra were obtained by using a Beckmann DU-40 spectrophotometer. The absorption data were obtained in a 1 cm path-length cell. All spectroscopic work was carried out at ambient temperatures.

RESULTS

RESULTS

Breakage of calf thymus DNA by curcumin and Cu(II)

Figure 2 shows the S_1-nuclease hydrolysis of native calf thymus DNA with increasing concentrations of curcumin in the presence of Cu(II). There was a gradual increase in DNA degradation with increasing curcumin concentration. Control experiments showed that heat denatured DNA underwent 100% hydrolysis whereas only about 8% of the native DNA was hydrolysed following treatment with S_1-nuclease at 37°C (data not shown). Curcumin does not significantly inhibit the S_1-nuclease activity at the concentrations tested. In the presence of Cu(II) (200 µM), curcumin generated a dose dependent increase in calf thymus DNA S_1-sensitive sites and maximum hydrolysis was observed following treatment with 200 µM curcumin.

The strand scission in DNA induced by curcumin and Cu(II) is also dependent upon Cu(II) concentration (Fig. 3). A similar progressive increase in DNA hydrolysis was observed when calf thymus DNA was incubated with 50 µM curcumin and increasing concentrations of Cu(II).

Cleavage of plasmid DNA by curcumin and Cu(II)

Supercoiled plasmid pBR322 DNA was examined as a

Figure 2 : Degradation of calf thymus DNA as a function of increasing curcumin concentration in presence of Cu(II) as measured by the degree of S_1-nuclease digestion.

DNA was incubated with increasing concentrations of curcumin (10-200 µM) in the presence of 0.2 mM Cu(II), overnight at room temperature.

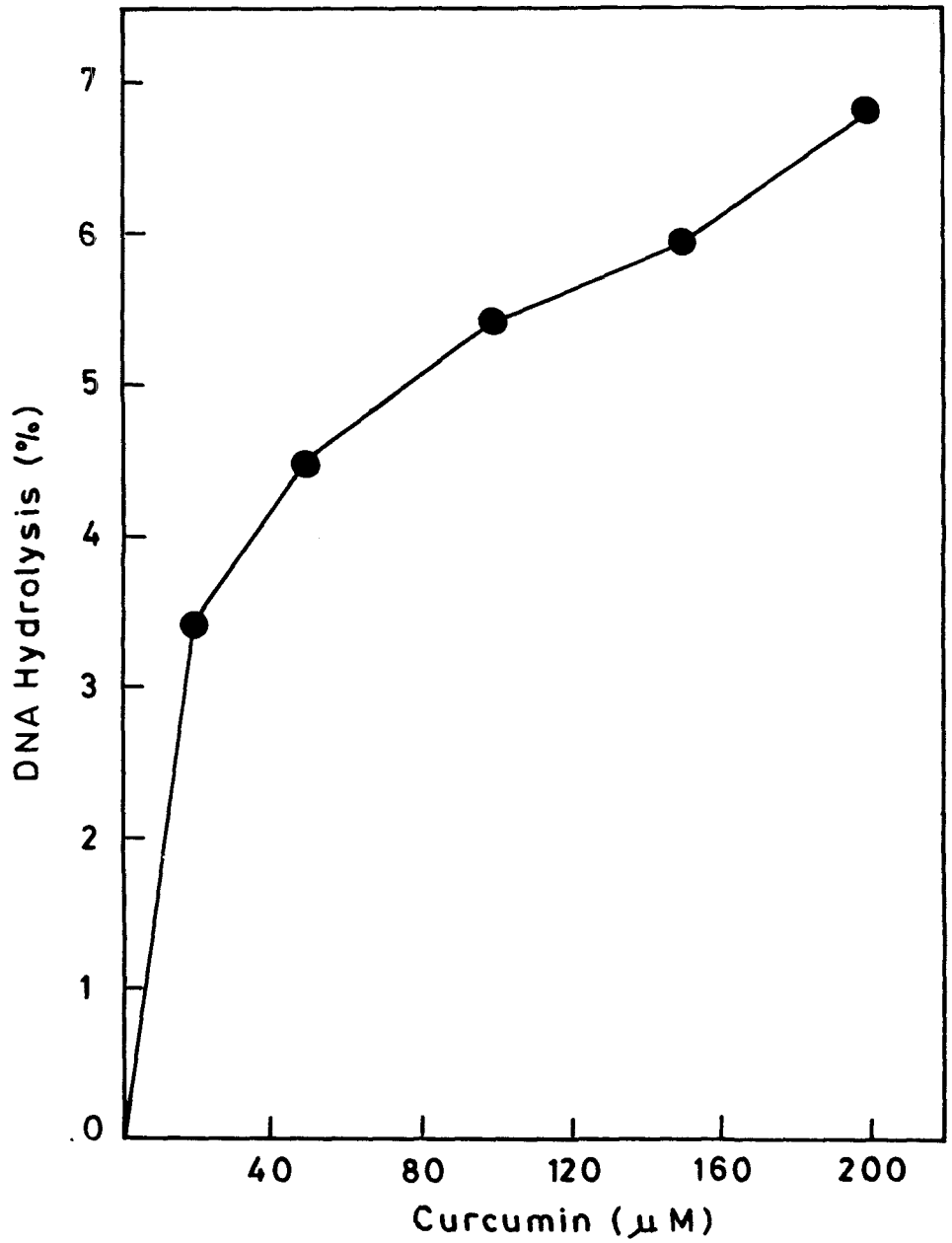
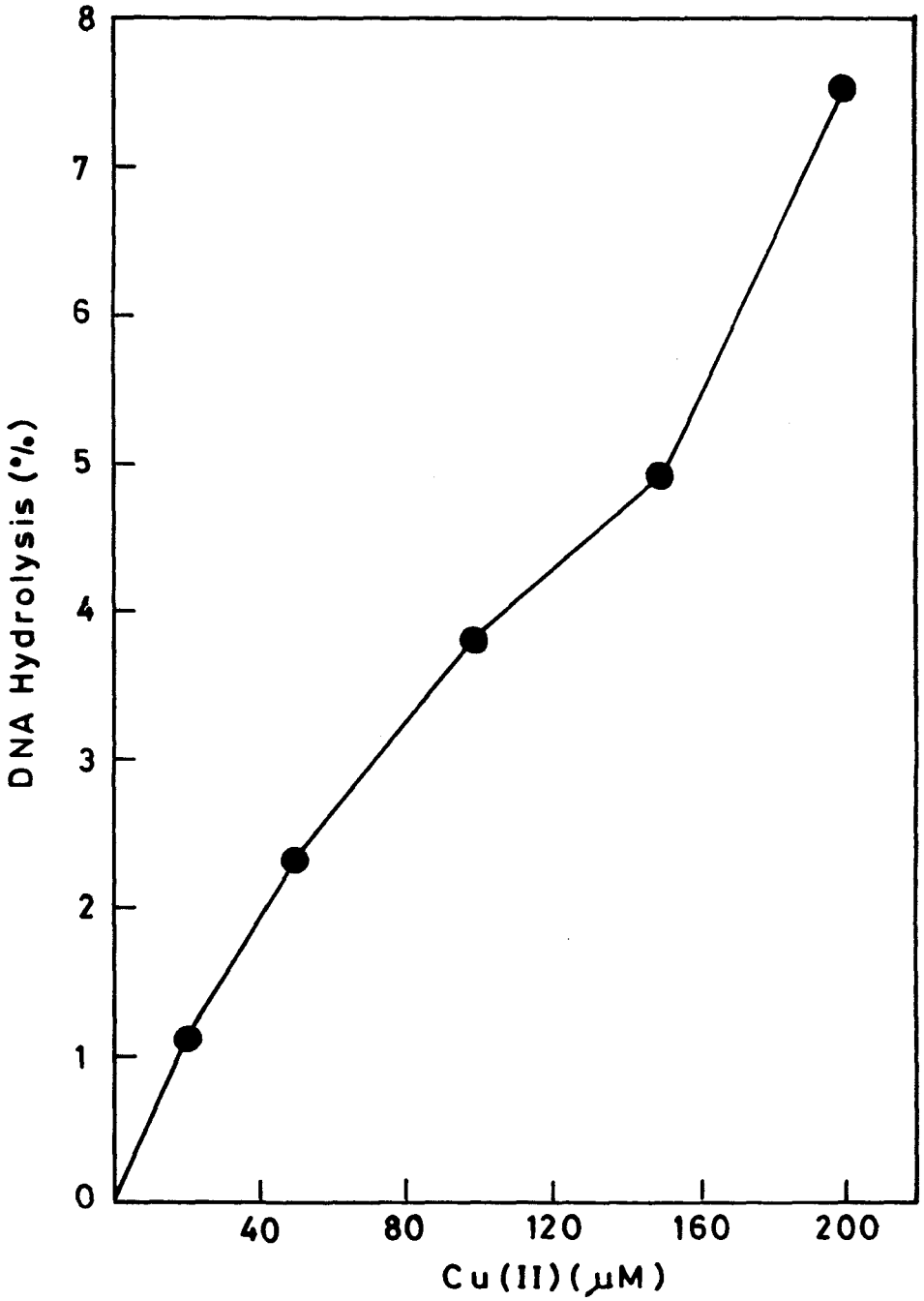

DNA Hydrolysis (%)
7
6
5
4
3
2
1
0
40
80
120
160
200
Curcumin (μM)

Figure 3 : Degradation of calf thymus DNA as a function of increasing Cu(II) concentration in the presence of curcumin measured by the degree of S_1-nuclease digestion.

DNA was incubated with increasing concentrations of Cu(II) in the presence of 50 μM curcumin at room temperature overnight.


DNA Hydrolysis (%)
0
1
2
3
4
5
6
7
8
40
80
120
160
200
Cu(II)(μM)

substrate as the relaxation of such a molecule is a sensitive test for just one nick per molecule, that results in its conversion to open circular form.

Curcumin converted supercoiled plasmid DNA to relaxed open circles in a Cu(II) dependent reaction. Figure 4 shows the effect of increasing curcumin concentration on supercoiled plasmid pBR322 DNA in presence of a fixed concentration of Cu(II). It may be noted that relatively higher concentrations of curcumin (400 μM and above) result in an inhibition of strand breakage activity. Similarly with increasing concentration of Cu(II), a dose dependent cleavage was observed at a curcumin concentration of 100 μM (Fig. 5). However, at higher concentration of Cu(II), the reaction was inhibited. Figure 6 shows the time dependent conversion of form I (SC) to form II (OC) DNA mediated by curcumin and Cu(II). A progressive increase in the concentration of form II DNA with a concomitant decrease in form I DNA is seen.

Effect of transition metal ions and incubation in light and dark

Figure 7 shows the effect of several metal ions on the degradation of plasmid DNA in presence of curcumin. It is

Figure 4 : Effect of increasing curcumin concentration on plasmid DNA in the presence of Cu(II).

Reaction mixtures containing 0.75 µg plasmid pBR322 DNA, 0.1 mM Cu(II) and increasing concentrations of curcumin were incubated for 2 hours at 37°C.

Lane 1 - DNA alone

2 - DNA, Cu(II)

3 - DNA, curcumin (0.8 mM)

4 - DNA, Cu(II), curcumin (0.05 mM)

5 - DNA, Cu(II), curcumin (0.1 mM)

6 - DNA, Cu(II), curcumin (0.2 mM)

7 - DNA, Cu(II), curcumin (0.4 mM)

8 - DNA, Cu(II), curcumin (0.8 mM)

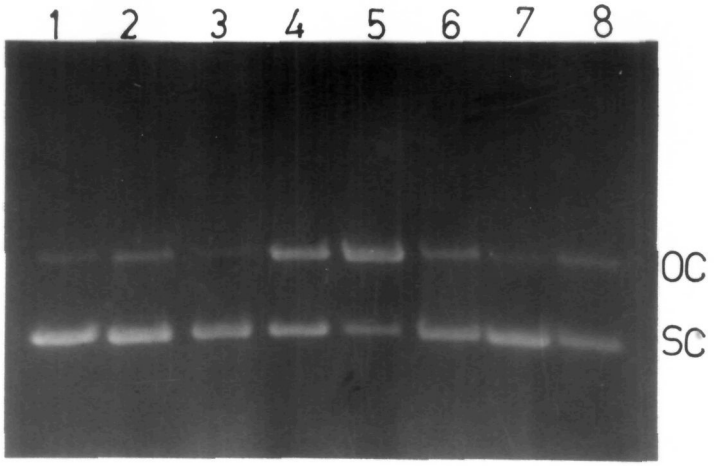

1 2 3 4 5 6 7 8
OC
SC

Figure 5 : Effect of increasing Cu(II) concentration on plasmid DNA in the presence of curcumin.

Reaction mixtures containing 0.75 µg plasmid pBR322 DNA, 0.1 mM curcumin and increasing concentrations of Cu(II) were incubated for 2 hours at 37°C.

Lane 1 - DNA alone

2 - DNA, Cu(II) (0.8 mM)

3 - DNA, curcumin

4 - DNA, curcumin, Cu(II)(0.05 mM)

5 - DNA, curcumin, Cu(II)(0.1 mM)

6 - DNA, curcumin, Cu(II)(0.2 mM)

7 - DNA, curcumin, Cu(II)(0.4 mM)

8 - DNA, curcumin, Cu(II)(0.8 mM)

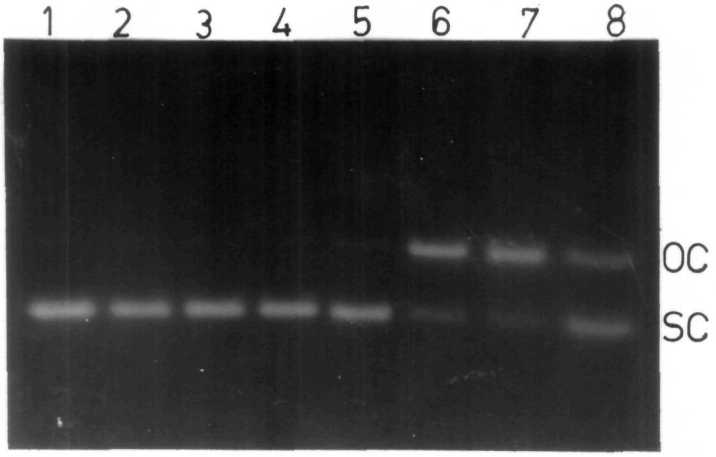

1 2 3 4 5 6 7 8
OC
SC

Figure 6 : Effect of increasing time on plasmid DNA in the presence of curcumin and Cu(II).

Reactions mixtures containing 0.75 µg plasmid pBR322 DNA, 0.1 mM curcumin and 0.2 mM Cu(II) were incubated at 37°C for different time periods.

Lane 1 - DNA alone

2 - DNA, Cu(II)

3 - DNA, curcumin

4 - DNA, Cu(II), curcumin, 0 min.

5 - DNA, Cu(II), curcumin, 10 min.

6 - DNA, Cu(II), curcumin, 30 min.

7 - DNA, Cu(II), curcumin, 60 min.

8 - DNA, Cu(II), curcumin, 120 min.

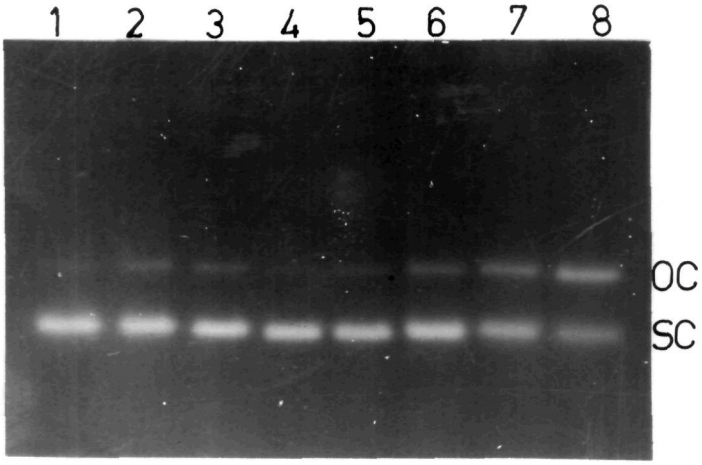

1 2 3 4 5 6 7 8
OC
SC

Figure 7 : Effect of different metal ions on plasmid DNA in the presence of curcumin.

Reaction mixtures containing 1 µg plasmid pBR322 DNA, 0.1 mM curcumin and 0.2 mM metal ions were incubated for 4 hours at 37°C.

Lane 1 - DNA alone

2 - DNA, curcumin, Cu(II)

3 - DNA, curcumin, Mg(II)

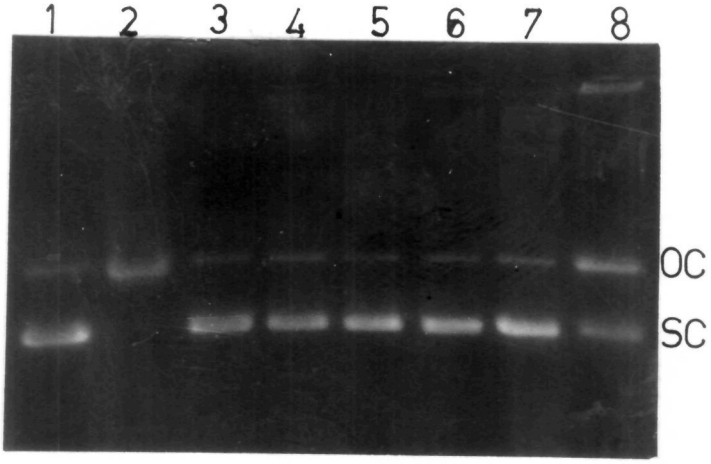
4 - DNA, curcumin, Co(II)

5 - DNA, curcumin, Mn(II)

6 - DNA, curcumin, Ni(II)

7 - DNA, curcumin, Ca(II)

8 - DNA, curcumin, Fe(II)


1
2
3
4
5
6
7
8
OC
SC

seen that only Cu(II) and to some extent Fe(II) complemented curcumin in the DNA breakage reaction. However, no breakage of supercoiled plasmid DNA occurs if the reaction is performed in both light and dark conditions but in the absence of Cu(II) (Fig. 8).

Inhibition of curcumin-Cu(II) induced DNA breakage by neocuproine

It was of interest to determine whether the production of Cu(I) from Cu(II) during curcumin-Cu(II) interaction was necessary for DNA breakage. For this purpose, a Cu(I) specific chelating agent, neocuproine was added to the curcumin-Cu(II)-plasmid DNA reaction mixture. Neocuproine forms a stable complex with Cu(I) in aqueous solution with a stoichiometry of 2:1. The inhibition of DNA breakage was examined by using a constant concentration of curcumin and Cu(II) and varying amounts of neocuproine. When increasing concentrations of neocuproine was added, a progressive decrease in the conversion of supercoiled plasmid DNA to relaxed open circles was observed (Fig. 9). In the experiment shown, 200 μM Cu(II) concentration was used in all samples. Complete inhibition of conversion of supercoiled DNA to relaxed form is seen at a neocuproine concentration

Figure 8 : Effect of curcumin on plasmid DNA in dark and in the presence of light [in absence of Cu(II)].

Reaction mixtures containing 1 µg plasmid pBR322 DNA and increasing curcumin concentration were incubated for 2 hours at room temperature.

Lane 1 - DNA alone

2 - DNA, curcumin (0.05 mM)

3 - DNA, curcumin (0.1 mM)

4 - DNA, curcumin (0.2 mM)

5 - DNA alone

6 - DNA, curcumin (0.05 mM)

7 - DNA, curcumin (0.1 mM)

8 - DNA, curcumin (0.2 mM)

First 4 lanes (1-4) correspond to reactions carried out in dark and the next 4 lanes (5-8) to reactions in presence of fluorescent light.

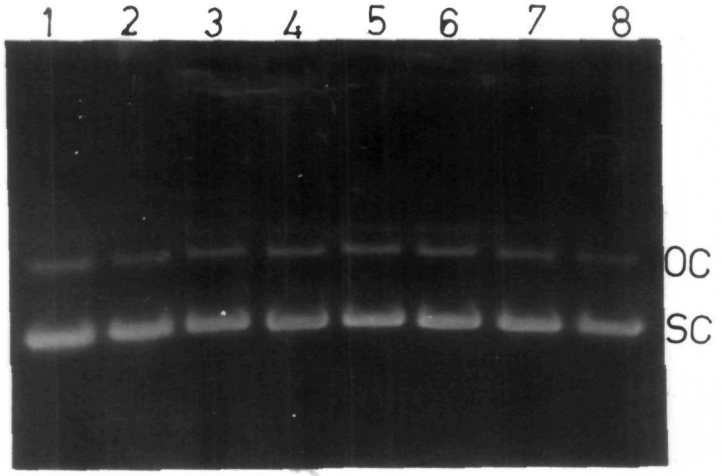

1 2 3 4 5 6 7 8
OC
SC

Figure 9 : Effect of increasing neocuproine concentration on curcumin-Cu(II) induced breakage of plasmid DNA.

Reaction mixture containing 1 µg plasmid pBR322 DNA, 0.2 mM curcumin, 0.2 mM Cu(II) and increasing concentrations of neocuproine were incubated for 3 hours at 37°C.

Lane 1 - DNA alone

2 - DNA, Cu(II)

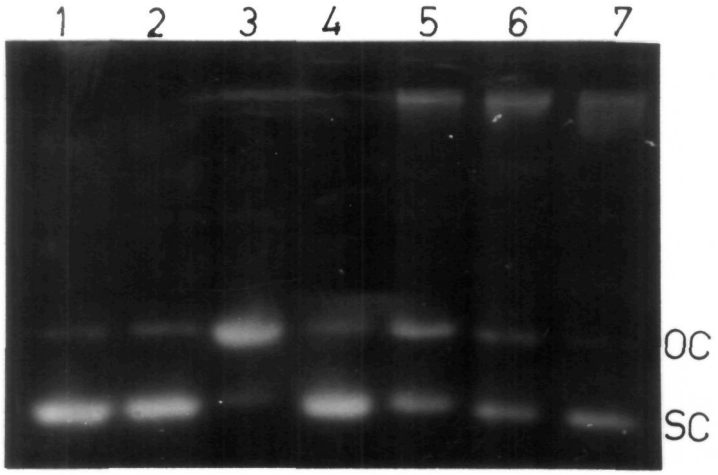
3 - DNA, curcumin, Cu(II)

4 - DNA, Cu(II), neocuproine (0.6 mM)

5 - DNA, curcumin, Cu(II), neo. (0.1 mM)

6 - DNA, curcumin, Cu(II), neo. (0.2 mM)

7 - DNA, curcumin, Cu(II), neo. (0.4 mM)


1
2
3
4
5
6
7
OC
SC

of 400 μM, indicating that when all the Cu(I) produced is bound by neocuproine, DNA degradation does not occur.

Effect of free radical scavangers on DNA breakage by curcumin and Cu(II)

Several polycyclic aromatic compounds such as flavonoids (Rahman et al., 1989), adriamycin (Eliot et al., 1984), bleomycin (Ehrenfield et al, 1989; Sugiura, 1979), phenanthroline (Gutteridge and Halliwell, 1982) have been shown to cleave DNA in the presence of a metal ion and molecular oxygen. In all these reactions, active oxygen species, such as hydroxyl radical and singlet oxygen were shown to be involved. For this reason, the effect of several free radical scavangers or quenchers on curcumin-Cu(II) mediated DNA degradation was examined. Sodium azide is a singlet oxygen ($^1O_2$) quencher; superoxide dismutase (SOD) and catalase scavange superoxide anion ($O^-_2{}^\bullet$) and hydrogen peroxide (H_2O_2), respectively; sodium benzoate, mannitol and potassium iodide eliminate hydroxyl free radicals ($OH^\bullet$). As shown in figure 10, curcumin-Cu(II) induced DNA degradation was nearly completely inhibited by the addition of catalase, confirming the involvement of H_2O_2 in the reaction. Potassium iodide and sodium azide also marginally inhibited the DNA breakage reaction indicating the involvement of

Figure 10 : Effect of free radical scavangers on curcumin-Cu(II) induced breakage of plasmid DNA.

Reaction mixtures containing 1 µg plasmid pBR322 DNA, 0.2 mM curcumin, 0.2 mM Cu(II) and 50 mM or 0.1 mg/ml quenchers were incubated for 3 hours at 37°C.

Lane 1 - DNA alone

2 - DNA, curcumin, Cu(II)

3 - DNA, curcumin, Cu(II), pot. iodide

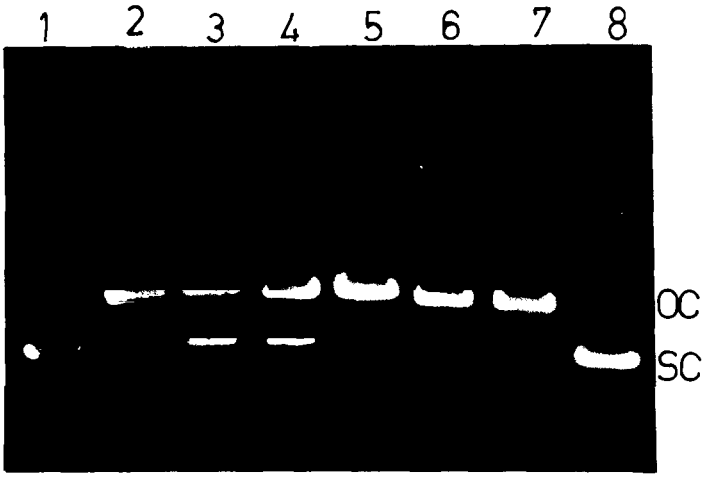
4 - DNA, curcumin, Cu(II), sodium azide

5 - DNA, curcumin, Cu(II), sod. benzoate

6 - DNA, curcumin, Cu(II), mannitol

7 - DNA, curcumin, Cu(II), SOD

8 - DNA, curcumin, Cu(II), catalase


1
2
3
4
5
6
7
8
OC
SC

hydroxyl radical and singlet oxygen, respectively.

Photogeneration of superoxide anion ($O^{\bullet}_2{}^-$) by curcumin

Figure 11 shows the generation of superoxide anion ($O^{\bullet}_2{}^-$) by curcumin in visible light. An increase in absorbance at 560 nm is observed on reduction of nitroblue tetrazolium (NBT) to a formazan by superoxide. The reaction is almost completely inhibited in presence of superoxide dismutase (SOD) indicating that the method genuinely assays the superoxide free radical.

Production of hydroxyl radical ($OH^{\bullet}$) by curcumin and Cu(II)

In this experiment, it is seen that hydroxyl radical ($OH^{\bullet}$) is generated by curcumin. Table I shows the effect of increasing curcumin concentration on the generation of hydroxyl radical as determined by the formation of hydroxylated salicylic acid. The formation of hydroxyl radical increases with an increase in concentration of curcumin. Cu(II) is able to produce nanomole amounts of hydroxylated product.

Formation of hydrogen peroxide (H_2O_2) by curcumin

The pathway for the generation of hydroxyl radical ($OH^{\bullet}$) involves hydrogen peroxide (H_2O_2) as intermediate.

Figure 11 : Generation of superoxide anion (O_2.-) by curcumin. Effect of incubation in presence of SOD.

The concentration of curcumin used was 50 µM. Reading of the sample is recorded at specified time periods.

(O) Effect of incubation time with curcumin alone.

(●) with superoxide dismutase.

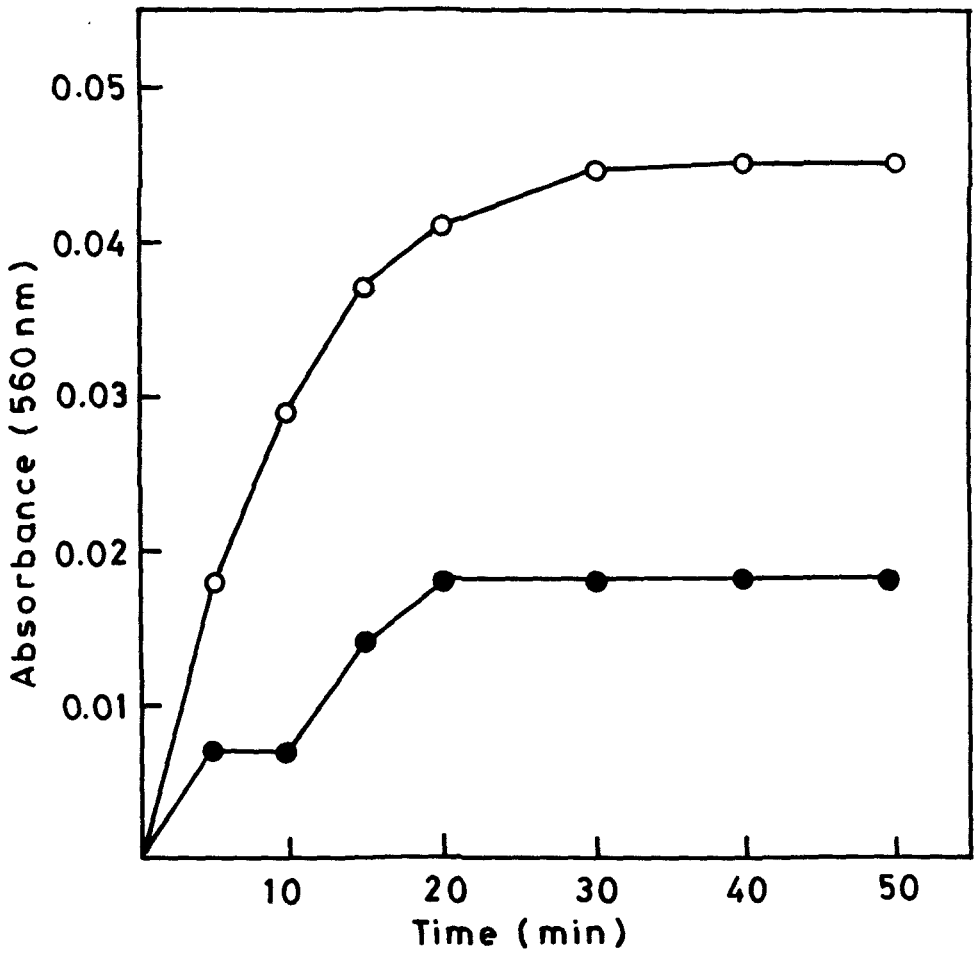

0.05
0.04
0.03
0.02
0.01
Absorbance (560nm)
10
20
30
40
50
Time (min)

Table I : Formation of hydroxyl radical ($OH^{\bullet}$) as a function of curcumin concentration.

The reaction mixtures containing increasing concentrations of curcumin (10-50 µM) were incubated for 2 hours at room temperature in the presence of light.

TABLE-I

Formation of hydroxyl radical as a function of curcumin concentration.

Curcumin (μM)	Hydroxylated product formed (n mol)
10	82.5
20	116.0
30	132.9
40	175.1
50	208.6

Values of curcumin shown are final reaction concentrations. Cu(II) was used at a final concentration of 0.1 mM. The incubations were done for 2 hours at room temperature in fluorescent light. Reaction conditions are described in 'Methods'.

Hydrogen peroxide in turn gives rise to OH radical either by Haber-Weiss or Fenton reaction.

i) $2\ O^{\bullet}_2{}^{-} + 2H^{+} \longrightarrow H_2O_2 + O_2$

$O^{\bullet}_2{}^{-} + H_2O_2 \longrightarrow O_2 + OH^{\bullet} + OH^{-}$

ii) $H_2O_2 + Cu(I) \longrightarrow OH^{\bullet} + OH^{-} + Cu(II)$

In this experiment, I have determined the hydrogen peroxide production capacity of curcumin. The method used involves the oxidation of titanium to pertitanic acid by hydrogen peroxide (Nakayama et al., 1983). Figure 12 shows the production of H_2O_2 with increasing concentrations of curcumin. It is seen that hydrogen peroxide formation increases with the concentration of curcumin. In the presence of catalase, H_2O_2 is not produced confirming that the method employed measures hydrogen peroxide in this assay.

Curcumin-DNA interaction

The absorbance of curcumin with a maximum at 420 nm is affected by addition of increasing DNA concentration (Fig. 13) showing both enhancement (hyperchromic shift) and quenching (hyperchromic shift) in absorption and also a shift in the absorption maxima (bathochromic shift) to about

Figure 12 : Generation of hydrogen peroxide (H_2O_2) by increasing concentrations of curcumin.

Incubation was done for one hour at room temperature. Reaction conditions are described in 'methods'.

(o) effect of increasing curcumin concentration.

(●) with catalase.

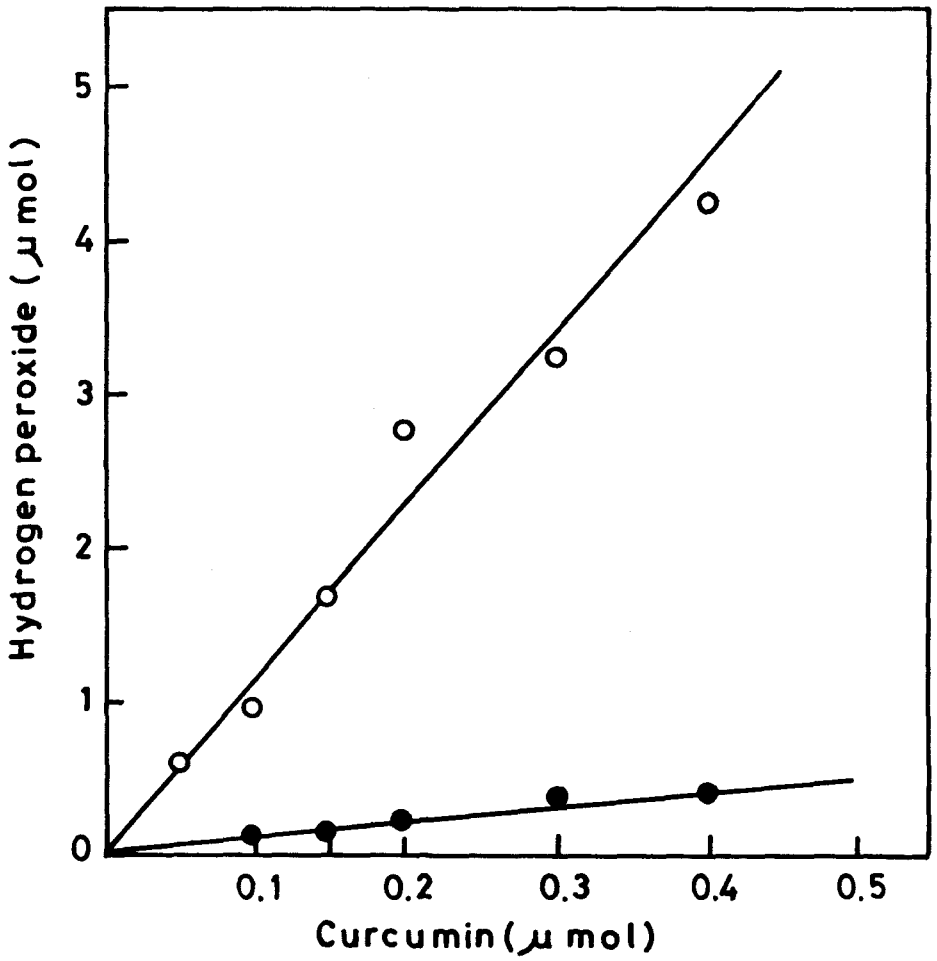

Hydrogen peroxide (μ mol)
0
1
2
3
4
5
0.1
0.2
0.3
0.4
0.5
Curcumin (μ mol)

Figure 13 : Effect of increasing concentration of DNA on the absorption spectrum of curcumin.

The concentration of curcumin and DNA in the reaction mixture was 25 μM and about 3 mM respectively, in 10 mM Tris-HCl buffer (pH 8.0).

Trace 1 (————) - curcumin alone

2 (—·——·-) - curcumin, DNA (0.125 mM)

3 (— — — —) - curcumin, DNA (0.25 mM)

4 (——···——) - curcumin, DNA (0.5 mM)

5 (·····) - curcumin, DNA (1 mM)

6 (——·····——) - curcumin, DNA (2 mM)

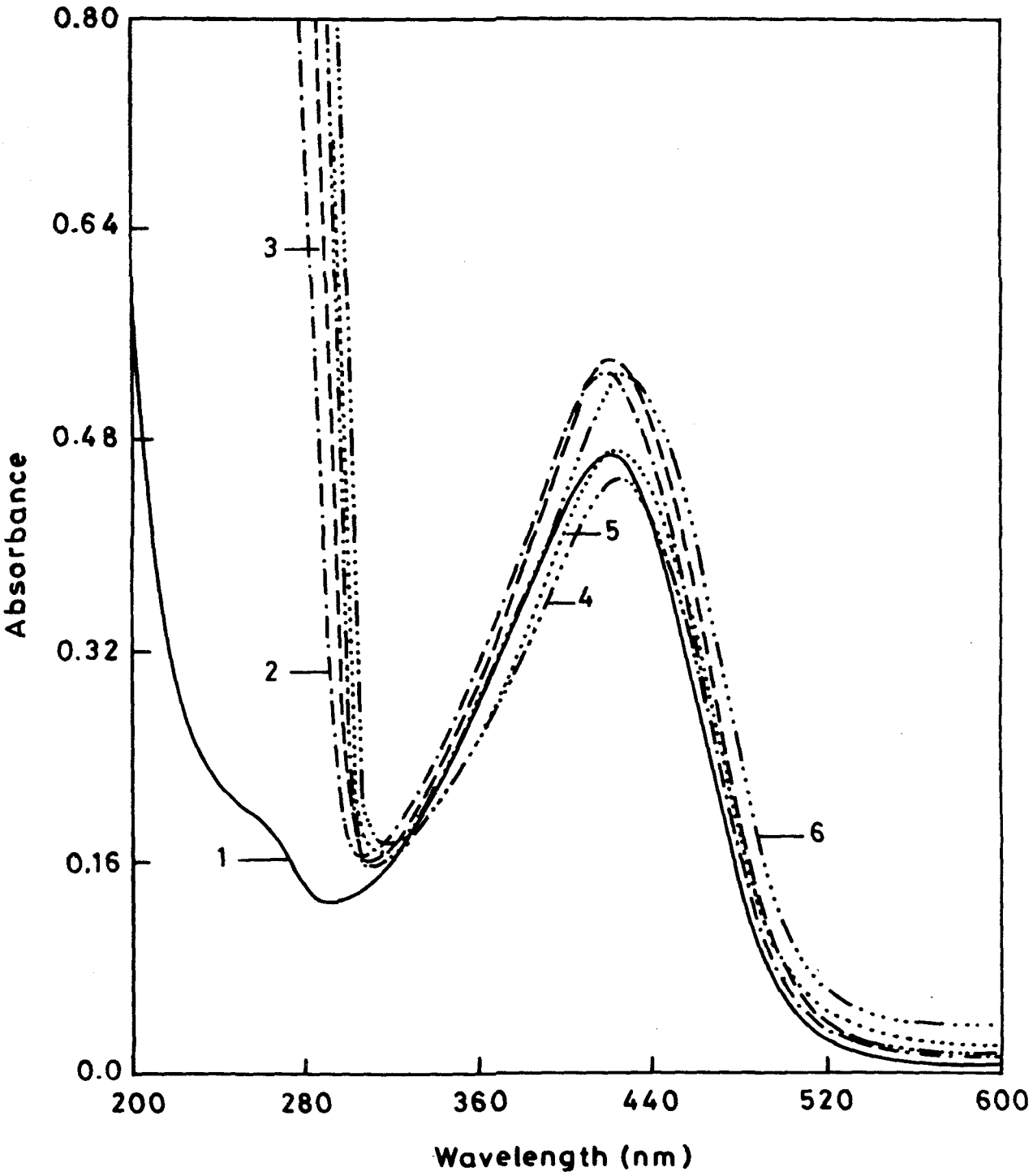

0.80
0.64
0.48
0.32
0.16
0.0
Absorbance
200
280
360
440
520
600
Wavelength (nm)
1
2
3
4
5
6

Figure 14 : Time course of absorption spectral change in curcumin induced by the addition of Cu(II).

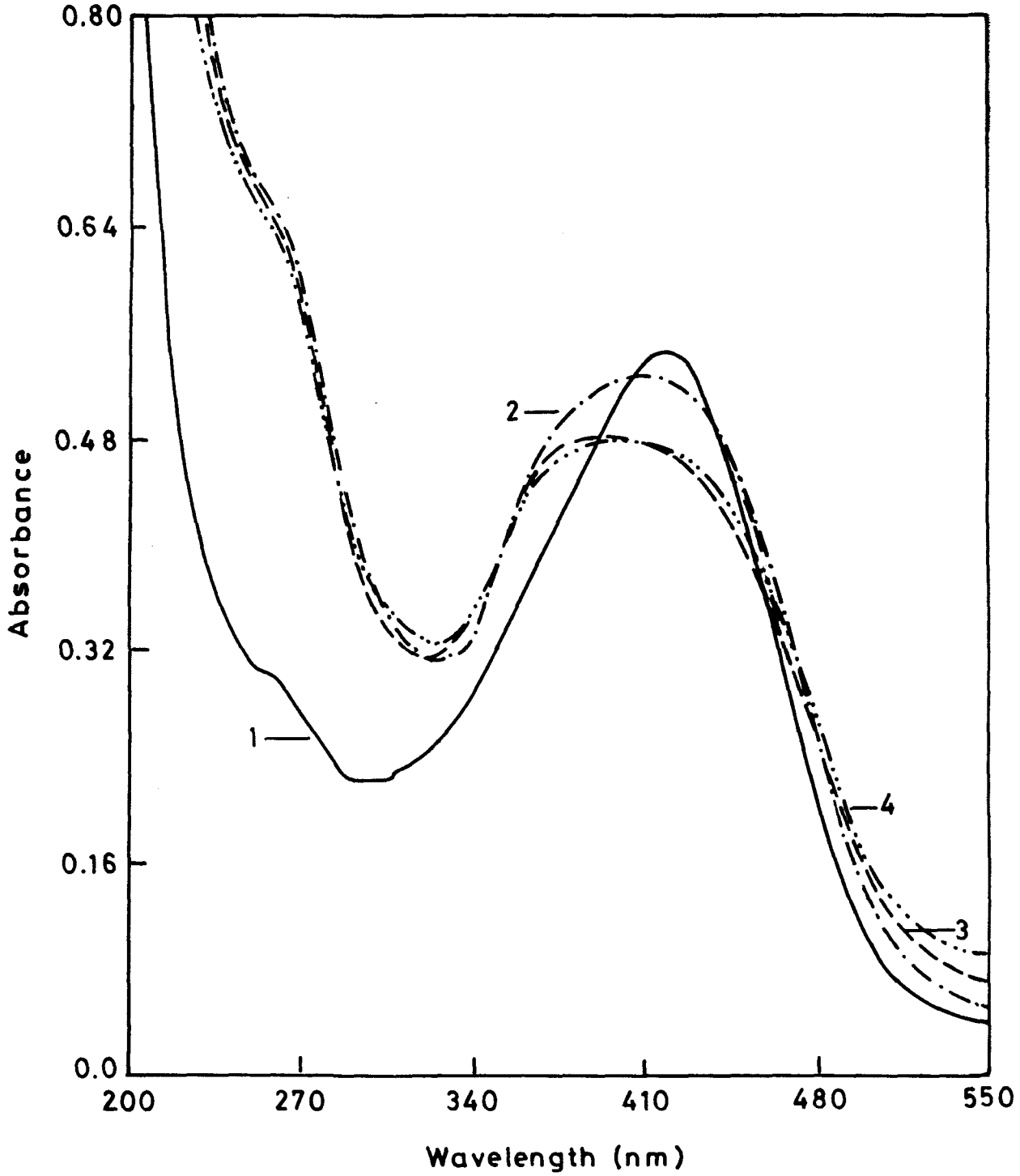
The concentrations of curcumin and Cu(II) were 25 µM and 100 µM respectively, in 10 mM Tris-HCl buffer, pH 8.0. Absorption spectra were recorded at different time periods [after addition of Cu(II)].

Trace 1 (———) - curcumin alone

2 (—·—·—) - after 1 min

3 (— — —) - after 10 min

4 (—···—) - after 30 min


0.80
0.64
0.48
0.32
0.16
0.0
Absorbance
200
270
340
410
480
550
Wavelength (nm)
1
2
3
4

440 nm. These results indicate that curcumin is capable of binding to double stranded DNA.

Curcumin-Cu(II) interaction

When curcumin and Cu(II) solutions were mixed, a shift occured in the λmax of curcumin from 420 nm to 390 nm (hypsochromic shift). The broad peak at 390 nm also decays with time (hypochromic shift) as seen in figure 14. These results are indicative of the binding of Cu(II) to curcumin. Taken together with the previous results it would appear that the formation of a ternary complex is possible between DNA, curcumin and Cu(II).

DISCUSSION

DISCUSSION

The results presented here lead to the following conclusions -

i) Curcumin in the presence of Cu(II) and molecular oxygen causes degradation of calf thymus DNA.

ii) Curcumin generates single stranded breaks in plasmid DNA in the presence of transition metal ions, mainly Cu(II) and to some extent Fe(II).

iii) Cu(II) is reduced to Cu(I), the latter being an essential intermediate in the DNA cleavage reaction.

iv) The proximal DNA cleaving agents are the active oxygen species; hydroxyl radicals, hydrogen peroxide and singlet oxygen appear to be involved.

The degradation of DNA by a number of DNA - binding drugs such as bleomycin (Ehrenfield et al., 1987), rifamycin (Quinlan and Gutteridge, 1987), adriamycin (Eliot et al., 1984) and the flavonoid quercetin (Rahman et al., 1989) is dependent upon metal ions and is considered to rely on the generation of oxygen derived free radicals (ROS). It appears that curcumin - Cu(II) induced DNA breakage is similar in

nature and does not require a reducing agent. It is proposed that a ternary complex of the drug curcumin, DNA and Cu(II) is formed, in which reduction of Cu(II) to Cu(I) occurs. The conversion of supercoiled molecule to the relaxed form was the result of a single nick in the DNA molecule. A second nick close to the first would give rise to the linear form. However, in the present case, no linear forms of plasmid DNA were observed.

There are two alternative routes for the generation of hydroxyl radical :

i) $O^{\bullet}{}_2^- + H_2O_2 \longrightarrow OH^{\bullet} + OH^- + O_2$

ii) $H_2O_2 + Cu(I) \longrightarrow OH^{\bullet} + OH^- + Cu(II)$

Reaction (i) is generally referred to as the Haber-Weiss reaction (modified) and (ii) as the Fenton reaction. It is known that the generation of superoxide anion may lead to the formation of hydrogen peroxide. Alternatively, the superoxide anion undergoes dismutation to form H_2O_2 and O_2, in aqueous solutions (Halliwell and Gutteridge, 1984) :

$$2O_2^{-\bullet} + 2H^+ \longrightarrow H_2O_2 + O_2$$

The formation of singlet oxygen may occur through the Haber-Weiss reaction or the interaction of superoxide anion with

hydroxyl radicals (Badway and Karnovsky, 1980).

$$O^{\cdot}{}_2^{-} + H_2O_2 \xrightarrow{\text{Metal}} {}^1O_2 + OH^{\cdot} + OH^{-}$$

$$O_2^{\cdot -} + OH^{\cdot} \longrightarrow {}^1O_2 + OH^{-}$$

Curcumin is capable of reducing Cu(II) to Cu(I), which being an intermediate in the DNA-degradation reaction. This drug catalysed reduction of transition metals has been implicated in DNA damage reactions by several other naturally occuring compounds (Bhat and Hadi, 1992; Rahman et al , 1989).

Ascorbic acid, flavonoids and curcumin are generally considered to be dietary antioxidants (Namiki, 1990; Perchellet and Perchellet, 1989). Curcumin, a polyphenol and a naturally occuring phytochemical, is a major chemical constituent of the ground and dried rhizome of Curcuma longa commonly called turmeric. Curcumin can stimulate nuclear DNA damage, protein degradation and lipid peroxidation in the presence of copper or iron and therefore acts as a prooxidant (Sahu and Washington, 1992). It seems that it probably undergoes autooxidation in the presence of oxygen and transition metal ions. This autooxidation generates ROS, and hence its prooxidant behaviour. Its molecular structure would allow it to react with oxygen free radicals, produced

by other compounds, providing it with radical-trapping properties.

Thus, curcumin has the potential of acting both as pro- and antioxidant, depending upon the redox state of the biological environment. In the cellular environment, these two opposing effects may be competitive; therefore, it may have a dual role in mutagenesis and carcinogenesis (Kahl, 1986).

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