MODE OF ACTION OF DNA REACTIVE MUTAGENS
(Mode of Action of Dietary Flavonoids)

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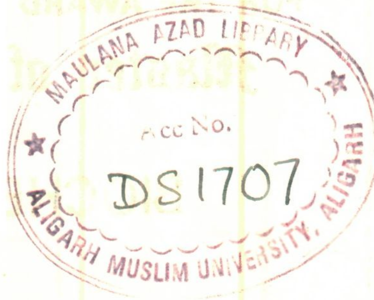
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Dedicated to my Aunt Mana
Without her Help and
Affection This Work Would
not have Seen the Dawn
of the Day

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CERTIFICATE

I certify that the work presented in this dissertation has been carried out by Mr. Mohamed Said Ahmed under my supervision. It is original in nature and has not been submitted for any other degree.

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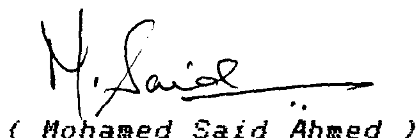

(Mohamed Said Ahmed)

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INTRODUCTION

It is generally accepted that a high proportion of human cancers is 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 occurring 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 past 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, fruits and a lower consumption of fat rich products and 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

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 ;

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., 1981a). 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 shown experimental carcinogenic activity for several species and various tissues. Wide 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 (Fenaroli, 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 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 bergapten. In addition to Umbelliferae, psoralen also occurs in plants from several other families (Ivie, 1978). Psoralen 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 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 been extensive for reasons 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* system (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 bioactivation of these alkaloids. This implies that there may be species difference in the carcinogenic potential of pyrrolizidine alkaloids.

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 commercial mushroom, Agaricus bisporus contains about 300 mg of agaritine, the -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-hydroxymethylphenyl-hydrazine (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 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).

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. (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, Stich et al, (1981b) 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.

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).

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 aflattoxins (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 is 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 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 (Stich et al., 1981b; 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 is 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 oesophageal 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 oesophageal 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, 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 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; Fink and Kritchevsky, 1981; Welsch and Aylsworth, 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 unit 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 can 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 :

Sugimura and his colleagues (1978, 1979) as well as others

(Pariza et al., 1983) have reported that the burnt and browned materials from heating protein during cooking is highly mutagenic. Pyrolysis of protein 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 amd wode;u ised 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 mutagenic pyrolysis product methylglyoxal (Sugimura and Sato, 1983). One cup of coffee also contains about 250 mg of the natural mutagen chlorogenic acid (Stich et al., 1981a) and about 100 mg 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 nitrosamines from amines, present in or derived from the diet, occurs by reaction with nitrous acid at acidic pH. In humans, gastric juice attains a pH of nearly 1.0. Such high concentration of hydrogen ions gives rise to the nitrosyl cation No^{+}, which is a highly reactive nitrosylating agentt. 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). 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*., 1977). 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 ($O_2^{\bullet -}$) 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 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 ($OH^{\cdot}$) (Wolff <u>et</u> <u>al</u>., 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 <u>et</u> <u>al</u>., 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 <u>et</u> <u>al</u>., 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 <u>et</u> <u>al</u>., 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 acid 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 DNA damage, 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 (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 (Pryor, 1976-1982). The enzymes that protect cells from oxidative damage are superoxidce 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 dimethylhydrazine 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 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 byproduct 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 an effective anticarcinogen 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 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 (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 rodents 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 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 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, in good part, to insufficient amounts of anticarcinogens and other protective factors in the diet (Maugh, 1979).

In the past 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 cancer. It is becoming increasingly clear from epidemiological 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 effect 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 22 gm. In addition, the endogenous conditions favour the formation of still more mutagens *in vivo* in humans (Oshshima and Bartsch, 1981).

Mutageniciy of flavonoids and the scope of the work presented :

Of late, there has been increasing interest in naturally occurring compounds, ingested as part of the normal diet, which are potentially mutagenic/carcinogenic (Ames, 1983). One such class of compounds is the flavonoids which occur in large amounts in a wide range of food plants including many fruits, vegetables, tea, skin of tubers and roots (Hermann, 1976). There are many flavonoids in plants and the mutagenicity of more than seventy naturally occurring flavonoids has been tested (Nagao et al., 1978). Of these, quercetin was the strongest mutagen, followed by kaempferol, rhamnetin, galangin, isorhamnetin and fisetin (Brown, 1980; Nagao et al., 1981). It has been estimated that the average daily intake of flavonoids in the American diet is about 1 gm and thus there is clearly a potential hazard (Singleton, 1981). All these compounds except querrcetin required metabolic activation by rat liver enzymes when tested in microbial systems (Nagao et al., 1978). The mutagenicity of quercetin was further enhanced by rat liver enzymes. This suggests that quercetin may interact directly with cellular DNA. Besides the microbial system, flavonoids especially quercetin and kaempferol have also been tested for mutagenicity in higher systems, such as rat, hamsters and Drosophila. However, in these systems there have been conflicting reports on the mutagenicity and carcinogenicity of quercetin (Hirono et al., 1981; Pamukcu et al., 1980;

Watson, 1982). Whereas studies of Ambrose et al. (1952) reported quercetin to be non-carcinogenic to rats fed 1% quercetin for 410 days, more recent definitive studies of Pamukcu et al. (1980) demonstrated 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 neoplastic cells (Podhajcer et al., 1980).

Quercetin in common with other flavonoids is a candidate substance for the development of antiviral agents (Vanden et al., 1986; Van Hoof et al., 1984) and is a promising compound for the inhibition of tumor invasion (Bracke et al., 1987). The mechanism by which quercetin exhibits its antitumor activity is not understood. Since it is a frame-shift mutation in *S. typhimurium* (Ames, 1972) it has been argued that it might be an intercalating agent (Bjeldanes, 1977). However, there are no chemical data to support this view. Previous studies have suggested that flavonoids function as scavengers of reactive species of oxygen such as singlet oxygen (Takahama et al., 1984), superoxide anion (Takahama et al., 1983), and H_2O_2 (Takahama, 1984). From the studies on the effect of metal ions, antioxidants and pH on the mutagenicity of quercetin in *S. typhimurium*, Hatcher and Bryan (1985) concluded that

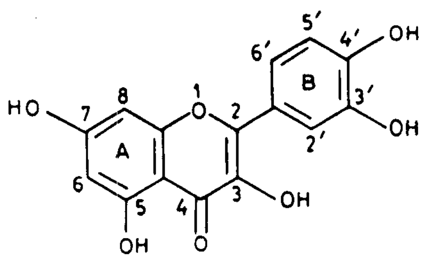
this reaction is antimutagenic.

Previous and on going studies in our laboratory on the flavonoids have shown that quercetin causes strand scission in DNA in vitro in the presence of Cu(II) and molecular oxygen (Rahman et al., 1989). In these studies calf thymus DNA, supercoiled pBR322 plasmid DNA and single stranded M13 phage DNA was shown to be degraded. In the case of plasmid, the products were relaxed circles or a mixture of these and linear molecules depending upon conditions. For the breakage reaction, Cu(II) could be replaced by Fe(III) but not by other ions such as Fe^{+2}, Co^{2}, Ni^{+2}, Mn^{+2}, and Ca^{+2}. In the case of quercetin– Cu(II) reaction, Cu(I) was shown to be an essential intermediate by using the Cu(I)– sequestering reagents, neocuproine and bathocuproine. Further, neocuproine inhibited the DNA breakage reaction. The involvement of the active oxygen species in the reaction was established by the inhibition of the DNA breakage by various scavengers of active oxygen species. Strauctorally related flavonoids rutin, galangin, apigenin, and fisetin, were ineffective or less effective than quercetin in causing DNA breakage. In a subsequent study (Rahman et al., 1990) quercetin was shown to bind to both double stranded and single stranded DNA. Quercetin and Cu(II) form a charge transfer complex that decays in an oxygen dependent reaction and this decay is accelerated by DNA. It was further shown (Fazal et al., 1990) that quercetin reduces oxygen to superoxide anion and that in the presence of

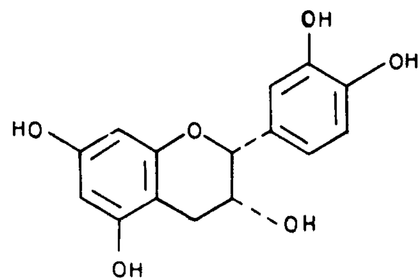
Cu(II) the hydroxyl radical is generated. Hydrogen peroxide is an intermediate and strand scission involved the hydroxyl radical. The strand scission reaction was shown to account for the biological activity for the quercetin as assayed by bacteriophage inactivation.

Salmonella mutagenicity data from several laboratories (Brown, 1980) have revealed the basic structural requirements for flavonoid mutagenicity in Salmonella typhimurium. The most important of these is the 3-OH group of the flavonol structure. The C-5 OH may also be important, although fisetin which lacks this hydroxyl is weakly mutagenic. Flavonoids lacking free hydroxyl group on the B-ring, eg. galangin, or having only 1-OH group, eg. kaempferol, show an absolute requirement for microsomal activation in order to exhibit mutagenicity for the salmonella tester strains. Quercetin, which possesses 3' and 4' hydroxyl groups of the B-ring, is directly mutagenic without metabolic activation. The mutagenicity data of various flavonoids when compared with the strand scission in vitro in the presence of Cu(II) show a striking similarity. For example fisetin (which lacks 5-OH) is less effective than quercetin in DNA breakage and galangin (which lacks 3' and 4' OH in B-ring) and apigenin (which has only one OH in B-ring at 4') catalyse DNA breakage to a negligible extent (Rahman et al., 1989). Thus, these findings suggest a direct relationship between mutagenicity and DNA degrading capability of various flavonoids. In addition to the

structural requirement mentioned above Macgregor and Jurd (1978) have further suggested that a double bond at the 2, 3, position and a structure which permits the proton of the 3-hydroxyl group to tautomerise to a 3-keto compound are essential for mutagenic activity. The present studies are designed to identify the structural requirement for the flavonoids essential for the DNA degrading activity in the presence of Cu(II), and to determine the specificity if, any, of DNA cleavage. The first of these objective will be met by using DNA molecules of a known sequence (plasmid pBR322, phage lamda) and measuring the extent of single strand breaks. The second objective will be attained by foot printing of the cleaved molecules. This M.Phil dissertation presents experiments dealing with first objective. The structures of various flavonoids used in these experiments are given in figure 1.



(a)



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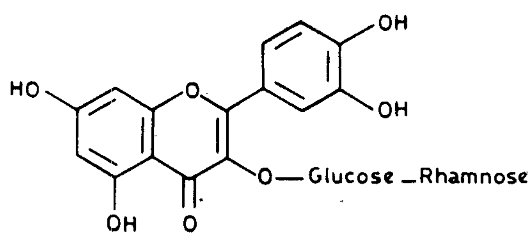
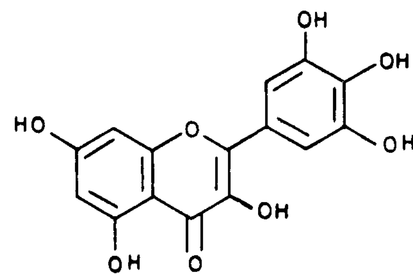
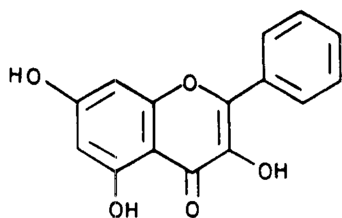


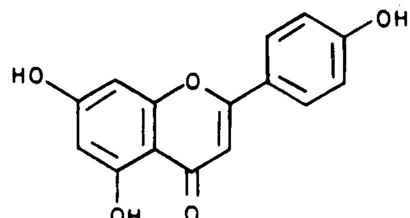
(d)



(c)



(e)



(f)

EXPERIMENTAL

MATERIALS

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

Chemical	Source
Apigenin	Aldrich Chemical Company, U.S.A.
Agarose	Koch-light Laboratory, England.
Bathocuproine disulphonate	Sigma Chemical Company, U.S.A.
Bovine Serum Albumin	Sigma Chemical Company, U.S.A.
Catalase	Sigma Chemical Company, U.S.A.
Deoxyribonucleic Acid (Calf thymus type I)	Sigma Chemical Company, U.S.A.
Dimethyl sulphoxide (DMSO)	Merck, India.
Diphenylamine	B.D.H., India.
Ethylenediamine tetra acetic acid (EDTA)	B.D.H., India.
Epicatechin	Sigma Chemical Company, U.S.A.
Galangin	Aldrich Chemical Company, U.S.A.
Myricetin	Sigma Chemical, Company, U.S.A.
Nitroblue tetrazolium (NBT)	Sisco Research Labs., Bombay.
Neocuproine hydrochloride	Sigma Chemical Company, U.S.A.
Quercetin	Sigma Chemical Company, U.S.A.

Supercoiled plasmid pBR-322 DNA	Prepared in this laboratory according to the procedure of Maniates *et al.* (1982).
Superoxide dismutase (SOD) (Bovine erythrocyte)	Sigma Chemical Company, U.S.A.
S_1 Nuclease	Sigma Chemical Company, U.S.A.
Tris (hydroxymethyl)-amino methane	Fluka, Switzerland.
Titanium sulphate	Department of Chemistry, A.M.U., Aligarh.
TritonX-100	B.D.H., India.

All other chemicals were commercial product of reagent grade.

METHODS

Stoichiometric titration of Cu(I) production :

The concentration of Cu(I) produced in the flavonoid– Cu(II) reaction mixture was determined by titrating with bathocuproine as follows : flavonoid (10 or 20 µM) in 10 mM Tris–HCl, pH 7.4 was mixed with varying concentration of Cu(II) and 120µl of 10mM stock bathocuproine aqueous solution was added to attain a final bathocuproine concentration of 400µM, in a total volume of 3.0 ml. Absorbance at 480 nm was recorded.

Reaction of flavonoids and Cu(II) with pBR322 DNA :

The reaction mixture (20 µl) contained 10 mM Tris–HCl, pH 7.5, 0.5µg pBR322 DNA and 0.10mM flavonoids in presence of 0.2 mM Cu(II). The mixtures were incubated at 37°C for 30 minutes, after which 8–10 µl of the solution containing 50% glycerol (V/V), 40µM EDTA and 0.05% bromophenol blue (V/V) were added. The samples were electrophoresed on 1.4% agarose gels. Which contained 0.5µg/ml ethidium bromide.

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 10mM Tris–HCl, pH 7.4 and 400µg calf thymus DNA (native,denatured or flavonoid treated DNA). The reactions were started by addition of Cu(II)(0.1 mM). In some experiments free radical scavengers were also added to the reaction mixtures.

To perform studies under anaerobic conditions nitrogen was bubled through the assay mixture containing the complete system [flavonoids (quercetin; myricetin; epicatechin; rutin; galangin; apigenin) + Cu(II) + H_2O_2 + DNA] for 2 minutes. All solutions were autoclaved before use. S_1 nuclease digestion of the mixture was carried out in a total volume of 1.0 ml by adding acetate buffer 0.1M pH 4.5, 1mM $ZnSO_4$ and 20–30 units of S_1 nuclease. The reaction mixtures were incubated for 2 hours at 48°C and the reactions stopped by addition of 0.2 ml of 10mg/ml bovine serum albumin and 1.0 ml of 14% percholoric acid. The acid soluble material was determined by the diphenylamine method (Schneider, 1957).

Detection of superoxide anion (O_2^-) :

Superoxide anion was detected by the reduction of nitroblue tetrazolium (NBT) essentially as described by Nakayama et al., (1983). A typical assay contained in a total volume of 3.0ml, 50mM potasium phosphate buffer pH 7.8, 33μM NBT, 0.1mM EDTA and 0.06% tritonX–100. The reaction was started by the addition of 0.04mM flavonoids (quercetin; myricetin; epicatechin; rutin; galangin; apigenin). Immediately after mixing the absorbance at 560nm was measured under various experimental conditions against a blank which did not contain flavonoids. To confirm the formation of O_2^-, superoxide dismutase (SOD) was introduced into the reaction mixture before adding flavonoids.

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

The assay is based on the ability of $OH^{\cdot}$ radical to hydorxylate (attack) aromatic rings 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 (2.0 ml) contained the following reagents at indicated concentrations: 2.0mM salicylate, 0.1mM EDTA, 0.1mM Cu(II) and 15.0mM KH_2PO_4-KOH buffer pH 8.0. The reactions were initiated by adding 0.06mM flavonoids (quercetin; myricetin; epicatechin; rutin; galangin; apigenin) and tubes were incubated at room temperature for 2 hours. Reactions were stopped by adding 80 µl of 11.6 N HCl and 0.5gms NaCl, followed by 4.0 ml of chilled diethylether. The contents were mixed by vortexing for 1 minute. Next, 3.0 ml of upper ether layer was pipetted off and evaporated to dryness in a boiling tube at 40°C. The tubes were cooled and the residue dissolved in 0.25 ml of cold distilled water to which, the following reagents were added in order stated: (a) 0.125 ml of 10% w/v TCA disolved in 0.5 N HCl, (b) 0.25 ml of 10% w/v sodium tungstate in water, (c) 0.25 ml of 10% w/v $NaNO_2$ (freshly prepared). After standing for 5 minutes, 0.5 ml of 0.5M KOH was added and absorbance at 510 nm was read exactly after 1 minute.

Estimation of H_2O_2 :

H_2O_2 was determined quantitatively according to the method of Hozumi (1969). A sample containing approximately 0.2 µmol

of flavonoid in 0.5 ml dimethyl sulfoxide, 1.5 distilled water and 2 ml of 50 mM sodium phosphate buffer pH 7.2 were mixed vigorously for 1 minute at room temperature. The reaction mixture was centrifuged at 9000 rpm for 10 minutes. One half milliliter of the supernant solution was added rapidly either to 2 ml of 1% titanium sulphate solution in 2.5N H_2SO_4 (as a sample) or to 2 ml of 2.5N H_2SO_4 (as a blank) and the absorbance were determined at 410 nm. The amount of H_2O_2 was calculated from a calibration curve of H_2O_2. In order to identify the H_2O_2, before addition of the titanium sulfate solution, 0.4 ml of catalase solution (1 mg/ml) or 0.4 ml of water (as a control) was added to 1.6 ml of the supernantant followed by incubation at 37°C for 10 minutes.

Spectrophotometric study on the oxidation of flavonoids by Cu(II) :

Cu(II) mediated oxidation of various flavonoids was followed spectrophotometricaly by recording the spectra between 280–500nm. The reaction mixture (3.0 ml) contained 50µM flavonoids, 150µM Cu(II) and 10mM Tris–HCl pH 8. The reactions were started by adding Cu(II) and absorption spectra were recorded at different time intervals.

RESULTS

RESULTS

The primary objective of the experiments described below is to determine the chemical features of flavonoids required for the DNA strand scission reaction as discussed in the "Introduction". A number of flavonoids have been chosen which differ in their chemical structure but are able to catalyse DNA cleavage to various degrees. In this dissertation, I have presented experiments which describe the rate of DNA degradation, the rate of generation of active oxygen species and the rate of spectral changes associated with the binding of Cu(II).

Rate of DNA degradation by various flavonoids and Cu(II) : Table I gives the rate of S_1 nuclease hydrolysis of calf thymus DNA following damage induced by various flavonoids and Cu(II). The percent DNA hydrolysed by S_1 nuclease is dependent on the extent of single strand breaks formed by the flavonoid and Cu(II). It may be seen that myricetin causes the largest degree of hydrolysis followed by quercetin and epicatechin. The lowest degree of hydrolysis is achieved by galangin. In order to further substantiate these result, conversion of supercoiled plasmid DNA to the relaxed circular form was used as an assay. This is a sensitive test for just one nick per genome. First, conversion of supercoiled plasmid pBR322 DNA to relaxed open circles by quercetin and Cu(II) was studied in a time dependent reaction (Fig. 2). As seen, complete conversion from supercoiled to the relaxed form occurs in 30 minutes.

Table-I :

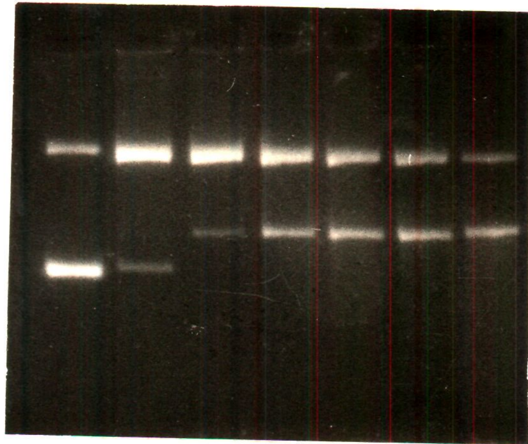
Comparison of S_1 nuclease hydrolysis following damage to DNA induced by flavonoids and Cu(II).

DNA nucleotide/ compound	% DNA hydrolysed
	DNA + flavonoid + Cu(II)
Denatured DNA	100
Native DNA	2.8
Quercetin	49.5
Myricetin	55.0
Epicatechin	41.3
Rutin	35.8
Galangin	24.8
Apigenin	35.8

Concentrations of flavonoids and Cu(II) were 0.1 mM each. Percent hydrolysis refers to the loss of precipitable DNA following S_1 nuclease treatment. Details of S_1 nuclease assay are given in the "Methods".

Figure 2. **Kinetic behaviour of DNA breakage induced by quercetin and Cu(II).**

Reaction mixtures containing 0.5µg pBR_{322} DNA, 0.1 mM quercetin and 0.2 mM Cu(II) were incubated at 37°C for the time periods indicated. Lanes 1–7: 0, 0.25, 0.5, 1.0, 1.25, 2.0, and 2.5 hours.


1 2 3 4 5 6 7

This period of incubation was subsequently used for cleavage by the other flavonoids (Fig. 3b and 3c). With 100μM flavonoids, complete conversion to open circular form of plasmid was seen with myricetin and epicatechin in addition to quercetin. With a lower concentration of 20μM flavonoids, complete conversion is seen only in the case of myricetin. Thus, these results agree with those of Table I where myricetin also showed maximum rate of DNA degradation.

Table II compares the effect of oxygen radical quenchers on flavonoid– Cu(II) mediated DNA degradation. Five quenchers were tested, viz; SOD ($O_2^{\cdot -}$), catalase (H_2O_2), sodium azide ($^1O_2$), Mannitol and thiourea ($OH^{\cdot}$ radical). As seen, all the quenchers used are effective in inhibiting the DNA degradation to various degrees with, thiourea being the most effective in all cases. Sodium azide is least inhibitory except in the case of epicatechin suggesting that singlet oxygen also contributes the DNA breakage activity although to a lesser extent than the hydroxyl radical.

Reduction of Cu(II) to Cu(I) by various flavonoids :

Bathocuproine and neocuproine are selective Cu(I) sequestering agents. These compounds have been earlier employed to quantitatively detect the reduction of Cu(II) to Cu(I) by quercetin (Rahman et al., 1989). The Cu(I) chelates have absorption maxima at 480 and 450 nm respectively (Jaselow and Dawson, 1951; Nabesar, 1964). Under the condition of our reactions, neither Cu(II) nor the flavonoids interfere with

Figure 3. **Comparison of the rate of DNA degradation by various flavonoids and Cu(II) at 0.1 mM (a) and 0.02mM (b), flavonoid concentration.**

Reaction mixtures containing 0.5µg pBR_{322} DNA, flavonoids, 0.2 mM Cu(II) (a) and 0.04 mM Cu(II) (b), were incubated at 37°C and terminated after 30 minutes. Other reaction conditions are given in the "Methods".

Lane 1: DNA alone

Lane 2: DNA + quercetin + Cu(II)

Lane 3: DNA + myricetin + Cu(II)

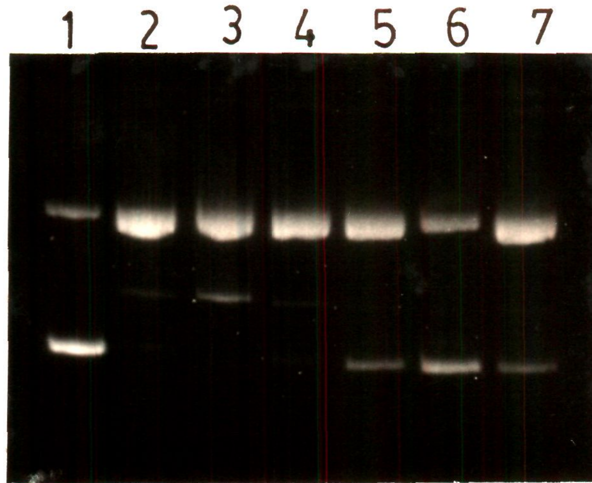
Lane 4: DNA + epicatechin + Cu(II)

Lane 5: DNA + rutin + Cu(II)

Lane 6: DNA + galangin + Cu(II)

Lane 7: DNA + apigenin + Cu(II)

(a)



(b)

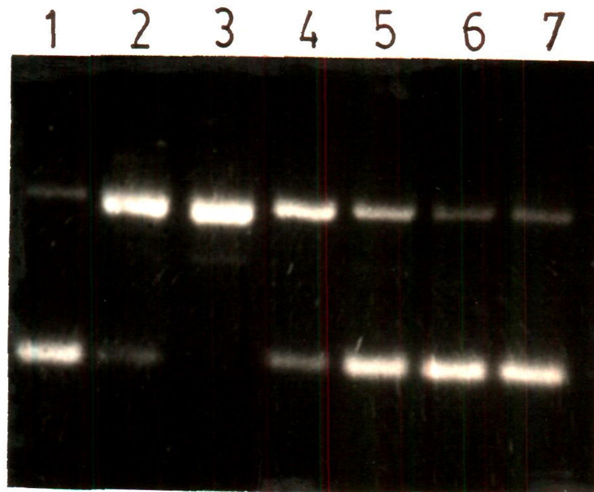


Table-II

Effect of quenchers of oxygen free radicals on the S^1 nuclease hydrolysis following damage to DNA induced by flavonoids and Cu(II).

Quenchers/ Flavonoids	Inhibition (%)				
	SOD (20µg/ml)	CATALASE (100µg/ml)	SODIUM AZIDE (10 mM)	MANNITOL (10 mM)	THIOUREA (5 mM)
Quercetin	56.8	56.8	28.4	51.2	85.2
Myricetin	40.3	72.3	0	40.3	72.3
Epicatechin	42.8	61.4	56.0	38.0	82.6
Rutin	43.0	33.9	19.8	43.0	82.6
Galangin	55.5	47.3	22.2	59.7	75.5
Apigenin	41.1	45.3	37.7	41.1	86.9

Concentrations of flavonoids and Cu(II) were 0.1mM each. Percent hydrolysis refers to the loss of precipitable DNA following S^1 nuclease treatment. Concentrations shown here are. The final reaction concentrations. Details of S^1 nuclease assay are given in the "Methods".

these maxima. However, flavonoids plus Cu(II) generate Cu(I). In Fig. 4 and 5, the generation of Cu(I) from Cu(II) by epicatechin is illustrated. Similar profiles are obtained by quercetin, myricetin, rutin, galangin, and apigenin. As the absorbance of bathocuproine—Cu(I) is not marked by the yellow flavonoid chromophore (Seen in Fig. 4), it is possible to calculate the concentration of Cu(I)—bathocuproine complex in such solution in the presence of the flavonoids from the absorbance at 480 nm and the molar extinction coefficient of the complex (13500) at this wavelength. A Job plot (Wong, et al., 1984) of absorbance versus Cu(II)/quercetin reveals maximum absorbance at the Cu(II)/quercetin molar ratio at which maximum conversion of Cu(II) to Cu(I) is achieved (Fig. 6a, b, c, d, e and f). Such ratios were determined to be 5, 4, 6, 5, 3 and 4 for quercetin, epicatechin, myricetin, rutin, galangin, and apigenin, respectively. Two different concentrations of flavonoids, namely 10μM and 20μM were used and the same ratio was obtained at both the concentrations. Thus, the ratio obtained with each flavonoid gives the stoichiometry for the reduction of Cu(II) by the free flavonoid. From the absorption data (A_{480}) shown in Fig. 6 for the different flavonoids, the amounts of Cu(I) produced were calculated by the formula: concentration = $A_{480(x)} - A_{480(0)}/13500$, where $A_{480(x)}$ and $A_{480(0)}$ are the absorbances at 480 nm of the samples with and without Cu(II) and 13500 is the molar absorptivity of the chromophore. The results are given in Table IIIa, b, c, d, e, and f. The data suggests that all

Figure 4. **Detection of epicatechin induced Cu(I) production by bathocuproine.**

The concentration of bathocuproine used was 50µM.

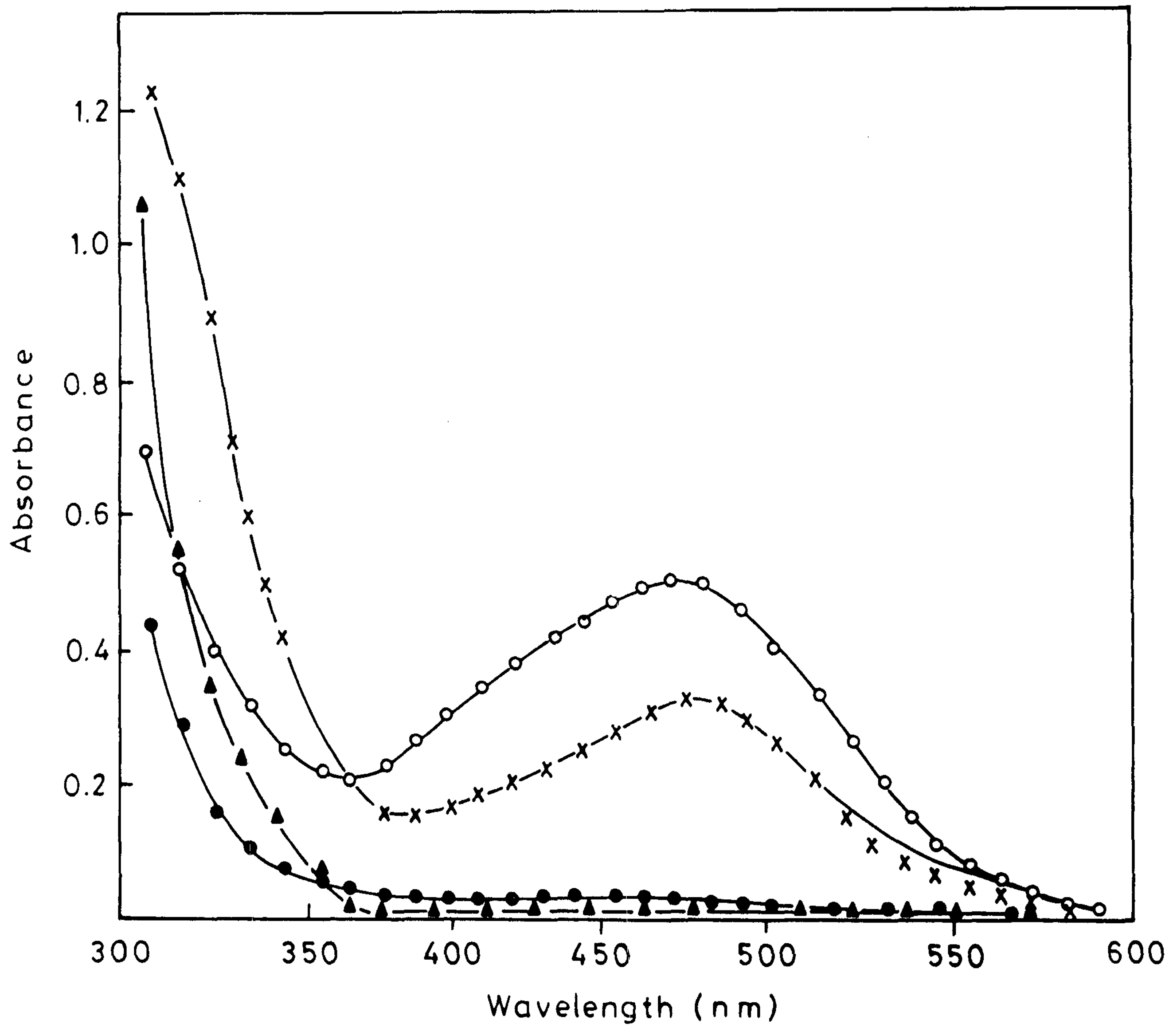
(▲) Bathocuproine + Cu(II) [100 µM]

(✕) Bathocuproine + Cu(I) [20µM]

(○) Bathocuproine + epicatechin (20µM) + Cu(II) [40 µM]

(●) Bathocuproine + epicatechin (20 µM)

In all cases the reaction was started by the addition of bathocuproine.



1.2
1.0
0.8
0.6
0.4
0.2
Absorbance
300
350
400
450
500
550
600
Wavelength (nm)

Figure 5. **Detection of epicatechin induced Cu(I) production by neocuproine.**

The concentration of neocuproine used was 50 µM.

(△) Neocuproine + Cu(II) [100 µM]

(○) Neocuproine + Cu(I) [20 µM)

(×) Neocuproine + epicatechin [20 µM]

(●) Neocuproine + epicatechin [20 µM] + Cu(II) [40 µM]

In all cases the reaction was started by the addition of neocuproine.

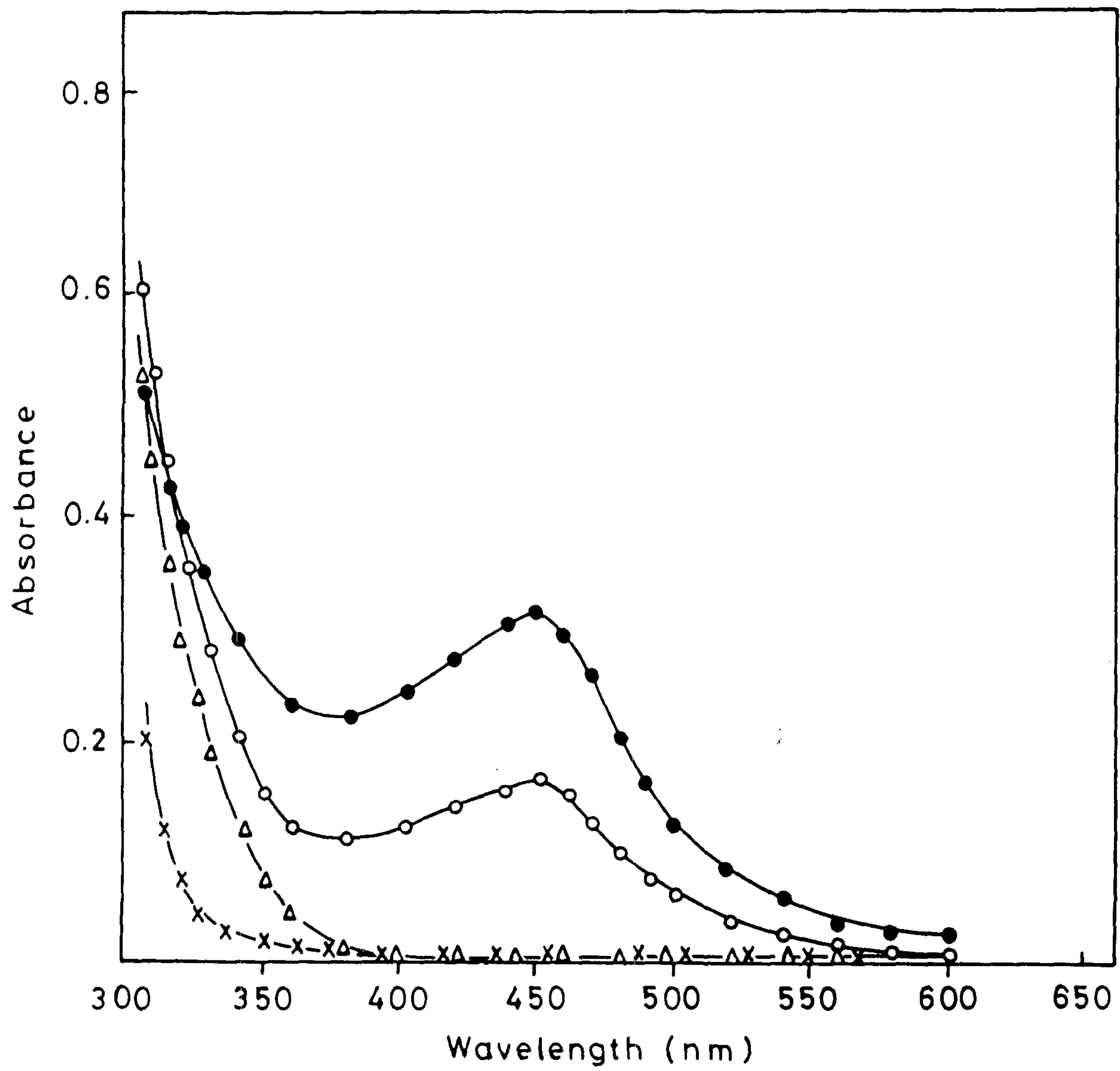
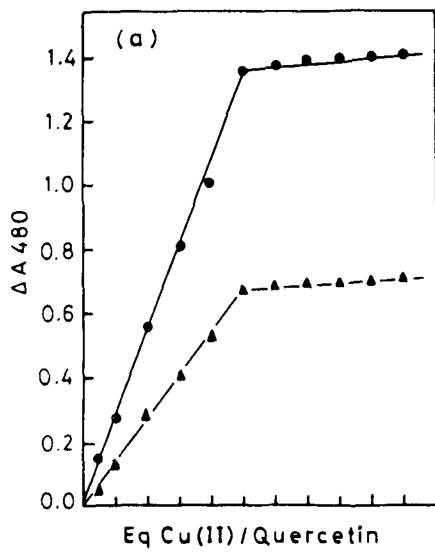
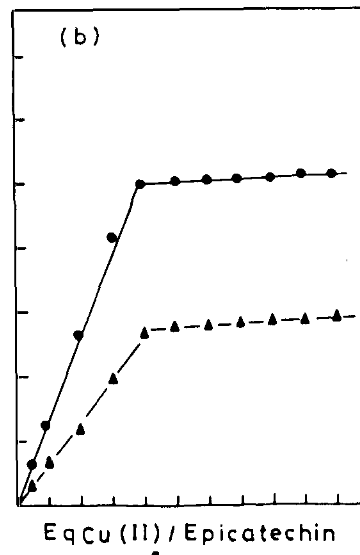
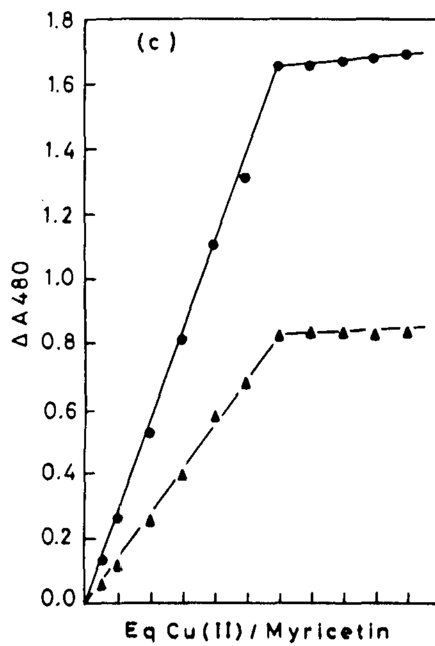

0.8
0.6
0.4
0.2
Absorbance
300
350
400
450
500
550
600
650
Wavelength (nm)

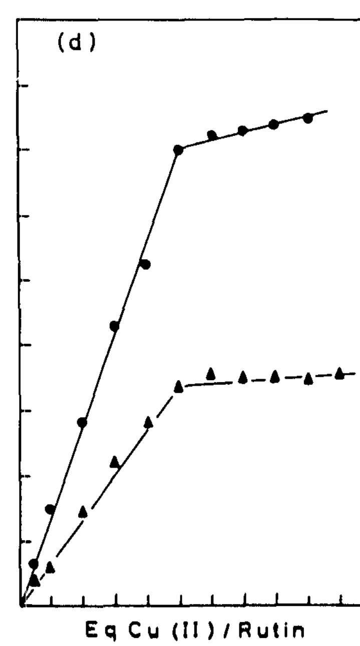
Figure 6. **Stoichiometry of flavonoid–Cu(II) interaction**

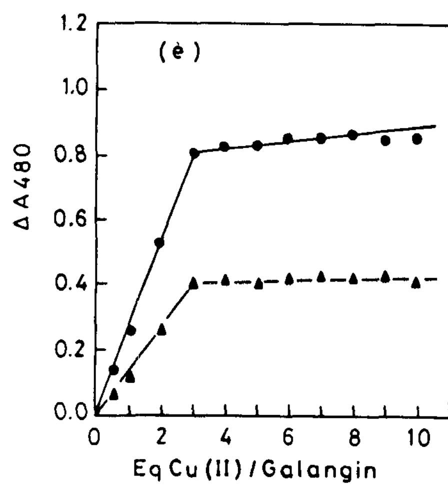
The concentration of flavonoids used were 10 µM (▲) and 20 µM (●). The difference in absorbance at 480 nm of samples with and without added Cu(II) is plotted versus molar ratio of Cu(II) and various flavonoids. The value of the independent variable at the intersection of the two lines is a measure of the moles of Cu(II) converted to Cu(I) per mole of flavonoids. The values obtained for various flavonoids are: (a) Quercetin, 1:5; (b) Epicatechin, 1:4; (c) Myricetin, 1:6; (d) Rutin, 1:5; (e) Galangin, 1:3; (f) Apigenin, 1:4.


(a)
ΔA 480
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Eq Cu(II) / Quercetin


(b)
Eq Cu (II) / Epicatechin


(c)
ΔA 480
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Eq Cu (II) / Myricetin


(d)
Eq Cu (II) / Rutin


(e)
ΔA 480
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
10
Eq Cu (II) / Galangin

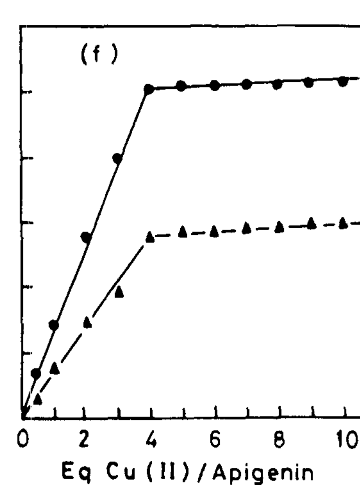

(f)
0
2
4
6
8
10
Eq Cu (II) / Apigenin

Table-IIIa :

Production of Cu(I) from quercetin-Cu(II) interaction as calculated from spectrophotometric data[a].

Cu(II) added (µM)	Cu(I) Produced (µM)	
	10 µM quercetin	20 µM quercetin
0	0	0
5	4.5	NT[b]
10	10.6	10.6
20	21.9	20.8
30	31.3	NT
40	39.6	40.8
50	50.3	NT
60	51.0	59.9
70	51.8	NT
80	51.9	78.5
90	52.5	NT
100	53.4	101.7
120	NT	102.4
140	NT	103.2
160	NT	103.9
180	NT	103.2
200	NT	104.9

Assays were carried out as described under "Methods".

[a]Concentrations of Cu(I) were calculated by using the equation : $A = \epsilon Cl$ in which $\epsilon = 13500$, $l = 1$ cm.

[b]NT = Not Tested.

Table-IIIb :

Production of Cu(I) from epicatechin–Cu(II) interaction as calculated from spectrophotometric data.

Cu(II) added (µM)	Cu(I) Produced (µM)	
	10 µM epicatechin	20 µM epicatechin
0	0	0
5	5.2	NT
10	10.3	9.7
20	18.5	18.4
30	29.6	NT
40	39.3	39.3
50	40.6	NT
60	41.4	61.7
70	42.2	NT
80	42.3	79.3
90	42.4	NT
100	42.7	81.6
120	NT	83.0
140	NT	82.3
160	NT	83.8
180	NT	84.5
200	NT	85.3

Table-IIIc :

Production of Cu(I) from myricetin-Cu(II) interaction as calculated from spectrophotometric data.

Cu(II) added (µM)	Cu(I) Produced (µM)	
	10 µM myricetin	20 µM myricetin
0	0	0
5	4.6	NT
10	9.4	9.6
20	18.9	19.3
30	29.3	NT
40	42.2	38.7
50	49.6	NT
60	60.8	59.6
70	61.2	NT
80	60.44	83.3
90	60.8	NT
100	60.7	98.7
120	NT	122.5
140	NT	120.3
160	NT	124
180	NT	125.5
200	NT	126.2

Table-IIId :

Production of Cu(I) from rutin–Cu(II) interaction as calculated from spectrphotometric data.

Cu(II) added (µM)	Cu(I) Produced (µM)	
	10 µM rutin	20 µM rutin
0	0	0
5	5.3	NT
10	9.11	9.85
20	19.3	22.0
30	33.7	NT
40	42.8	42.4
50	49.3	NT
60	53.85	63.3
70	52.4	NT
80	50.4	77.6
90	51.2	NT
100	53.6	103.3
120	NT	109.1
140	NT	109.8
160	NT	111.9
180	NT	111.1
200	NT	112.6

Table-IIIe :

Production of Cu(I) from galangin-Cu(II) interaction as calculated from spectrphotometric data.

Cu(II) added (µM)	Cu(I) Produced (µM)	
	10 µM galangin	20 µM galangin
0	0	0
5	4.8	NT
10	9.3	9.9
20	19.3	19.5
30	29.6	NT
40	31.9	39.5
50	29.6	NT
60	32.0	59.4
70	32.9	NT
80	31.4	61.3
90	32.9	NT
100	29.6	61.5
120	NT	64.7
140	NT	62.8
160	NT	63.6
180	NT	61.8
200	NT	61.9

Table–IIIf :

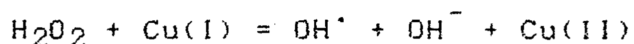
Production of Cu(I) from apigenin–Cu(II) interaction as calculated from spectrophotometric data.

Cu(II) added (µM)	Cu(I) Produced (µM)	
	10 µM apigenin	20 µM apigenin
0	0	0
5	5.2	NT
10	11.9	9.6
20	21.4	21.5
30	29.3	NT
40	41.8	40.8
50	41.9	NT
60	42.0	59.4
70	42.3	NT
80	42.4	78.7
90	42.6	NT
100	42.7	79.0
120	NT	79.4
140	NT	79.85
160	NT	79.4
180	NT	80.1
200	NT	82.4

the flavonoids on addition of 1 equivalent of Cu(II) produced 1 equivalent of Cu(I) at concentrations below the equilibrium levels.

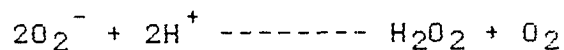
Generation of active oxygen species by flavonoids :

Recent work in this laboratory has shown that quercetin-Cu(II) mediated strand scission of DNA in the presence of molecular oxygen involves, the generation of active oxygen species such as superoxide anion ($O_2^{\bullet -}$) and hydroxyl radical ($OH^{\bullet}$) (Rahman _et al_., 1989). Quercetin was shown to reduce oxygen to superoxide. In the presence of Cu(II), the hydroxyl radical was formed. The strand scission of DNA was shown to occur under conditions in which Cu(II), quercetin and either hydrogen peroxide or oxygen were present and superoxide was not necessary intermediate. Strand scission involved the hydroxyl radical and a radical DNA intermediate (Fazal _et al_., 1990). These results indicated that hydrogen peroxide is an essential intermediate in the reaction. It was further concluded that the hydroxyl radical is produced by the Fenton reaction -



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

The formation of H_2O_2 by O_2 can occur by the following mechanism. Addition of a second electron to $O_2^{\bullet -}$ gives the peroxide ion (O_2^{2-}) which has no unpaired electron and is not a radical. However, peroxide ion O_2^{2-} at pH around neutrality immediately protonates to give hydrogen peroxide (H_2O_2). In aqueous solution O_2^{2-} also undergoes dismutation to form H_2O_2 and O_2 (Halliwell and Gutteridge, 1984).



$$2O_2^- + 2H^+ \text{--------} H_2O_2 + O_2$$

The capacity of various flavonoids to generate superoxide anion ($O_2^{\cdot -}$) was compared and the results are given in Table IV. The formation of superoxide anion was determined by reduction of nitroblue tetrazolium and the values are expressed as n moles of formazan formed as a function of incubation period in visible light and dark. As seen, the reaction is enhanced by light and is completely inhibited by superoxide dismutase indicating that the method genuinely assays the superoxide anion. The result shows that myricetin is the most efficient producer of superoxide anion followed by quercetin and epicatechin. The data are also in agreement with the DNA cleavage rates where myricetin was the most efficient DNA cleaving flavonoid.

The capacity of various flavonoids to generate hydroxyl radical in the presence of Cu(II) was also compared (Table V). This assay uses salicylate as the reporter molecule (Richmond _et al_., 1981). That this assay genuinely measures hydroxyl radical was previously demonstrated by inhibition with hydroxyl radical scavenging agents (Fazal _et al_., 1990). The rate of hydroxyl radical formation by different flavonoids is similar to the superoxide anion generation given in Table IV.

As described above, the pathway for the generation of $OH^{\cdot}$ hydroxyl radical involves H_2O_2 as intermediate which in turn is given rise to by the superoxide anion. Therefore,

Table-IV :

Rate of production of superoxide anion from solution of flavonoids as assayed by reduction of nitroblue tetrazolium to a formazan.

Formazan (n mole)

Time/ min	Light						Dark					
	Quercetin	Myricetin	Epicatechin	Rutin	Galangin	Apigenin	Quercetin	Myricetin	Epicatechin	Rutin	Galangin	Apigenin
5	4.8	10.8	2.0	4.8	2.8	2.0	2.2	12.4	2.2	4.8	1.0	1.8
10	9.8	19.4	6.6	6.8	3.8	3.8	6.6	16.6	6.6	5.8	1.0	1.8
20	17.2	27.4	16.8	10.8	5.8	7.8	7.8	19.4	7.8	5.8	1.0	3.8
30	26.6	37.8	21.2	14.0	9.8	9.8	10.2	22.4	8.0	5.8	1.0	3.8
40	33.0	49.6	28.4	16.0	11.8	14.0	11.4	22.4	8.0	5.8	1.0	3.8
60	55.8	74.6	44.8	18.4	15.0	16.0	11.4	22.4	8.0	5.8	1.0	3.8
Light + SOD	0	0	0	0	0	0	0	0	0	0	0	0

The final concentrations of flavonoids were 40 µM. The incubations were performed under illumination of ≈ 500 lux from a fluorescent strip light. The reading were converted to n mol by using the extinction coefficient of the formazan (15000) at its absorption maximum of 560 nm (Auclair and Voisin, 1985).

Table V :

Formation of hydroxyl radical as assayed by the hydroxylation of salicylate by structurally related flavonoids.

Flavonoid	Hydroxylated product formed (n moles)
Quercetin	153.8
Myricetin	158.5
Epicatechin	126.2
Rutin	98.5
Galangin	61.5
Apigenin	70.7

Concentrations of flavonoids in the reaction mixtures were 60 µM. Data are the means of triplicate samples. Details of other reaction conditions are given in the "Methods".

it was of interest to compare the rate of production of H_2O_2 by different flavonoids. The method involves the oxidation of titanium to pertitanic acid by hydrogen peroxide (Hozumi, 1969). The results given in Table VI again demonstrate that myricetin, followed by quercetin and epicatechin are the most efficient producers of hydrogen peroxide. In the presence of catalase, H_2O_2 is not produced confirming that the procedure employed genuinely measures H_2O_2.

Rate of DNA degradation by flavonoids in the presence of H_2O_2 under anaerobic conditions :

As mentioned earlier, quercetin—Cu(II) mediated DNA cleavage does not occur in the absence of oxygen. However, oxygen can be replaced by hydrogen peroxide. The anaerobic incubation with hydrogen peroxide is inhibited by catalase but not with superoxide dismutase (Fazal et al., 1990). These results were interpreted to suggest that H_2O_2 is an essential intermediate which is reduced by Cu(I) (Fenton reaction) to generate the hydroxyl radical. In order to determine whether the same pathway operates in DNA degradation by other flavonoids, the experiments shown in Table VII was done, the table shows the rates of S_1 nuclease hydrolysis of DNA following incubation with different flavonoids in the presence of Cu(II) and H_2O_2 under anaerobic conditions. In agreement with previous results, most efficient DNA cleavage was seen with myricetin, quercetin and epicatechin, and galangin was the least effective.

Table-VI :

Generation of H_2O_2 by structurally related flavonoids.

Compound[a]	μ mole H_2O_2 Produced	
	without catalase	with catalase
Quercetin	2.25	0
Myricetin	2.75	0
Epicatechin	2.00	0
Rutin	1.65	0
Galangin	1.20	0
Apigenin	1.50	0

[a]Concentration of flavonoids were 0.2μmol.

Data are the means of triplicate samples.

Table-VII :

Comparison of S_1 nuclease hydrolysis following damage to DNA induced by flavonoids and Cu(II) under anaerobic conditions.

Incubation conditions	% DNA hydrolysed
Native (aerobic)	1.6
Myricetin + Cu(II) + DNA (aerobic)	28.3
Myricetin + Cu(II) + DNA (anaerobic)	7.9
DNA + H_2O_2 (anaerobic)	12.6
DNA + H_2O_2 + Cu(II) (anaerobic)	14.0
Quercetin + H_2O_2 + Cu(II) + DNA (anaerobic)	70.7
Myricetin + H_2O_2 + Cu(II) + DNA (anaerobic)	72.3
Epicatechin + H_2O_2 + Cu(II) + DNA (anaerobic)	70.7
Rutin + H_2O_2 + Cu(II) + DNA (anaerobic)	64.4
Galangin + H_2O_2 + Cu(II) + DNA (anaerobic)	55.0
Apigenin + H_2O 2 + Cu(II) + DNA (anaerobic)	61.3

Percent Hydrolysis refers to the loss of precipitable DNA following S^1 nuclease treatment.

Final concentrations of flavonoids, Cu(II) and H^2O^2 were 0.1 mM. 0.1mM and 5mM respectively.

Details of other reaction conditions are given in the "Methods".

Changes in the absorption of flavonoids upon reaction with Cu(II) :

The flavonoids used in this study, excluding epicatechin, exhibit a visible absorption spectrum below 500 nm with a major peak around 380 nm. In the case of quercetin, the addition of Cu(II) results in a instantanious disappearance of a major band and the appearance of broad peak around 450 nm, which is characteristic of the formation of a charge transfer complex. The absorption spectrum further changes as a function of time with gradual disappearance of the peak at 450 nm and the appearance of a new species absorpting at 330 nm. Possibly this represents the Cu(II) catalysed oxidation products of quercetin (Fazal et al., 1990). Fig. 7a, b, c, d and e depict similar experiments and show the decay with time of the charge transfer complexes of quercetin, myricetin, rutin, galangin, and apigenin, respectively. It is seen that whereas the behaviour of myricetin is similar to that of quercetin, the decay of the charge transfer complex of rutin and galangin is extremely slow. On the other hand, it appears that in the case of apigenin there is no formation of discernible charge transfer complex. It is not certain how these differences in the spectral behaviour of different flavonoids may be related to the relative DNA cleavage efficiencies of different flavonoids.

It was suggested that the peak appearing at 330 nm on decay of charge transfer complex represents the oxidation products of quercetin (Fazal et al., 1990). Such a peak is

Figure 7a. **Time course of absorption spectral changes in quercetin induced by the addition of Cu(II)**

Quercetin and Cu(II) concentrations were 50 µM and 150 µM respectively in 10 mM Tris-HCl buffer, pH 8.0. Absorption spectra were recorded at time periods after addition of Cu(II).

0 time	(—)
1.0 minute	(▲)
5.0 minute	(Δ)
10 minute	(●)
15 minute	(O)

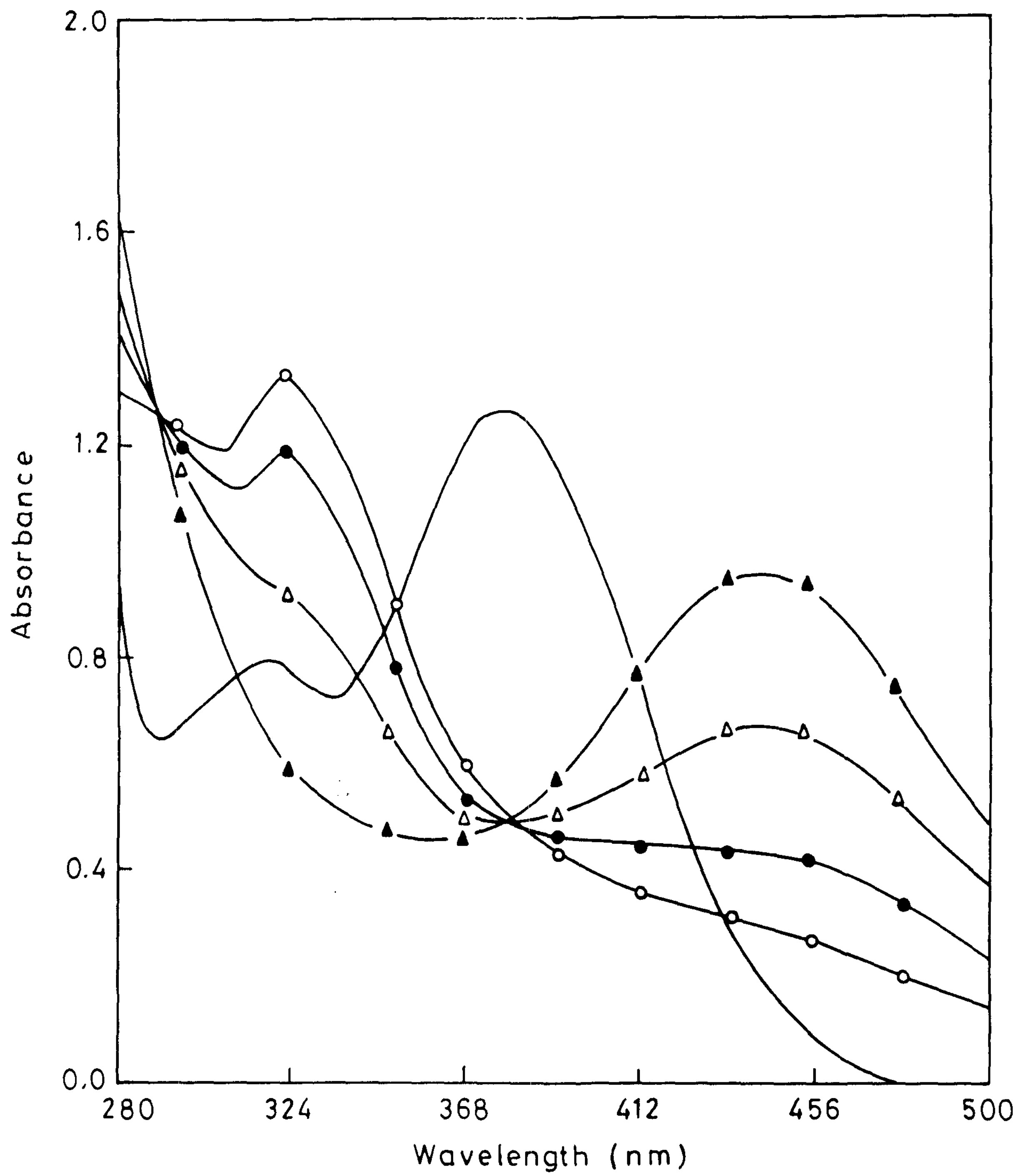
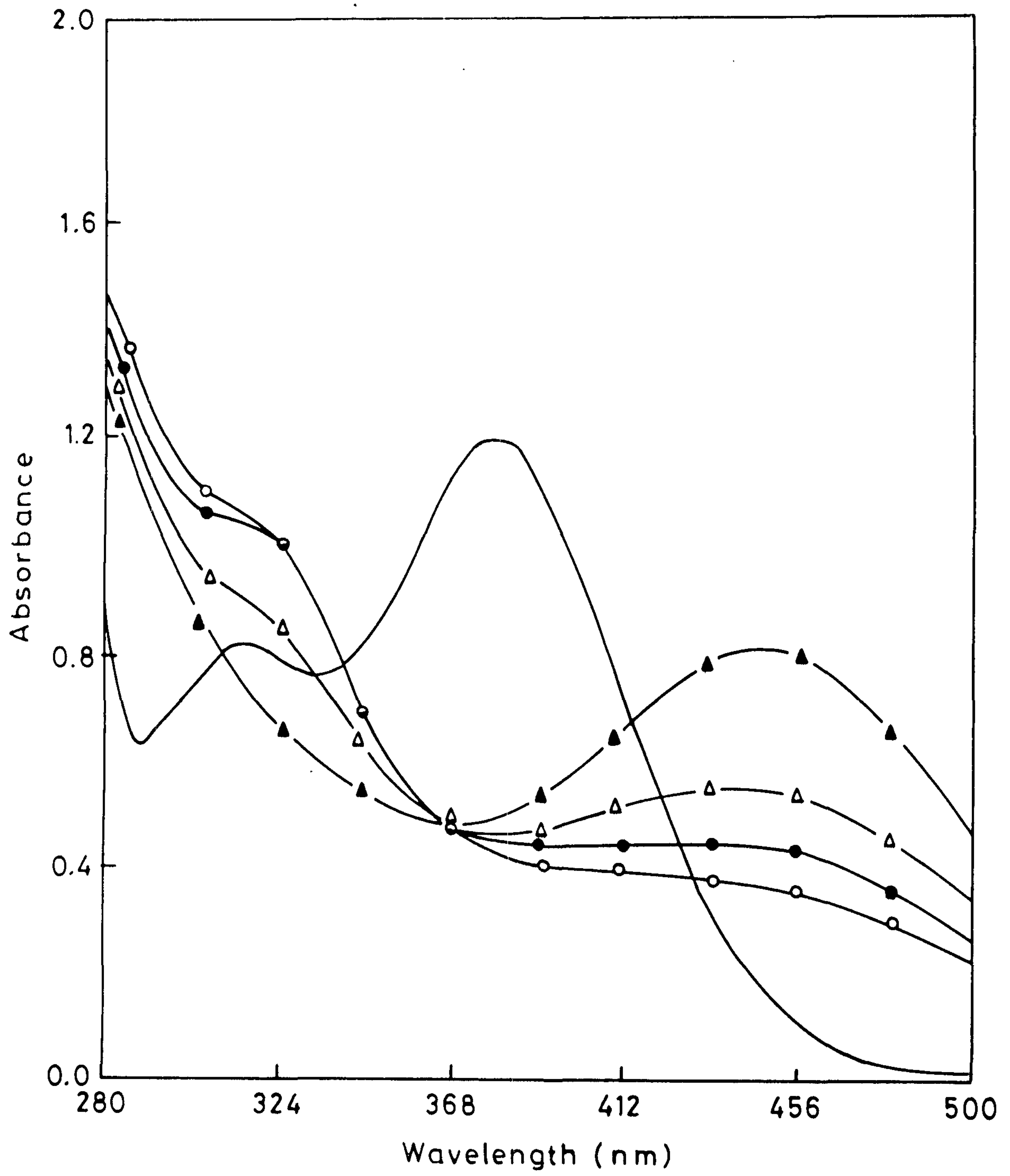

2.0
1.6
1.2
0.8
0.4
0.0
Absorbance
280
324
368
412
456
500
Wavelength (nm)

Figure 7b. **Time course of absorption spectral changes in myricetin induced by the additon of Cu(II).**

Myricetin and Cu(II) concentrations were 50µM and 150 µM respectively in 10 mM Tris-HCl buffer, pH 8.0. Absorption spectra were recorded at different time periods after addition of Cu(II).

0 time	(—)
1.0 minute	(▲)
5.0 minute	(Δ)
10 minute	(●)
15 minute	(O)

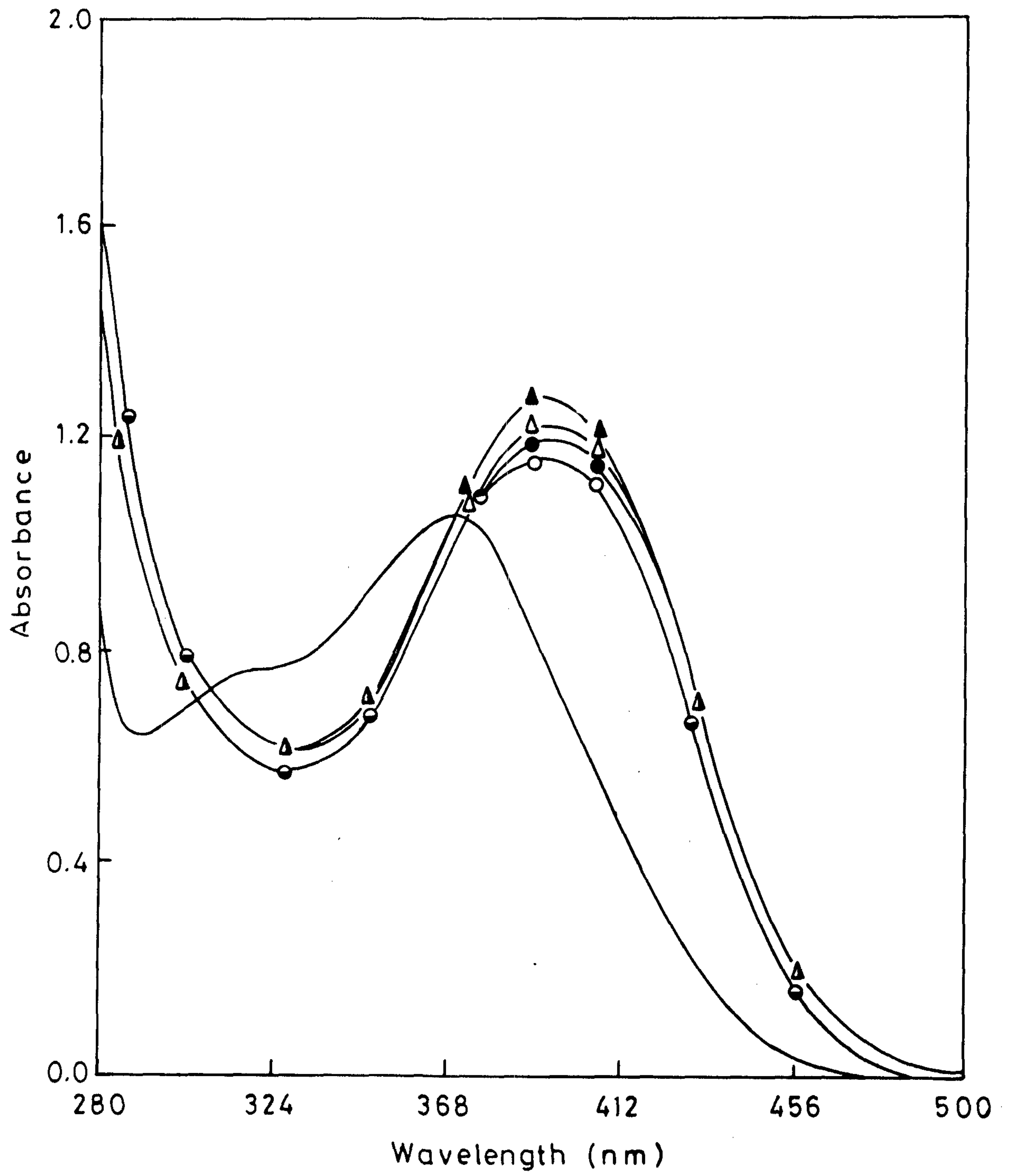


2.0
1.6
1.2
0.8
0.4
0.0
Absorbance
280
324
368
412
456
500
Wavelength (nm)

Figure 7c. **Time course of absorption spectral changes in rutin induced by the addition of Cu(II).**

Rutin and Cu(II) concentrations were 50 µM and 150 µM respectivey in 10 mM Tris-HCl buffer, pH 8.0. Absorption spectra were recorded at different time periods after addition of Cu(II).

0 time	(―)
1.0 minute	(▲)
5.0 minute	(△)
10 minute	(●)
15 minute	(○)



2.0
1.6
1.2
0.8
0.4
0.0
Absorbance
280
324
368
412
456
500
Wavelength (nm)

Figure 7d. **Time course of absorption spectral changes in galangin induced by the addition of Cu(II).**

Galangin and Cu(II) concentrations were 50 μM 150μM respectively in 10 mM Tris-HCl buffer, pH 8.0. Absorption spectra were recorded at different time periods after addition of Cu(II).

0 time	(—)
1.0 minute	(▲)
5.0 minute	(△)
10 minute	(●)
15 minute	(○)

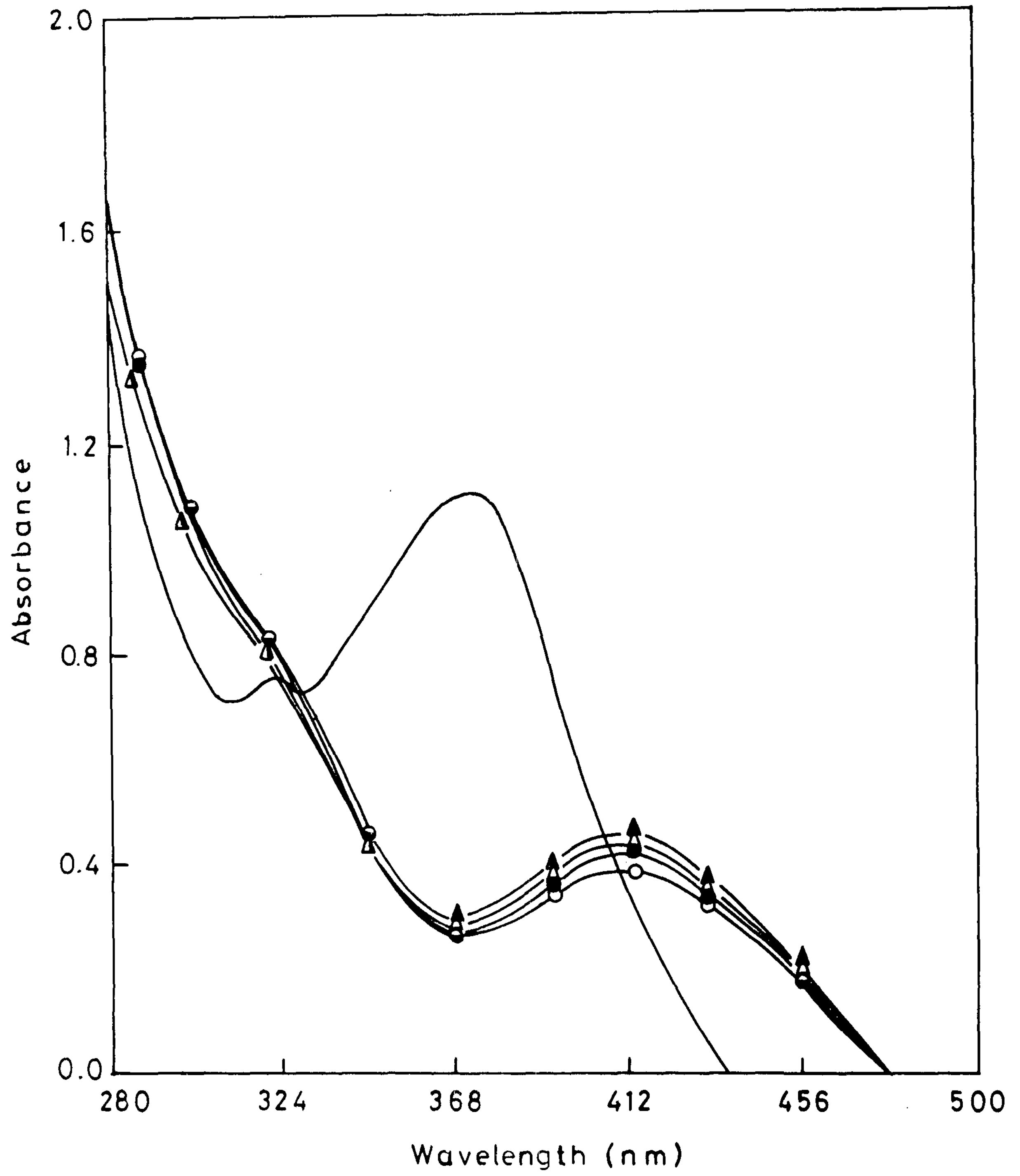
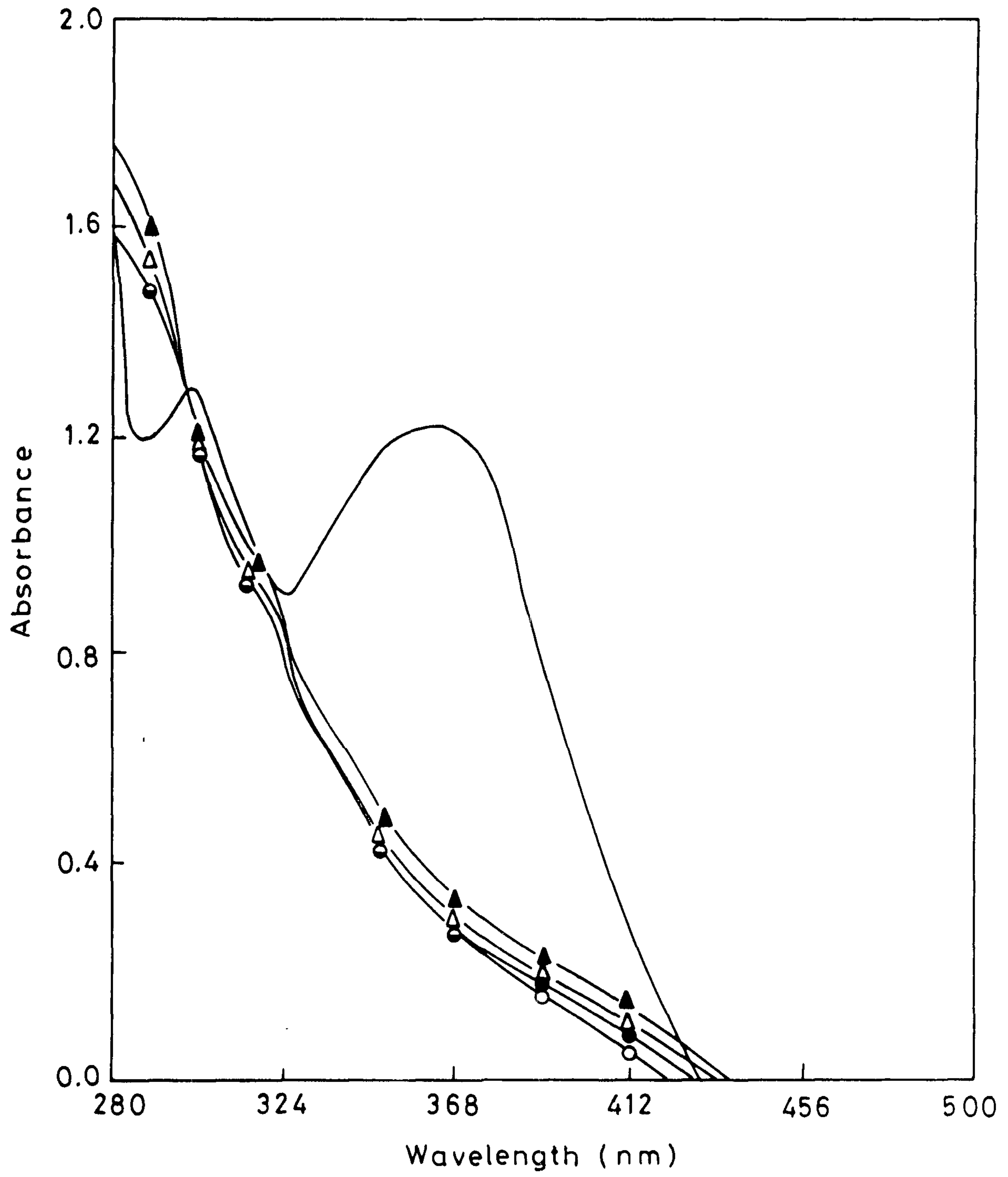

2.0
1.6
1.2
0.8
0.4
0.0
Absorbance
280
324
368
412
456
500
Wavelength (nm)

Figure 7e. **Time course of absorption spectral changes in apigenin induced by the addition of Cu(II).**

Apigenin and Cu(II) concentrations were 50 µM and 150 µM respectively in 10 mM Tris-HCl buffer, pH 8.0. Absorption spectra were recorded at different time periods after addition of Cu(II).

0 time	(—)
1.0 minute	(▲)
5.0 minute	(△)
10 minute	(●)
15 minute	(○)



2.0
1.6
1.2
0.8
0.4
0.0
Absorbance
280
324
368
412
456
500
Wavelength (nm)

also observed on oxidation of rutin by horse radish peroxidase (Takahama, 1986).

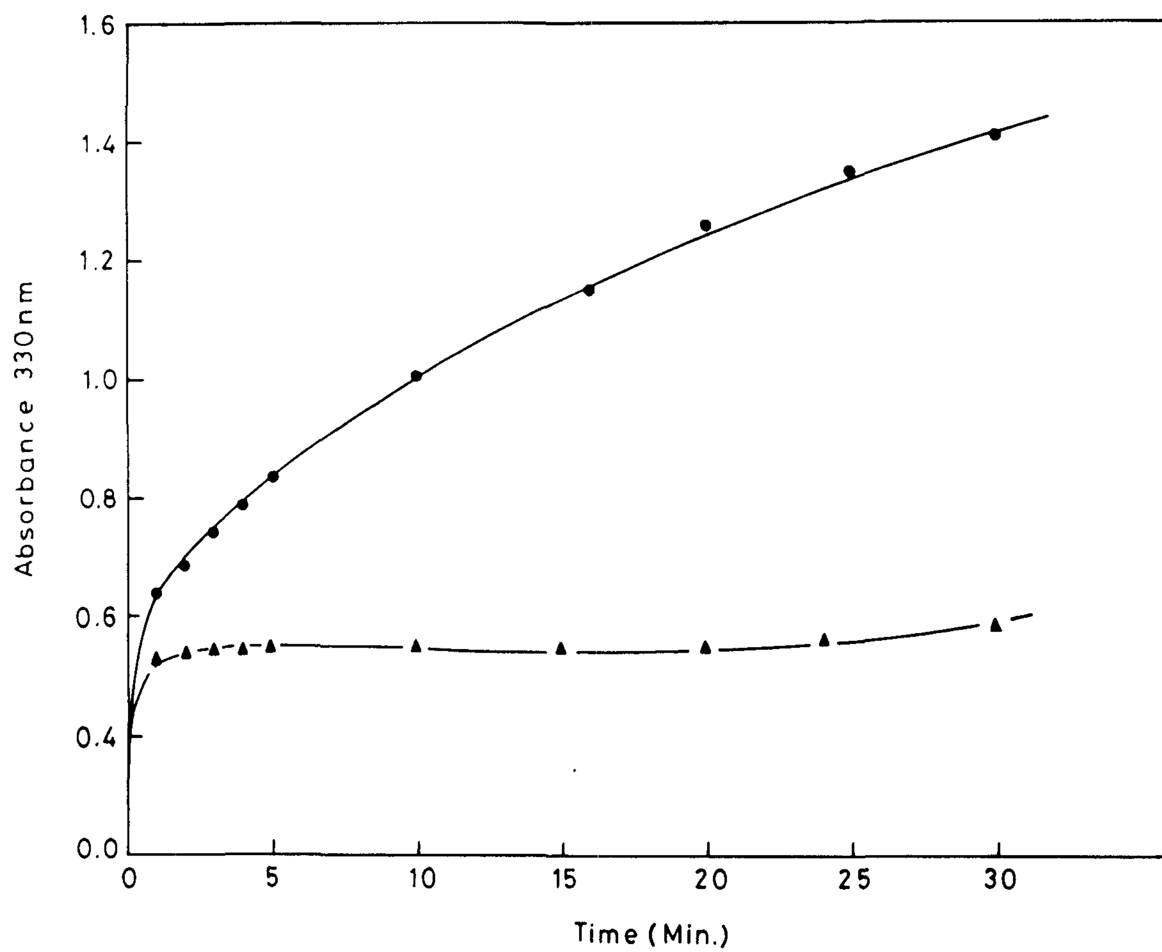
In order to confirm that the oxidation of quercetin results in a peak at 330 nm, the Cu(II) mediated oxidation was followed in the presence of ascorbate as a reducing agent. The results given in Fig. 8 show that ascorbate inhibits such oxidation confirming that the band at 330 nm is due to the formation of oxidized products of quercetin.

Figure 8. **Inhibition of Cu(II) mediated oxidation of quercetin by ascorbate.**

The reaction mixture (3.0 ml) contained 50 µM quercetin, 300 µM Cu(II) and 1 mM ascorbate in 10 mM Tris–HCl, pH 8.0. Absorbance at 330 nm was recorded at different time intervals. Reactions were started by addition of Cu(II).

(▲) + Ascorbate

(●) – Ascorbate



1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.0
Absorbance 330nm
0
5
10
15
20
25
30
Time (Min.)

DISCUSSION

DISCUSSION

The major conclusions of the experiments described in this dissertation may be stated as follows : (i) the rate of DNA cleavage by various flavonoids correlates with increasing number of OH-groups in the B-ring of the flavonoid molecule. It also correlates with the stoichiometry of Cu(II) reduction by the free flavonoid i.e. the greater the moles of Cu(II) reduced per mole of free flavonoid, the greater the rate of DNA cleavage; (ii) the increasing extent of hydroxylation of B-ring also gives rise to increased production of the superoxide anion, the hydroxyl radical and hydrogen peroxide.

As mentioned earlier (page 23), it was concluded by Brown (1980) from mutagenicity data that the most important structural requirement for mutagenicity of flavonoids is the 3-OH group of the flavonol structure (see fig. 1). In a study on Cu(II) catalysed oxidation of quercetin and 3-hydroxy flavone, Utaka and Takada (1985) observed that the oxidatioon of the latter required a higher temperature and a longer reaction time whereas, the former could be oxidized at room temperature. This was explained by the fact that quercetin possesses electron donating hydroxyls at 3' and 4' positions in the B-ring which makes C-2 carbon electron dense, thereby facilitating the oxidation of quercetin to 2-alkoxyflavan-3-4 diones. Possibly the same mechanism explaines the present

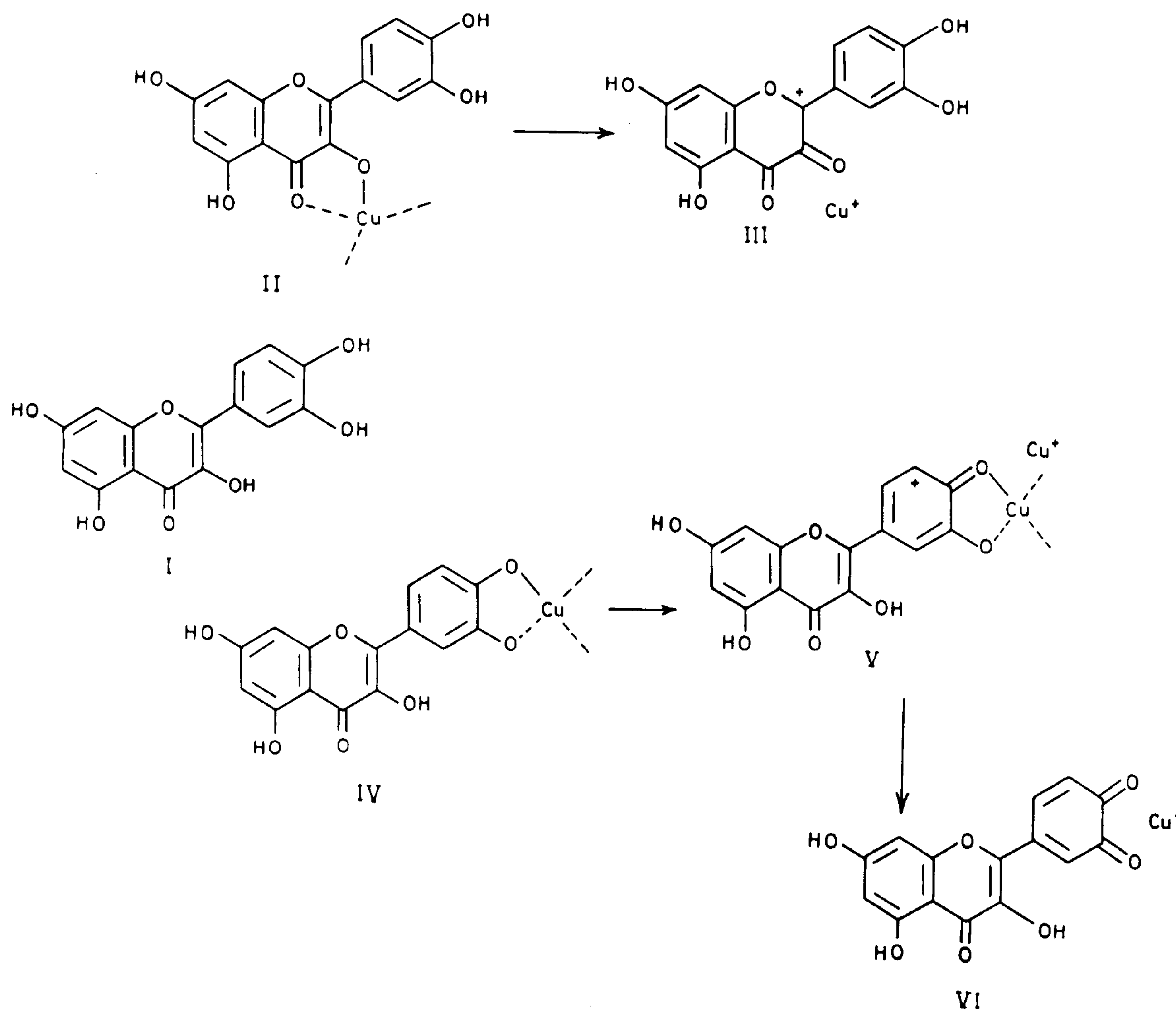
observation of increased hydrolysis of DNA with increasing hydroxyl groups in the B-ring (Fig. 9, top panel). Possible routes for the generation of Cu(I) from Cu(II) and the formation of the oxidation products of quercetin is given in (Fig. 9). It may be mentioned that there may be several oxidized forms of quercetin and the nature of these may change during the subsequent reactions.

The above mechanism of Cu(II) mediated oxidation of flavonoids would be plausible only in the case of galangin, quercetin, and myricetin with none, two and three hydroxyl groups respectively in the B-ring. In rutin the 3-OH is glycosylated with rutinose (a dissacharide of glucose and rhamnose). The significant DNA breaking activity of rutin could possibly be due to the hydrolysis of the glycoside during the course of incubation or (more likely) due to its contamination with quercetin. Thin layer chromatograms of rutin samples suggest the presence of a contaminant with the same chromatographic characteristics as quercetin. The case of apigenin however, is more intriguing as it does not possess 3-OH and has only one OH group in the B-ring. Here the only conceivable groups for sequestring Cu(II) leading to its reduction would be the 5-OH and the carbonyl function at position 4. On the other hand, the generation of Cu(I) from Cu(II) by epicatechin can possibly occur through sequestration of Cu(II) by 4'-OH and 5'-OH in the B-ring (Fig. 9, bottom panel).

From the above, it would appear that reduction of

Figure 9. **Possible routes for the generation of Cu(I) from Cu(II) in the presence of quercetin.**

Quercetin (I) can sequester Cu(II) to generate II which, on oxidation of the quercetin, leads to intermediate cation (III). Alternatively, sequestration by OH groups at positions 3' and 4' (Figure 1a) gives IV. This can lead, in principle, to the reduction of two Cu(II) species with quercetin being oxidized via an intermediate (such as V) to the quinone, VI.



II
III
I
IV
V
VI
Cu⁺

Cu(II) to Cu(I) by various flavonoids is the critical event in DNA degradation. As seen in "Results" the degree of Cu(I) formation is directly related to the rate of DNA cleavage. We have earlier proposed (Rahman, et al., 1989) and subsequently established (Rahman, et al., 1990) that a ternary complex of Cu(II)-quercetin-DNA is formed. Free flavonoids can catalyse the reduction of Cu(II) to Cu(I) in the absence of DNA through generation of superoxide anion. However, the formation of the ternary complex is essential for subsequent reoxidation of Cu(I) (to generate active oxygen species) and the accompanying cleavage of DNA. The generation of oxygen radicals in the proximity of DNA is well established as cause of strand scission (Wong, et al., 1984; Ehrenfield, et al., 1987; Eliot, et al., 1984). It is recognised by most workers that hydroxyl radical reactions with DNA are preceded by the association of a complex with DNA, followed by the production of the hydroxyl radical at that particular site. A water soluble fraction from cigarette tar has been shown to associate with DNA and produce the hydroxyl radical in situ (Borish, et al., 1987). Thus, the nature of binding and selectivity of the ligand may determine the type of reaction and DNA damage by oxygen rdicals (Pryor, 1988).

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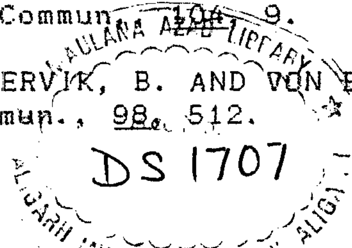
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