THE CLASTOGENIC ACTIVITY OF
PHENOLIC OXIDATION PRODUCTS

by

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Several epidemiological studies have demonstrated the importance of diet in the development of gastro-intestinal carcinomas in man. This study examines the role of plant phenolics, major components of the human diet.

Employing a CHO cell test system, it was observed that phenolics with at least two hydroxyl groups in the ortho position, relative to each other were particularly clastogenic. This activity was abolished by the addition of S9, a rat liver microsomal preparation. The clastogenic activity of these compounds was found to increase with time, alkaline pH, and the presence of transition metals. It was therefore deduced that the source of activity might be an oxidative by-product.

High-pressure liquid chromatography was used to separate out these oxidative products. No activity was found to reside in any of the separated components or combinations of components. Further study therefore centred on oxidative products not retained by chromatography and on those labile to this process.

Under oxidative conditions, the presence of hydrogen peroxide was detected. Levels measured were sufficient to explain the clastogenic activity of completely oxidized solutions of phenolic acids. Addition of the enzyme, catalase, appeared to abolish all activity of completely oxidized solutions. Hydrogen peroxide could not, however, account for the genotoxic effects measured in freshly prepared solutions.

The presence of superoxide was detected in actively oxidizing solutions of plant phenolics. Its production appeared to be pH-dependent. Addition of superoxide dismutase increased the clastogenic activity of compounds tested, presumably by converting superoxide to peroxide, a more stable oxidative by-product.
Addition of tyrosinase, a monophenol oxidase, also increased the clastogenic activity of freshly prepared solutions. Since this enzyme catalyzes the oxidation of several phenolics without subsequent generation of peroxide, it was deduced that phenolic free radicals must also be present which could be at least partially responsible for the enhanced biological activity. Electron spin resonance proved this was the case.

Using electron spin resonance, the primary oxidative products were characterized both at high pH and by enzymatic activation. The results obtained agree with those published in the literature.

Several reports in the literature have suggested that phenolics may also act as free radical scavengers. The importance of plant phenolics in diet may therefore depend on the oxidative conditions of the system to be tested. Under oxidative conditions, free radicals appear to be generated, which are capable of causing mutations and chromosomal rearrangements. Phenolic oxidation products may therefore play a role as initiators and promotors of carcinogenesis. However, under alternate conditions, phenolics may also act to scavenge free radicals, and could therefore be classed as inhibitors of carcinogenesis.
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INTRODUCTION

In 1964, the World Health Organization released a report on the prevention of cancer in man. This report concluded that a majority of common fatal cancers were a result of lifestyle and other environmental factors and were therefore in principle, preventable. The report was a culmination of epidemiological research which suggested that as many as 80 to 90% of all human tumours could be found in this category. The implications of such a statement were and are enormous.

Included in lifestyle and other environmental factors are the components: diet, culture (drug, tobacco, and alcohol use), occupation, radiation (sunlight) exposure, as well as air and water quality effects. Of these components, diet appears to have the most far reaching implications in the prevention of cancer. It is through diet that man comes in contact with a majority of chemicals (for reviews see, Stich et al., 1981c; Hietanen, 1981; Miller and Miller, 1979; Sugimura, 1979; and Clayson, 1975), and it is through diet modulation that the frequency of tumours at specific sites can be altered. Epidemiology supports this view (Hirayama, 1981; Higginson, 1979; Sugimura, 1979; Wynder and Gori, 1977; Wynder et al., 1977).

The purpose of this study was to examine a major component of diet, the plant phenolics. These compounds have a unique structure enabling them to play a variety of roles in cellular development. Because of their high reactivity and ability to permeate cell membranes, they have been found to induce significant toxicity in cells. This study examines some of the reaction mechanisms involved in inducing genetic damage.

Biological Significance of Phenolics:

Phenolic compounds make up a considerable fraction of plant material consumed as food. It has been estimated that a minimum of 600
mg of phenolic material is ingested each day in the normal human diet (Powell et al., 1974). Interestingly, higher animals cannot themselves synthesize compounds with benzenoid rings from aliphatic precursors (phenolic steroids being one notable exception). Plants are therefore the source of nearly all phenols found in animals. Even such essential phenols as tyrosine, catecholamine, thyroxine, vitamin E tocopherols, and vitamin K menadiones are drawn directly or indirectly from plant sources (Singleton, 1981).

The reactive nature of phenolic compounds makes them important intermediaries in metabolism. Their ability to participate in oxidation-reduction reactions allows them to play both a genotoxic and beneficial role in cellular development. Phenolics form the backbone of main biosynthetic pathways in aromatic amino acid synthesis. They may also play a role as inhibitors of carcinogenesis. Gallic and tannic acids, for example, have both been found to be effective inhibitors of nitrosation at gastric pH (Mirvish et al., 1975a; 1975b).

At the same time, some are potent toxins capable of inducing dietary reactions in animals. Naturally occurring phenolic substances found to be carcinogenic in animal tests include the potent aflatoxins (for a review see Miller and Miller, 1979), safrole (Miller et al., 1979; Wislocki et al., 1977), quercetin (Pamukcu, 1980), and tannins (Körpassy, 1961). They may also act as cocarcinogens. The chlorogenic acid content of coffee, for example, is sufficient to catalyze formation of N-nitrosamine carcinogens from nitrate and secondary amines (Challis and Bartlett, 1975; Mori and Hirono, 1977).

The function of phenols in plants is unknown. One hypothesis is to confer protection of plants from diseases caused by bacteria, fungi, and viruses. Reasons for this assumption include the fact that many phenolic compounds have anti-microbial activity, phenolics are widely distributed
in plants, and that there are often large increases in phenolic synthesis in plants after infection with plant pathogens (Friend, 1981).

In animals, phenylalanine (α-aminohydrocinnamic acid) and its metabolite tyrosine (α-amino-p-hydroxyhydrocinnamic acid) are important amino acids required for protein synthesis. Low food intake of these phenols causes adverse effects in animals similar to protein deficiency (Harper, 1973). The phenol, 3,4-dihydroxyphenylalanine or dopa is an important intermediate in both the synthesis of epinephrine and melanin, in man. It is a derivative of tyrosine and has been used as an antitumour drug for the treatment of melanomas (Wick, 1977; Parsons and Morrison, 1982).

Rare autosomal recessive genetic deficiencies in the metabolism of these phenolics may lead to conditions known as phenylketonuria, tyrosinemia, alkaptonuria, and albinism. Interestingly, tyrosinemia which is believed to result from a deficiency in the enzyme, p-hydroxyphenylpyruvate hydroxylase, often leads to hepatic carcinomas in patients at a very young age (Weinberg, et al., 1976).

Vitamins K and E are essential components of man's diet. Vitamin K is broadly distributed in plants and is required in the diet of most higher animals. It is believed to play a role in prothrombin synthesis. Vitamin E consists of a group of closely related vitamers present in vegetable oils, known as tocopherols. Nutritional deficiency in this vitamin leads to sterility, muscular weakness, and atrophy in rodents (Lehninger, 1970). Strong evidence suggests that its "antioxidant" properties prevent the destructive non-enzymatic attack of molecular oxygen on the double bonds of polyunsaturated fatty acid components of tissue lipids. It also appears to prevent oxidation of other components of the diet. This will be discussed in some detail in the Discussion part of this thesis.
Although phenols are found in almost all plants, there is great diversity in the type and content for each species. However, the foods actually consumed by higher animals appear to be low in both variety and content of the various forms of phenolics. This is most likely due to taste selection in animals. Given a choice, animals choose foods with a low phenolic content from a lignin (toughness) or tannin (astringency) viewpoint, but perhaps a relatively high anthcyanin content (colour ripeness) (Bate-Smith, 1972).

Classification of Phenolics:

Phenolics are compounds of the general formula, ArOH, where Ar is a phenyl and OH, a hydroxyl group. Often these compounds are liquids or low melting solids and because of hydrogen-bonding, have relatively high boiling points. Typically they exhibit acidity with a high reactivity of their ring structure toward electrophilic substitution. Most are only sparingly soluble in water (Morrison and Boyd, 1973). For simplicity, these compounds have been divided into the following classification: a) simple phenols, b) phenolic acids; i) benzoic acids and ii) cinnamic acids, c) flavonoids, d) tannins, and e) lignins.

A. Simple Phenols:

Simple phenols may be defined as molecules containing only the benzenoid ring structure with an adjacent hydroxyl group(s).
Appendix 1 lists the molecular structures of some of the more commonly found simple phenols. Because of their extreme toxicity, they do not tend to be found in their parent form in plants. They do, however, exist as breakdown products of larger, more highly structured phenols and nonphenols. Pyrolysis of catechin (from gum catechu) and gallates were the historical sources of catechol, a simple phenol of high reactivity (Singleton, 1981). Simple phenols can also be generated by pyrolysis of carbohydrates (Higman et al., 1970). All phenols exhibit some degree of anti-microbial activity (Friend, 1981).

B. Phenolic Acids:
   i) Benzoic Acids:
   These compounds possess the same basic phenolic structure; the benzenoid ring with a hydroxyl group, but with the addition of a carboxylic acid group immediately adjacent to the ring structure.

The molecular structures and names of commonly found benzoic acids are shown in Appendix 2. Benzoic acids are widely distributed in plants and can be found in both angiosperms and gymnosperms (Ribéreau-Gayon, 1972). The most important mechanism of formation of benzoic acids in plants is the side chain degradation of cinnamic acids (Gross, 1981).
p-Hydroxybenzoic, vanillic and syringic acids are constituents of lignin, from which they are liberated by alkaline hydrolysis (Ribéreau-Gayon, 1972). Protocatechuic and gallic acid are believed to have a different function. The former is widely distributed while the latter is rarer and often found as its dimer, ellagic acid. Gallic and ellagic acids are important components of many tannins. Bound forms of gallic acid, notably epicatechin gallate or galloyl glucoses and tannic acids are associated with and are the main source of gallic acid in foods (Singleton, 1981). The toxicity levels for benzoic acids are relatively low in animal studies (Singleton, 1981). They do possess, however, considerable anti-microbial activity (Friend, 1981).

ii) Cinnamic Acids:

Cinnamic acids are similar in molecular structure to benzoic acids, but possess an ethylene group between the benzene ring and the carboxylic acid group. Their structures are illustrated in Appendix 2.

Like benzoic acids, these compounds are widely distributed in nature. The four cinnamic acids most commonly known and distributed in plants are: p-coumaric, caffeic, ferulic, and sinapic acids. At least one of these is believed to be found in all plants. According to Bate-Smith
(1959), p-coumaric acid is the most commonly found phenolic of all phenolic constituents (Ribéreau-Gayon, 1972).

Since cinnamic acids possess a double bond, they can exist in two isomeric forms, cis and trans-cinnamic acid.

![trans-cinnamic acid](image)

The naturally occurring cinnamic acids are the trans isomers. However, these isomers can be interconverted by the action of light (Ribéreau-Gayon, 1972). Agriculturally important foods contain high levels of cinnamic acid derivatives. These include caffeic, p-coumaric, ferulic, and sinapic acids (or 3,4-dihydroxy-, 4-hydroxy-, 3-methoxy-4-hydroxy-, and 3,5-dimethoxy-4-hydroxy-cinnamic acids, respectively). Often these cinnamates occur as esters of quinic acid (chlorogenic acid and analogues) or other nonphenolic hydroxyl compounds such as glucose, shikimic acid and tartaric acid (reviewed by Singleton, 1981).

The common phenolic cinnamic acids do not occur freely in plants but are acetylated to quinic and other hydroxylic substances. In animals, quinic and shikimic acid derivatives are converted to saturated cyclohexane carboxylic derivatives by intestinal microorganisms. They are then metabolized by the liver to the benzoic acid conjugate,
hippuric acid (Brewster et al., 1977). This illustrates the facility animals have for detoxifying some of the more predominant phenolic compounds.

C. Flavonoids:

The chemical structures of flavonoids is based on a $C_{15}$ skeleton with a chromane ring bearing a second aromatic ring B in position 2, 3, or 4.

![Chemical structure of flavonoids](image)

Subgroups of flavonoids are classified according to the substitution patterns of ring C. Both the oxidation state of the heterocyclic ring and the position of ring B are important in the classification (Hahlbrock, 1981). Examples of the six major subgroups (chalcones and the isomeric flavones, flavones, flavonols, anthocyanins, and isoflavonoids) are illustrated in Appendix 3. Several reviews exist on the structure, occurrence, and methods of identification of flavonoids (Harborne et al., 1975; Ribéreau-Gayon, 1972; Mabry et al., 1970).

Common plant foods contain from traces to several grams of flavonoids per kilogram fresh weight. A few hundred milligrams per kilogram is common for many vegetables and fruits (Singleton, 1981). The human dietary intake has been estimated to be as high as 1 gram per day (Kuhnau, 1976).

Flavonoids found in agriculturally important foods include the common natural anthocyanins: cyanidin and delphinidin, the flavones:
apigenin and luteolin, the flavonols: kaempferol and quercetin, the flavan-3-ols: (+)-catechin and (-)-epicatechin, the flavanone: naringenin, and the flavanonol: taxifolin (reviewed by Singleton, 1981). Flavonoids are responsible for the colour and flavour in food. They also constitute the pigmentation in flowers. Most flavonoids show low toxicity, however, a number have been shown to induce mutagenicity. The most extensively reported, quercetin, is a component of the carcinogenic braken fern (Pamukcu, A.M., 1980).

D. Tannins:

The tannins are a diverse and chemically difficult group to classify. Their two main characteristics are a polyphenolic chemical structure, and their ability to precipitate protein from aqueous media. Much of the difficulty in their classification has arisen from the fact that early biochemists confused their ability to produce leather from hide, the tanning process, with their polyphenolic nature. In this way the accepted meaning of the word, tannin, was enlarged in the general botanical and biochemical literature to include a range of compounds which are polyphenols, but not tannins (Haslam, 1979). The most widely accepted division is that first proposed by Freudenberg in 1920. It separates the tannins into two classes, the hydrolysable and the nonhydrolysable or condensed tannins.

i) Hydrolysable Tannins:

The hydrolysable tannins, as their name infers, are complex phenols which can be degraded under hydrolytic conditions (acid, alkali, or hydrolytic enzymes). Subdivision is usually made on the basis of the phenolic acid(s) liberated by hydrolysis. Those yielding gallic acid as the only phenolic component are defined as gallotannins and those
liberating besides gallic acid, its derivative ellagic acid, are named ellagitannins.

Gallotannins are esters of sugars, such as glucose, and gallic acid or m-digallic acid. They are not widely distributed in plants (Haslam, 1981). Ellagitannins, on the other hand, do not necessarily combine with glucose in the original tannin. These tannins are more widely distributed (Haslam, 1981).

ii) Condensed Tannins:

These tannins are the most commonly found and are of the greatest commercial significance (Haslam, 1981). They are widespread in fruits and certain grains as well as in such beverages as cider, cocoa, tea, and red wine (Haslam, 1975). Relatively little is known, however, about their structure and molecular formation.

Freudenberg (1920) first put forth his "catechin hypothesis" in which (+)-catechin formed the sole basis of tannin chemistry. Wide ranging studies with flavan-3-ols and flavan-3,4-diols as models has led to proposals for their self-condensation to the complex polymers known as condensed tannins (Haslam, 1966).
OH

flavan-3-ols
(catechins)

flavan-3,4-diols
(leucoanthocyanidins)

For a review of this hypothesis, see Haslam, 1981. The distribution in nature of condensed tannins, based on leucoanthocyanidins has been examined by Bate-Smith and Metcalfe (1957) and by Bate-Smith and Ribéreau-Gayon (1958). These compounds were found to be so prominent, that their property of giving anthocyanidins upon heating with acid could be used as a diagnostic tool for their presence (Swain, 1975).

Condensed tannins have a molecular weight of between 500 and 3000. Since the flavans, diagrammatically represented above, have molecular weights in the order of 250-300, the condensed form would contain 2 to 10 monomers and can thus be considered as oligomers (Ribéreau-Gayon, 1972). Polymerization plays a role in the properties of these compounds. Whether dimers, trimers or tetramers are present during formation, has been found to affect the shape of the subsequent molecule, and thus its properties of stability, distribution, and reaction with protein (Ribéreau-ayon, 1972).

Tannins have played an important role in folk medicines. Because of their potent antimicrobial activity, they have been used as antibiotics (Chan et al., 1978), as a burn treatment, and as an enema (Singleton,
1981). Their property of binding protein enhanced coagulation of a burned surface and produced local inflammation, a property which enabled them to be used as a form of enema. However, at the time of the second world war, they were also found to induce hepatotoxicity and have since lapsed into disuse. This hepatotoxicity is most likely related to findings of carcinogenicity by Körpassy, 1961. Kirby, 1960, has suggested that condensed tannins are bound to body proteins, where, as hydrolysable tannin or fragments, they may be translocated to the liver.

Epidemiological studies have attempted to evaluate the role of tannins in terms of human cancer. Some reports have correlated the frequency of mouth and esophageal tumours with the consumption of tannin-rich material (Morton, 1970; 1972; 1973; Raghava and Baruah, 1958). These correlations have been examined in the cases of betel nut chewing in parts of Asia and the consumption of sorghums in parts of Africa, and herbal teas on other areas (Singleton, 1981).

E. Lignins:

Lignin is found as an integral cell wall constituent of all vascular plants. It is comprised of a heterogeneous group of large, insoluble, three-dimensionally linked polymers. Unlike polysaccharides such as cellulose it lacks an ordered structure. It is believed that it is formed by enzymatic dehydrogenation of cinnamyl alcohols (example, 4-coumaryl alcohol), coniferyl alcohol, and sinapyl alcohol (Grisebach, 1981).
Lignin, or lignin fibres have never been shown to be toxic to man (Singleton, 1981) They are a component of dietary fibre and as such add bulk to the diet of higher animals. They also appear to absorb bile steroid and dietary cholesterol, as well as bind nitrite thereby inhibiting nitrosamine formation in the gut (Inglett and Falkehag, 1979). Lignin's role in diet may therefore be favorable, or at least inert (Singleton, 1981).

Testing of Phenolics:

Plant phenolics to be screened in a short term test for clastogenic activity were those found to be present in foods from a North American diet. Of particular interest were those present in phenolic-rich beverages which have been linked in epidemiological studies to human cancers. Coffee has been implicated as an etiologically important factor in the development of tumours of the urinary bladder (Cole and MacMahon, 1971; Simon et al., 1975; Howe et al., 1980) pancreas (MacMahon et al., 1981) and ovary (Trichopoulos et al., 1981). The use of herbal teas has been implicated with the development of
nasopharyngeal cancers in China (Hirayama and Ito, 1981) and Curacao (Hecker, 1981). "Chagayu", a rice gruel boiled with tea leaves, has been associated with an elevated risk of esophageal tumours (Segi, 1975, Hirayama, 1979).

These phenolic-rich beverages are particularly high in content of chlorogenic acid and its breakdown product, caffeic acid (the average cup of coffee contains 260 mg or about 10 mg/ml) (Challis and Bartlett, 1975) and polyphenols (teas may contain up to 30% per dry weight) (Kaiser, 1967). These compounds were also readily available and showed considerable ease of handling.

The CHO Cell Chromosome Aberration Test:

The introduction of short-term tests for the screening of potentially hazardous substances has greatly aided in the identification and classification of carcinogens. They accomplish this by circumventing the enormous cost and time required for live animal testing. At present, the most widely used assay is the Ames' bacterial mutation system. In surveys conducted to correlate the results of the Ames test with those of in vivo tests, the Ames test was found to correctly identify about 90% of the compounds tested (McCann et al., 1975; Coombs et al., 1976; Anderson and Styles, 1978). It should be pointed out, however, that these tests are not effective for screening all carcinogens. One such group of compounds are the oxidative mutagens, substances capable of giving rise to activated oxygen species. Testing in this lab demonstrated that the CHO cell chromosome aberration test was capable of detecting these substances. Recently, the Ames group has developed a new strain, TA102, which is sensitive to these compounds; however it was unavailable at the time of testing (Levin et al, 1982).
The CHO cell chromosome test assays for the induction of chromosomal damage. Data on chromosome breakage can therefore be compared with the bacterial mutation assays. In a paper by Ishidate and Odashima (1977) 134 chemicals were assayed for their cytogenetic effects. Thirty four of the test chemicals had been previously demonstrated to be carcinogenic. The chromosome aberration test correctly identified 25 of the 34 (73.5%) and incorrectly identified 9 (26.5%). These results were comparable with the Ames system which correctly identified 28 (82.4%) and incorrectly identified 6 (17.6%).

Aside from being sensitive to oxidative clastogens, the CHO cell test system offers many other advantages. Almost all cell lines derived from Chinese hamster tissues have a stable karyotype with a low diploid number (2n = 22, Au and Hsu, 1982). Although this cell line tends to become aneuploid with time, this does not pose a serious problem, since clastogen effects do not require analyses of chromosome numbers. Cell cycle times for this particular line is about 14 hours, a time limit which is easy to incorporate into a laboratory schedule.

Scoring for this test was done by recording the number of breaks, gaps and translocations per cell in mitosis (see Figure 1). Application of the test chemical was for 3 hours with a subsequent 16 hour incubation period. We would, therefore, anticipate only cells damaged originally in late S phase to be scored in mitosis. One would therefore only expect chromatid as opposed to chromosome aberrations. While longer incubation times with test chemicals would illustrate if damage could be induced earlier in the cell cycle, it was not in the realm of this study to examine stage-specific agents. The CHO cell test system employed was one for the rapid identification of oxidative clastogens.
Figure 1:

Orcein-stained metaphase CHO cells which have been exposed to a clastogenic agent for 3 hours. The cells were then washed, cultured for an additional 16 hours, exposed to colchicine for 4 hours and stained and mounted. "A" refers to control chromosomes, and "B" are chromosomes which have been treated with test chemicals. Note chromatid breaks and translocations (marked with arrows).
Identification of Hydrogen Peroxide:

Several techniques have been employed for the measurement of $H_2O_2$ in solution. Cytochrome c peroxidase in the presence of $H_2O_2$ forms a stable enzyme substrate complex with ferrocytochrome c. Inhibition of the reaction is accomplished by the addition of cyanide or low azide concentrations. This technique is extremely sensitive (capable of detecting formation at a rate of 1 $\mu$M/min.) although slightly complex (Boveris et al., 1972; Yonetani, 1965). Because of the large quantities of $H_2O_2$ produced under our experimental conditions, this technique was deemed unsuitable. Another technique measures $H_2O_2$ production by the detection of $O_2$ generated in the presence of catalase (Jensen, 1966). This is accomplished with an oxygen electrode, a piece of equipment not available at the time of this study. Production of $H_2O_2$ was therefore measured in a relatively inexpensive fashion as described below.

In this study, the assay employed for the identification of $H_2O_2$ was one modified from Wang and Nixon, 1978. This assay contains potassium iodide (KI) and trace amounts of ammonium molybdate at pH 5.0. Under these conditions, there is virtually no absorbance at 360 nm in an absorbance spectrophotometer. Peroxides, catalyzed by the presence of ammonium molybdate react with the iodide to generate $I_3^-$ which absorbs light at a maximum of 360 nm. Controls for this reaction were obtained by preparing identical samples in the presence of catalase, an enzyme which catalyzes the destruction of $H_2O_2$ to $H_2O$ and $O_2$. The production of $I_3^-$ could then be obtained using the net absorbance readings at 360 nm. Conversion of this value to molar quantities of $H_2O_2$ was obtained by comparing absorbance values obtained from test chemicals with a standard curve generated in the presence of commercially available $H_2O_2$. This system is capable of
measuring quantities of $H_2O_2$ in the $0 - 100 \mu M$ range.

**Identification of Superoxide:**

Because of the rapidity of spontaneous dismutation of $O_2^\cdot\cdot$, steady state concentrations of this radical, whether enzymatically or chemically produced, tend to be relatively low. Chemical methods for the detection of $O_2^\cdot\cdot$ are integrative and allow great sensitivity for its detection. In these methods, $O_2^\cdot\cdot$ is trapped with a chosen scavenger. The reaction is then followed by changes in absorbance as the scavenger alters its electronic configuration. The scavenger is used at concentrations which compete with the dismutation reaction so that virtually all $O_2^\cdot\cdot$ present is trapped by the scavenger.

Several forms of scavengers have been employed for the detection of $O_2^\cdot\cdot$. The $O_2^\cdot\cdot$ dependent oxidation of epinephrine to adrenochrome may be followed spectrophotometrically (McCord and Fridovich, 1969). However, the specificity of this reaction is relatively poor and the autooxidation of epinephrine must be controlled by lowering the pH (Bors et al, 1978). This prohibits any study of rates of production of $O_2^\cdot\cdot$ at different pH values. The rate of reduction of nitrobluetetrazolium has also been employed as an indication of $O_2^\cdot\cdot$ production but this method also appears to lack specificity (Beauchamp and Fridovich, 1972).

The method of choice, then is the measurement of reduction of ferricytochrome c. The rapidity of this reaction makes cytochrome c an excellent quantitative trap (Forman and Fridovich, 1973). Acetylation of the cytochrome molecule has been employed for greater specificity in mitochondrial measurements of $O_2^\cdot\cdot$ production (Azzi et al., 1975). However, this was not required in this present study, because the test conditions used lacked any electron transport chain enzymes which might
reduce the specificity of the reaction.

In this reaction, superoxide anion radicals reduce native ferricytochrome c(cyt 3+) by univalent electron transfer:

$$\text{O}_2^- + \text{cyt}^{3+} \rightarrow \text{cyt}^{2+} + \text{O}_2$$

(from Kuthan and Ullrich, 1982). The reduced cytochrome molecule has a characteristic absorption maximum at 550 nm. Since the molar extinction coefficient for the reduced form is well known, and one molecule of ferricytochrome C2+ is produced for each molecule of superoxide present, the concentration of superoxide anion radicals can be estimated. Generally, the rate of production is expressed as the rate of conversion at half maximum absorbance, or $K_{0.5}$ (Finkelstein et al., 1981) (see Results).

Measurement of Phenolic Free Radicals:

The electron spin resonance, or ESR measurement of free radicals was first performed by Zavoisky in 1945 (Zavoisky, 1945). Since then, greater sensitivity has been obtained through better instrumentation and techniques of handling samples. ESR is now considered the single most important experimental approach to research on free radicals (Borg, 1976).

The phenomenon of ESR occurs because all electrons have identical values of mass, charge, intrinsic angular momenta and magnetic moments which permit them to interact with their atomic or molecular surroundings in ways characteristic of the chemical structure of the molecule to be studied. The critical property which underlies these measurements is the quantized value of the electron's magnetic moment, a value derived from its spin.
The electron to be measured is a moving charge and therefore generates a magnetic field, the axis of which has an associated magnetic dipole moment. Since quantum restrictions require all electrons to be the same, relative to any axis of reference, only two orientations of spin are allowed for each atom. These two orientations are energetically equal in the absence of an external magnetic field. However, in an applied magnetic field they act to align in either a parallel or antiparallel fashion. In the parallel state, the electron is energetically more stable and is thus at lower energy than the antiparallel state. The equation which describes the energy difference between states is given below:

\[ \Delta E = gBH \]

where

- \( E \) = energy
- \( g \) = g-factor (a proportionality constant)
- \( B \) = Bohr magneton (a constant)
- \( H \) = magnetic field

In an idealized "free electron" sample, one would expect the value of \( \Delta E \) to be constant from one electron to another. However, in the presence of other electrons and their magnetic states, the value of \( \Delta E \) becomes characteristic of the molecule. Electron spin resonance spectroscopy takes advantage of the fact that only paramagnetic molecules - or odd electron sites - can be made to interact with appropriate external fields. Thus, this method allows one to examine the molecular surroundings of the free radical in a bulk sample matrix.

ESR spectra provide information in a number of ways. First of all, the applied magnetic field at which resonance occurs is largely determined by the effective spectroscopic splitting factor, the
g-factor. This value is a constant of the free-radical studied and can be used for comparison of the experimental conditions used by other groups. All g-factors measured in this study were obtained using the marker diphenylpicrylhydrazyl (DPPH) which has a known g value of 2.0036 ± 0.0002 (Kittel, 1971). Calculations of g-factor were based on the equation:

\[
g_x = \frac{g_{\text{DPPH}}}{1 + \frac{\Delta H_{\text{corr}}}{H_D}}
\]

where \( g_{\text{DPPH}} = 2.0036 \pm 0.0002 \)
\( \Delta H_{\text{corr}} = \text{corrected field (G)} \)
\( H_D = \text{field of DPPH (G)} \)

The number of spectral lines produced in an ESR spectrum can also provide valuable information about the radical. These reflect interactions of unpaired electrons with localized magnetic fields which are not a result of the applied external field, and are referred to as the nuclear hyperfine interactions. This splitting of resonance lines is dependent on atoms which possess inherent nuclear spin angular momenta such as \(^1\text{H}, ^2\text{D}, ^1\text{H}, ^1\text{N}, \) and \(^1\text{C}\). Since nuclear spin moments are quantized properties, the number of splittings is discrete and may be defined by the equation:

\[
N_{\text{Hfs}} = (2I + 1)
\]

where \( N_{\text{Hfs}} = \text{number of hyperfine splittings} \)
\( I = \text{nuclear spin quantum number} \)

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In the phenolic samples measured, only $^1\text{H}$ contributed to the hyperfine splitting phenomena. The separation of these spectral lines is a reflection of the strength of the nuclear hyperfine interaction. Values for $N_{\text{HFS}}$ for the phenolics measured in this study were available in the current literature and it was possible to correlate the values obtained with those published.

Finally, the intensity of the signal provides information about the number of free radicals present. Signals obtained by autooxidation at alkaline pH-values were observed to be significantly stronger than those generated enzymatically. However, both appeared to exhibit similar hyperfine patterns and g-values. Using the electron spin resonance technique it was possible to delineate some of the characteristics of the phenolic free radical molecules.

It is the contention of this thesis that the oxidative products of plant phenolics, namely, hydrogen peroxide, superoxide and phenolic free radicals, contributed to the clastogenic activity of phenolic molecules in a CHO cell chromosome aberration test. Since these oxidative products have been shown to cause chromosomal damage, it is hypothesized that phenolic molecules under oxidative conditions may act as promoters of carcinogenesis.
MATERIALS AND METHODS

Cell Cultures:

CHO cells were grown in MEM (Eagle's Minimal Essential Medium, Grand Island Biological Co.) supplemented with 10% fetal calf serum, antibiotics (streptomycin sulphate, 29.6 µg/ml; penicillin, 125 µg/ml; kanamycin, 100 µg/ml; fungizone, 2.5 µg/ml;) and sodium bicarbonate (1 mg/ml). The stock cultures were maintained in 240 ml plastic culture flasks (Falcon) at 37°C in a water-saturated CO₂ incubator.

Chemicals:

Simple phenols (catechol, resorcinol, pyrogallol), phenolic acids (p-hydroxybenzoic acid, protocatechuic acid, gallic acid, p-coumaric acid, caffeic acid, ferulic acid, chlorogenic acid), flavonoids (quercetin, (+)-catechin and (-)-epicatechin) and tannic acid were purchased from the Sigma Chemical Co. (St. Louis, MO.). The purity of these compounds was ascertained by reversed-phase high pressure liquid chromatography analyses at the wavelength maximum for each of these compounds. No impurities could be detected at this level of analyses. Horse radish peroxidase (Type II, from horse radish, 200 purpurogallin units per mg solid), catalase (purified powder from bovine liver, 11000 Sigma units per mg solid), superoxide dismutase (Type I, from bovine blood, 2900 Sigma units per mg protein), and tyrosinase (Grade III from mushrooms, 2000 Sigma units per mg solid) were also purchased from the Sigma Chemical Co., as were Aflatoxin B1, ferricytochrome c (Type VI, from horse heart), xanthine, xanthine oxidase (Grade III from buttermilk) potassium iodide, and ammonium molybdate. All other chemicals were reagent or equivalent grade.
Cell Cultures and Assay for Chromosome Aberrations:

The method employed was that previously described by Stich et al., 1979. CHO cell cultures were grown as 40-60% confluent cultures on 22 mm square coverslips in 35 mm diameter tissue culture dishes. These were exposed to chemicals for a period of 3 hours at 37° C. Chemicals were routinely dissolved in 2.5% MEM (MEM supplemented with 2.5% fetal calf serum) adjusted to pH 7.4, and added to culture dishes in 1 ml aliquots. Tests involving an S9 activating mixture, used 0.5 ml aliquots added to each petri dish prior to the addition of 0.5 ml of a double strength test chemical. Transition metals and enzymes were added to serially diluted stock solutions in 10 ul aliquots just prior to incubation. After incubation, test solutions were removed by gentle suction and cultures were washed twice with warmed fresh MEM, then cultured a further 16 hours in fresh medium supplemented with 10% fetal calf serum.

Chromosome aberrations were estimated after a 4 hour incubation period with 0.1 ml of colchicine (0.1% in 2.5% MEM) added to the existing 1 ml volume of 10% MEM. Cells on the 22 mm coverslips were treated with a 1% sodium citrate solution for 20 minutes, then fixed in a 3:1 ethanol/glacial acetic acid solution for 20 minutes. Air-dried slides were stained with a 2% solution of orcein in 50% acetic acid/water, dehydrated, and mounted in permount. For each sample, a minimum of 100 metaphase plates were analyzed, scoring a cell as positive if it contained at least one chromatid break or exchange. Exchanges scored included chromatid and chromosome exchanges, mono- and multi-radials, and rings (see Figure 1).

Statistical variance in the response of the CHO cell chromosome aberration test is sufficiently large to make conventional testing for standard deviation impossible. To provide some idea of the reproducibility of the test, sample compounds were tested on the same
day and again on alternate days in double blind experiments. These results are tabulated in Table 1, and provide the background spontaneous breakage rate of a cell line scored under the experimental conditions listed above.

Controls for CHO Cell Conditions:

The activity of the S9 activation mixture was assayed with the following controls. A stock solution of $2 \times 10^{-2}$ M Aflatoxin B1 (Sigma Chemical Co., St. Louis, MO.) was serially diluted to concentrations of $2 \times 10^{-4}$ M, $2 \times 10^{-5}$ M, and $1 \times 10^{-5}$ M in 2.5% MEM. 0.5 ml of these solutions were then added with 0.5 ml of the S9 mixture or MEM to a final concentration of $1 \times 10^{-4}$ M, $1 \times 10^{-5}$ M, and $5 \times 10^{-6}$ M aflatoxin B1. The S9 was deemed satisfactory if at least 80% of the metaphase plates had at least one chromosome break at a concentration of $5 \times 10^{-6}$ aflatoxin B1. Aflatoxin B1 at the same concentration, but without S9 present should not elevate the frequency of chromosome aberrations over that found in non-treated control cultures. The enzymes horseradish peroxidase, catalase, superoxide dismutase, and tyrosinase were also added separately at the same concentrations as were used with test chemicals, to check for any increase in the spontaneous frequency of chromosome breaks. A similar procedure was used for transition metal controls of copper sulphate and manganese chloride.

Preparation of S9:

The S9 mixture was prepared as described by Ames et al., 1975. Microsomal preparations were obtained from the livers of male Swiss rats that had been pretreated with Aroclor 1254. The S9 mixture consisted of liver supernatant (0.3 ml per mix), 0.4 M MgCl₂, 1.65 M KCl, glucose-6-phosphate (1.3 mg/ml mix), NADP (2.55 mg/ml mix) and 0.2 M
phosphate-buffered saline. This solution was freshly prepared and 0.5 ml was added to each petri dish prior to the addition of 0.5 ml of double strength test chemical.

**Preparation of Metal Solutions:**

0.1 M solutions of cupric sulphate, and manganese chloride were prepared with glass distilled water. These stock solutions were then diluted 10-fold in a 0.5 M stock solution of glycine. 10 µl of the resulting solution was added to each 1 ml aliquot of the test compound to give a final concentration of $1 \times 10^{-4}$ M transition metal content.

**High Pressure Liquid Chromatography:**

High Pressure Liquid Chromatography (HPLC) chromatograms were obtained using a Perkin Elmer Series 2 gradient pump, under the direction of a Perkin Elmer Sigma 10 data system. Absorption was monitored at 260 nm with a Perkin Elmer LC-55 absorption spectrophotometer. Chromatography was carried out at a flow rate of 2 ml/min on a Vydac 201 TP 5 micron reversed phase column, 4.6x250 mm. A linear gradient starting at 2% methanol, 98% glass distilled water and rising by 2% methanol per minute was employed. All solvents contained 0.1% phosphoric acid to suppress ionization of phenolic acids and permit reversed phase chromatography. This level of phosphoric acid was not found to cause chromosome aberrations in control CHO cultures.

Separation of oxidation products for testing on CHO cell chromosomes was accomplished using a Waters preparative reversed phase column on a Waters Prep LC/System 500A preparative HPLC system. Samples were eluted at a flow rate of 100 ml/min at an isocratic methanol concentration of 20%. Concentrates were obtained by rotary evaporation at 30°C until all methanol was removed. The samples so obtained were
then run on Vydac 201 TP 5 micron columns as described above for identification and purity purposes.

**Assay for Hydrogen Peroxide:**

Hydrogen peroxide \( \left( \text{H}_2\text{O}_2 \right) \) was measured using a modification of the procedure described by Wang and Nixon (1978). Colour reagent was prepared fresh each day, and contained 6% potassium iodide, 0.05 M acetic acid, and \( 2 \times 10^{-5} \) M ammonium molybdate. Aliquots (0.4 ml) of the sample to be assayed were pipetted into two separate tubes. Catalase (0.1 µg in 10 µl) was added to one sample, and the sample allowed to incubate at room temperature for a minimum of 10 minutes. This period of time was sufficient to destroy \( \text{H}_2\text{O}_2 \) in concentrations of up to 100 µM. Aliquots (0.4 ml) of colour reagent were then added to each sample, and the samples incubated at room temperature for a further 5 minutes. Absorbance resulting from the molybdate catalyzed release of \( \text{I}_3^- \) by \( \text{H}_2\text{O}_2 \) was determined at 360 nm in a dual beam Perkin Elmer Lambda 3 spectrophotometer, using the peroxide-free catalase-treated sample as a blank. \( \text{H}_2\text{O}_2 \) levels were estimated from a standard curve prepared using phosphate buffered saline/dextrose samples containing 0-100 micromolar \( \text{H}_2\text{O}_2 \) (see Figure 2). When necessary, samples were diluted before the assay to bring the \( \text{H}_2\text{O}_2 \) levels to within the range of the standard curve. Samples containing high levels of caffeic acid were also diluted before assay to bring the blank absorbance at 360 nm to within the range of the spectrophotometer.

**Assay for Superoxide:**

The method employed was a modification of that described by McCord and Fridovich, 1969. Superoxide, \( \text{O}_2^- \), formation was measured as the rate of reduction of ferricytochrome c (Type VI, from horse heart).
Figure 2:
Reference curve for the determination of H$_2$O$_2$ concentration. Known quantities of commercially-available H$_2$O$_2$ were assayed with the potassium iodide colour reagent and plotted vs. absorbance at 360 nm. The dotted line insert is a magnification of values below 6 $\mu$M of H$_2$O$_2$. This assay was linear between 1 and 80 $\mu$M, any measurements above this level had to be diluted back into this range.
reflected as an increase in absorbance at 550 nm. Caffeic acid was freshly prepared as a 1x10^{-3} M solution in 0.05 M phosphate buffer at a final pH of 5.0. One half of this mixture was taken and to it was added solid superoxide dismutase (Type I, from bovine blood) to a final concentration of 50 µg/ml. This mixture was allowed to incubate at room temperature for a minimum of one hour.

Absorbance spectra were scanned from wavelengths of 400 nm to 700 nm (the visible region) immediately prior to initiation of the assay. This precaution ensured that no oxidation of the caffeic acid solutions had occurred, since oxidation products of caffeic acid tend to absorb maximally in this range.

A cytochrome c stock solution was prepared at a concentration of 1.1x10^{-5} M in 0.05 M phosphate buffer at a pH of 7.0, wrapped in aluminum foil, and stored on ice. Using a Perkin Elmer dual beam Lambda 3 absorption spectrophotometer, 2 cuvettes (1 cm light path, 2 ml working volume) were zeroed at 550 nm with 0.9 ml of the cytochrome c mixture in each. To one of the cuvettes 0.1 ml of 0.05 M phosphate buffer pH 7.0 was added. To the other cuvette, 0.1 ml of the caffeic acid solution pH 5.0 was added. The final concentrations were thus 1x10^{-4} M caffeic acid, and 1x10^{-5} M cytochrome c in the sample cuvette and 1x10^{-5} M cytochrome c in the reference cuvette. The final pH of the solution was measured to be 6.99. Neither freshly prepared caffeic acid nor superoxide dismutase had any appreciable absorbance at 550 nm. The lid was closed and the chart recorder was begun immediately. The chart recorder was run at 60 mm/min with a full absorbance range of 0.5 optical density (OD) units.

This procedure was repeated for the caffeic acid stock solution containing superoxide dismutase. Subsequent measurements were made in an identical fashion but with cytochrome c stock solutions of pH 8.0 and
9.0. The addition of 0.1 ml of the buffered caffeic acid solution was found to decrease the pH of the cytochrome solution to pH 7.98 and 8.97 respectively.

Rates of reduction of ferricytochrome c were calculated using the equation of Kuthan and Ullrich (1982):

\[
\frac{\Delta \text{Abs} \times 1}{t} = \frac{c}{\varepsilon} \text{ at half maximum} = K_{0.5}
\]

where \( \Delta \text{Abs} = \) change in half maximum absorbance at 550 nm

\( t = \) time (in minutes) at which half maximum absorbance was reached.

\( \varepsilon = \) molar extinction coefficient of ferricytochrome c.

= \(21 \text{ mM}^{-1} \text{ cm}^{-1}\) (Van Gelder and Slater, 1962).

To test for responsiveness of the ferricytochrome c assay to the production of \(\text{O}_2^-\) and its destruction by superoxide dismutase, the following technique was employed (McCord and Fridovich, 1969). A stock solution of \(1 \times 10^{-5}\) M ferricytochrome c and \(5 \times 10^{-5}\) M xanthine was prepared in 0.05 M phosphate buffer pH 7.8 and stored on ice. 2 ml of the stock solution was pipetted into each of two cuvettes and the absorbance level was zeroed for 550 nm. To the sample cuvette was added sufficient xanthine oxidase to provide a rate of reduction of ferricytochrome c of 0.025 optical density units per minute. (20 ul of a \(10^{-7}\) M solution of xanthine oxidase). Addition of superoxide dismutase was made in 10 ul aliquots of a 0.10 \(\mu\)g/ml solution in order to determine the amount required to inhibit this rate by 50% (see Figure 3).
Figure 3:

Reference curve for the measurement of $O_2^{-}$ and its inhibition by superoxide dismutase. Curve "a" was prepared by mixing a stock solution of xanthine and xanthine oxidase with a known concentration of buffered ferricytochrome c and monitored as change in absorbance at 550 nm. This served as a $O_2^{-}$ generating system. Curve "b" was generated by adding a sufficient quantity of superoxide dismutase to inhibit the conversion of cytochrome c by 50%. This provided an assay for the activity of the enzyme and confirmed that our ferricytochrome c was active.
Electron Spin Resonance:

Electron Spin Resonance, ESR, studies were performed on a Varian E-Line Century Series Model E-109 EPR System with a TM110 cavity. The microwave cavity was tuned initially by setting the Varian E102 Microwave Bridge from standby to tune mode, with a 9.52 GHz frequency, 0.2 mW of power with the reference arm in the "off" position and the phase in the mid range. The field controller module was set with a field of 03400 gauss, and a scan range of 4.0 x 100 gauss. Using the high frequency module, the gain was set at 1.25 x 100, with a function 100 KHz and a modulation of 0.80 x 10 gauss peak to peak, and a time constant of .250 seconds.

Phenolics were observed to resonate in the range of 3290-3310 gauss field setting at a microwave frequency of 9.38 GHz. All measurements were on aqueous solutions at room temperature in Varian quartz flat cells which had been cleaned with concentrated sulphuric acid and rinsed with distilled water then acetone and blown dry.

The reference arm was locked in by inserting the flat cell and tuning the microwave cavity to maximum efficiency. The phase control was adjusted to maximum, and the bridge was switched to the operate position. The microwave power was then increased to 100 mW and the wand adjusted to bring the detector into the range of 200 KHz. The power was then reduced to 0.2 mW and the reference arm was turned until the "lock" position light came on. The microwave power could then be adjusted to the desired setting without causing the receiver level to increase.

As a control for the accuracy of the field controller module, two standards of known g-value were run under identical conditions. These were di-test-butyl nitroxide in CCl4 and crystalline diphenylpicryl-hydrazyl (DPPH). The separation of peaks could then be recorded and the field sweep tuned so that they were in agreement.
For g-value measurements, diphenylpicrylhydrazyl was used as an external standard. The DPPH sample was loaded in a quartz capillary tube and taped to a flat cell. Concurrent measurements of test chemical and DPPH were run in a dual wave cavity. Correction for slight variations in magnetic field for each side of the cavity was measured by placing samples of DPPH on both sides of the dual cavity and recording the field separation between peaks.

Phenolic samples which were oxidized enzymatically required significantly higher power to generate signals. For these samples, ESR spectra were run at 20 mW. Alkali generated signals were recorded at 1 mW of power.
RESULTS

The Clastogenic Activity of Plant Phenolics:

The ability of plant phenolics to induce chromosome damage was monitored with a conventional CHO cell chromosome short term test. Illustration of the background spontaneous breakage rate of the cell line, may be found in Table 1. Statistical variance in the response of the CHO cells to chemicals tested, is sufficiently large to make conventional testing for standard deviation impossible. Table 1 illustrates, however, that the response of the cells is consistent if the dose level is considered. The clastogenic activity (ie. the ability to cause chromosome damage) of compounds were therefore compared on the basis of the minimum dose found to illicit activity.

The clastogenic activity of some of the phenols, phenolic acids, flavonoids, and tannins in a CHO cell test system may be found listed in Table 2. This table represents a generalized survey of commonly occurring phenolics, and the dose level at which they were found to cause activity.

In the group of simple phenols, the activities of catechol and resorcinol contrast each other. At dose levels of 0.063 mg/ml catechol, a compound with two adjacent hydroxyl groups, was found to be strikingly active. This activity was virtually lost with the addition of S9 liver microsomal preparation. Resorcinol, a phenol with hydroxyl groups in the meta positions, was not active until a much higher dose of 2.0 mg/ml was reached. This activity was enhanced by the addition of S9.

A similar trend was observed within the phenolic acid groups of benzoic and cinnamic acids. p-Hydroxybenzoic acid, a benzoic acid with a single hydroxyl group in the para position, was virtually inactive even at doses as high as 25.0 mg/ml. S9 slightly enhanced its effect. Gallic acid however, with three adjacent hydroxyl groups, was extremely
TABLE 1: Reproducibility of the CHO cell chromosome aberration test.

<table>
<thead>
<tr>
<th>Plate Number</th>
<th>% Metaphases with Chromosome Aberrations</th>
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<tr>
<td></td>
<td>day 1</td>
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<tr>
<td>1</td>
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</tr>
<tr>
<td>10</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Control test: CHO cells were exposed for 3 hours to 2.5% MEM in the absence of test chemical. The background spontaneous breakage rate of the cell line on day 1, 2, and 3, may be calculated to be 0.1%, 0.2%, and 0.1%, respectively.

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>% Metaphases with Chromosome Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>day 1</td>
</tr>
<tr>
<td>2.0</td>
<td>T</td>
</tr>
<tr>
<td>1.0</td>
<td>T/MI</td>
</tr>
<tr>
<td>0.5</td>
<td>65.9</td>
</tr>
<tr>
<td>0.25</td>
<td>50.0</td>
</tr>
<tr>
<td>0.125</td>
<td>7.5</td>
</tr>
<tr>
<td>0.06</td>
<td>3.2</td>
</tr>
<tr>
<td>0.03</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Control test: CHO cells were exposed to the same serial dilutions of caffeic acid concurrently, and on separate days. T represents toxicity, and T/MI is used to describe levels of test chemicals which caused toxicity and mitotic inhibition in metaphase chromosomes which were scored.
<table>
<thead>
<tr>
<th>Phenolic Group</th>
<th>Compound</th>
<th>Concentration (mg/ml)</th>
<th>Activation</th>
<th>% Metaphases with Chromosome Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple Phenols</td>
<td>Catechol</td>
<td>0.063</td>
<td>-S9</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>Resorcinol</td>
<td>2.0</td>
<td>-S9</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>10.0</td>
</tr>
<tr>
<td>Phenolic Acids</td>
<td>p-Hydroxybenzoic</td>
<td>25.0</td>
<td>-S9</td>
<td>0.0</td>
</tr>
<tr>
<td>i)</td>
<td>Acid</td>
<td></td>
<td>+S9</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>Gallic Acid</td>
<td>0.032</td>
<td>-S9</td>
<td>50.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>3.1</td>
</tr>
<tr>
<td>ii)</td>
<td>p-Coumaric Acid</td>
<td>5.0</td>
<td>-S9</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>Caffeic Acid</td>
<td>0.25</td>
<td>-S9</td>
<td>50.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Chlorogenic Acid</td>
<td>0.25</td>
<td>-S9</td>
<td>45.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>2.2</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>Quercetin</td>
<td>0.125</td>
<td>-S9</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>Rutin</td>
<td>2.5</td>
<td>-S9</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Catechin</td>
<td>2.5</td>
<td>-S9</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>Epicatechin</td>
<td>0.8</td>
<td>-S9</td>
<td>24.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>4.1</td>
</tr>
<tr>
<td>Tannins</td>
<td>Tannic Acid</td>
<td>0.125</td>
<td>-S9</td>
<td>51.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+S9</td>
<td>12.4</td>
</tr>
</tbody>
</table>

CHO cells were exposed for 3 hours to the phenolic compounds and sampled 20 hours later for chromosome analysis. The concentrations tabulated are the minimum concentrations of test chemical observed to illicit chromosome damage in a dose response experiment.
clastogenic at a dose level as low as 0.032 mg/ml. This activity was abolished by S9. The cinnamic acid, p-coumaric acid with one hydroxyl group, was low in clastogenicity. The addition of S9 increased its effect slightly at dose levels of 5.0 mg/ml. Caffeic and chlorogenic acids shared virtually the same chromosome damaging effect. Both were active at low doses of 0.063 mg/ml and this effect was abolished when S9 was present. Structurally they are similar, with chlorogenic acid containing an additional quinic acid group attached to its carboxylic acid group. Ferulic acid, similar to caffeic acid in structure but with a methyl group substituted for one of the ortho position hydroxyl groups, was significantly less active. Aberrations were produced at a concentration of 25.0 mg/ml, and these could be eliminated by the addition of S9.

In the flavonoid group of phenolics, the flavonol, quercetin, was relatively active at a concentration of 0.125 mg/ml. This activity was lost in the presence of S9. Rutin, a flavonol glycoside, was not significantly active, even at a concentration of 2.5 mg/ml. Because of its insolubility in aqueous solution, no testing of higher concentrations could be attempted within the experimental guidelines set down in Materials and Methods.

The flavan-3-ols, (+)-catechin and (-)-epicatechin, are important components in tannin chemistry. They represent the two most common forms found in plants, of the four possible stereoisomers of the compound. Interestingly, (-)-epicatechin is significantly more active than (+)-catechin, causing chromosome aberrations at a concentration of 0.8 mg/ml. S9 appeared to decrease this activity. Commercially available tannic acid, which is primarily composed of hydrolysable tannins, was quite active at a concentration of 0.125 mg/ml. The addition of S9 reduced this activity, but did not entirely eradicate it.
Oxidation Effects:

The activity of some of these compounds was found to alter if the pH of the reaction mixture was allowed to become alkaline before bringing it back to pH 7.4 or if the preparation time was increased to several hours or even days. Caffeic and chlorogenic acids may be used as examples of this effect. In Tables 3 and 4, the activities of freshly prepared chemical is compared with reaction mixtures allowed to incubate 1 and 3 days at room temperature. These activities are also compared with solutions dissolved at pH 10 and allowed to return to pH 7.4 after a 1 hour incubation period at room temperature. The activity of oxidized solutions was observed to be two to four-fold greater in both cases. This oxidation effect was only observed to occur in phenolics possessing adjacent hydroxyl groups.

That some change had occurred could be readily observed by the dramatic change in colour of the compounds. Figure 4 illustrates the change in the visible absorbance spectrum of caffeic acid solutions at various pH values within 30 minutes of preparation. An aqueous solution of caffeic acid at high pH was observed to change from a colourless to a bright yellow liquid and finally to a brown coloured solution with some precipitate. The rate at which these colour changes took place was pH dependent. At higher pH values (pH greater than 12.0) the entire process was complete in about 3 hours.

Transition metals were observed to enhance both the colour change and the clastogenic activity of these compounds. In Table 5, the clastogenic activities of several phenolics are shown in comparison with their activity in the presence of copper and manganese metals. In all cases of compounds containing adjacent hydroxyl groups, the clastogenic activity of these compounds was significantly increased.
TABLE 3: The relative clastogenic activity of oxidized solutions of Caffeic Acid.¹

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>% Metaphases with Chromosome Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>freshly prepared</td>
</tr>
<tr>
<td>2.0</td>
<td>T</td>
</tr>
<tr>
<td>1.0</td>
<td>T/MI</td>
</tr>
<tr>
<td>0.5</td>
<td>65.9</td>
</tr>
<tr>
<td>0.25</td>
<td>50.0</td>
</tr>
<tr>
<td>0.125</td>
<td>7.5</td>
</tr>
<tr>
<td>0.063</td>
<td>3.2</td>
</tr>
<tr>
<td>0.032</td>
<td>0.0</td>
</tr>
<tr>
<td>0.016</td>
<td>0.0</td>
</tr>
</tbody>
</table>

¹ CHO cells were exposed to freshly prepared and autooxidized solutions of caffeic acid for 3 hours and sampled 16 hours later for chromosome analysis.

² Caffeic acid was prepared as for the freshly prepared solution, at pH 7.0, but allowed to incubate at room temperature for 1 and 3 days.

³ pH altered solutions were prepared by increasing the pH level of the caffeic acid solution to 12.0 for 1 hour at room temperature, before returning it to pH 7.0.
TABLE 4: The relative clastogenic activity of oxidized solutions of Chlorogenic Acid.¹

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>% Metaphases with Chromosome Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>freshly prepared</td>
</tr>
<tr>
<td>2.0</td>
<td>T</td>
</tr>
<tr>
<td>1.0</td>
<td>T/MI</td>
</tr>
<tr>
<td>0.5</td>
<td>55.0</td>
</tr>
<tr>
<td>0.25</td>
<td>45.0</td>
</tr>
<tr>
<td>0.125</td>
<td>2.1</td>
</tr>
<tr>
<td>0.063</td>
<td>0.0</td>
</tr>
<tr>
<td>0.016</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ CHO cells were exposed to freshly prepared and autooxidized solutions of chlorogenic acid for 3 hours and sampled 16 hours later for chromosome analysis.

² Chlorogenic acid was prepared as for the freshly prepared solution at pH 7.0, but allowed to incubate at room temperature for 1 and 3 days.

³ pH altered solutions were prepared by increasing the pH level of the chlorogenic acid solution to 12.0 for 1 hour at room temperature, before returning it to pH 7.0.
Caffeic acid absorbance spectrum at various pH values. Curves a, b, c, d, e, and f were generated at pH values of 10.0, 9.0, 8.0, 7.0, 6.0 and 5.0 respectively. All solutions were at equal concentration and were measured within 30 minutes of preparation. Solutions at higher pH levels were observed to turn to a bright yellow colour which faded to a dark brown with time.
### TABLE 5: Relative clastogenic activities of phenolics in the presence of transition metals.\(^1\)

<table>
<thead>
<tr>
<th>Phenolic Group</th>
<th>Compound</th>
<th>Concentration (mg/ml)</th>
<th>% Metaphases with Chromosome Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>none(^2)</td>
</tr>
<tr>
<td>Simple Phenols</td>
<td>Catechol</td>
<td>0.063</td>
<td>32.0</td>
</tr>
<tr>
<td></td>
<td>Resorcinol</td>
<td>2.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Phenolic Acids</td>
<td>p-Hydroxybenzoic Acid</td>
<td>25.0</td>
<td>0.0</td>
</tr>
<tr>
<td>i) benzoic</td>
<td>Gallic Acid</td>
<td>0.032</td>
<td>50.5</td>
</tr>
<tr>
<td>ii) cinnamic</td>
<td>p-Coumaric Acid</td>
<td>5.0</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Caffeic Acid</td>
<td>0.25</td>
<td>50.0</td>
</tr>
<tr>
<td></td>
<td>Chlorogenic Acid</td>
<td>0.25</td>
<td>45.0</td>
</tr>
<tr>
<td></td>
<td>Ferulic Acid</td>
<td>25.0</td>
<td>10.2</td>
</tr>
</tbody>
</table>

1 CHO cells were exposed for 3 hours to the phenolic compounds with or without the transition metals present. They were then sampled 20 hours later for chromosome analysis.

2 Cu\(^{+2}\) represents a 10\(^{-4}\) M final concentration of CuSO\(_4\) during the 3 hour incubation period with CHO cells. This concentration was not found to induce chromosome aberrations when treated alone.

3 Mn\(^{+2}\) represents a 10\(^{-4}\) M final concentration of MnCl\(_2\) during the 3 hour incubation period with CHO cells. This concentration was not found to induce chromosome aberrations when treated alone.

T/MI is used to describe dose levels of test chemicals which caused toxicity and mitotic inhibition in metaphase chromosomes which were scored.
This effect was not observed in phenolics with single hydroxyl groups, hydroxyl groups in the meta or para positions with respect to each other, or with groups possessing methyl substituted hydroxyl groups. Manganese appeared to show a more dramatic enhancing effect than when copper was present. All levels of metals tested were within dose ranges not found to cause aberrations by themselves (10^-4 M).

**Enzymatic Oxidation:**

Oxidizing enzymes were subsequently used to observe their effect on the induction of chromosomal damage in CHO cells by phenolic compounds. Caffeic, chlorogenic, and gallic acids (compounds with two or more adjacent hydroxyl groups) were incubated in the presence of horseradish peroxidase, superoxide dismutase, tyrosinase (monophenol oxidase), and catalase, each at a concentration of 1.0 µg/ml. This concentration was not found to induce genetic damage, when used alone.

Table 6 lists the percent cells with metaphase chromosomes altered in the presence of these compounds and enzymes. Similar effects were observed with all three phenolics. Catalase was observed to reduce some of the toxicity of the three compounds. Its effect was only slight, however, in freshly prepared solutions. Superoxide dismutase, and tyrosinase, on the other hand, appeared to enhance the clastogenic activity of these compounds. Tyrosinase was observed to change the colour of all three compounds to a brown colour within one hour of its addition at this concentration.

Concurrent application of catalase and superoxide dismutase, both at concentrations of 1.0 µg/ml, was found to reduce the clastogenic activities of caffeic, chlorogenic, and gallic acids. Table 7 lists the results of this experiment. These activities were observed to be below levels measured when only one of the enzymes was present. More
TABLE 6: Relative clastogenic activities of phenolics in the presence of enzymes.¹

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mg/ml)</th>
<th>% Metaphases with Chromosome Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Without Cat.</td>
</tr>
<tr>
<td>Caffeic Acid</td>
<td>0.5</td>
<td>T/MI</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>T/MI</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>8.0</td>
</tr>
<tr>
<td></td>
<td>0.063</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>0.032</td>
<td>0.0</td>
</tr>
<tr>
<td>Chlorogenic Acid</td>
<td>0.5</td>
<td>T/MI</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>52.0</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>0.063</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>0.032</td>
<td>MI</td>
</tr>
<tr>
<td>Gallic Acid</td>
<td>0.5</td>
<td>T</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>T/MI</td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>T/MI</td>
</tr>
<tr>
<td></td>
<td>0.063</td>
<td>MI</td>
</tr>
<tr>
<td></td>
<td>0.032</td>
<td>51.2</td>
</tr>
<tr>
<td></td>
<td>0.016</td>
<td>31.6</td>
</tr>
<tr>
<td></td>
<td>0.008</td>
<td>0.0</td>
</tr>
</tbody>
</table>

¹ CHO cells were exposed to freshly prepared solutions of caffeic, chlorogenic, and gallic acids with and without enzyme for 3 hours. They were then sampled 20 hours later for chromosome analysis.

T, and T/MI are used to describe dose levels of test chemicals which caused toxicity and mitotic inhibition in metaphase chromosomes which were scored.

Abbreviations are as follows: Cat. for catalase, SOD for superoxide dismutase, HRP for horseradish peroxidase, and Tyro. for tyrosinase. All enzymes were at a concentration of 1.0 μg/ml, a concentration found not to induce chromosome aberrations when treated alone.
TABLE 7: Relative clastogenic activities of caffeic acid in the presence of concurrent applications of catalase and superoxide dismutase, and catalase with horseradish peroxidase.\(^1\)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mg/ml)</th>
<th>% Metaphases Without Enzyme</th>
<th>With CAT &amp; SOD</th>
<th>Aberrations</th>
<th>With CAT &amp; HRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caffeic Acid</td>
<td>0.5</td>
<td>T/MI</td>
<td>MI</td>
<td>MI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>65.0</td>
<td>21.3</td>
<td>64.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>8.0</td>
<td>4.0</td>
<td>21.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.063</td>
<td>3.4</td>
<td>0.0</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.032</td>
<td>0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chlorogenic Acid</td>
<td>0.5</td>
<td>T/MI</td>
<td>MI</td>
<td>MI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>52.0</td>
<td>11.2</td>
<td>32.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>2.8</td>
<td>3.1</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.063</td>
<td>0.0</td>
<td>0.1</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.032</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Gallic Acid</td>
<td>0.5</td>
<td>T</td>
<td>T/MI</td>
<td>T/MI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>T/MI</td>
<td>MI</td>
<td>T/MI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.125</td>
<td>T/MI</td>
<td>38.9</td>
<td>T/MI</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.063</td>
<td>MI</td>
<td>21.4</td>
<td>34.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.032</td>
<td>51.2</td>
<td>7.5</td>
<td>26.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.016</td>
<td>31.6</td>
<td>0.0</td>
<td>5.4</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\) CHO cells were exposed to freshly prepared solutions of caffeic, chlorogenic, and gallic acids, in the presence of catalase with either superoxide dismutase or horseradish peroxidase for 3 hours. They were then sampled 20 hours later for chromosome analysis. The enzymes were present at concentrations of 1.0 μg/ml, a concentration found not to cause aberrations when present alone. Abbreviations are as follows: CAT for catalase, SOD for superoxide dismutase and HRP for horseradish peroxidase.

T, and T/MI are used to describe dose levels of test chemicals which caused toxicity and mitotic inhibition in metaphase chromosomes which were scored.
importantly, much of the toxicity of superoxide-treated samples is reduced by the presence of catalase. Also listed in Table 7 are the results of concurrent application of 1.0 μg/ml each of catalase and horseradish peroxidase. These results appeared very similar to ones produced by catalase alone.

It is interesting to note that these enzymes were not sufficient to completely eliminate the clastogenic activity of the phenolic compounds tested. This would suggest that peroxide and superoxide levels were in excess of 500 μM and 100 μM/min, respectively, in the presence of catalase and superoxide dismutase for caffeic acid, chlorogenic acid and gallic acid. Concurrent application of the enzymes alone was found not to induce chromosome damage.

In a separate experiment, caffeic acid that had been allowed to incubate at room temperature for 3 days was tested in the presence of catalase (1.0 μg/ml). Here, catalase completely eliminated its activity on CHO cell chromosomes. The results are listed in Table 8.

Chromatographic Separation:

Oxidation products were chromatographically separated from caffeic acid using high pressure liquid chromatography (HPLC). Figure 5 demonstrates HPLC profiles of freshly prepared, 1 day, 3 day, and 6 day aqueous solutions of caffeic acid prepared at neutral pH. Here it can be seen that the main peak at a retention time of 13.8 minutes in the freshly prepared solution diminishes in size with subsequent formation of multiple peaks over the time period examined. Autooxidation appeared to reduce the main peak from greater than 98% of the total solution chromatographed, to approximately 45% of the final total, as judged by absorbance at 260 nm, over a period of 6 days. Samples incubated for the
<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>% Cells with Aberrations no catalase</th>
<th>+catalase</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>T</td>
<td>0.5</td>
</tr>
<tr>
<td>0.5</td>
<td>22.0</td>
<td>1.0</td>
</tr>
<tr>
<td>0.25</td>
<td>23.0</td>
<td>0.5</td>
</tr>
<tr>
<td>0.125</td>
<td>22.6</td>
<td>-</td>
</tr>
<tr>
<td>0.063</td>
<td>3.0</td>
<td>-</td>
</tr>
<tr>
<td>0.032</td>
<td>1.0</td>
<td>-</td>
</tr>
<tr>
<td>0.016</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

CHO cells were exposed to oxidized preparations of caffeic acid with and without catalase enzyme for 3 hours. They were then sampled 20 hours later for chromosome analysis. Caffeic acid was allowed to autooxidize at room temperature at pH 7.0 for 3 days before incubation with CHO cells. Catalase was at a concentration of 1.0 µg/ml, a concentration found not to induce chromosome aberrations.
Figure 5:

High pressure liquid chromatography separation of oxidized solutions of caffeic acid. Chromatograms were prepared by measuring the absorbance at 260 nm of products separated on a Vydac 201 TP 5 micron column. Separation was obtained with a 2% methanol per minute gradient starting with 2% methanol, 98% glass distilled water. All solvents contained 0.1% phosphoric acid. The solution assayed was a 1 mg/ml preparation of caffeic acid in water, which was allowed to incubate at room temperature for the times specified. Note the reduction of the main caffeic acid peak at 13 minutes, with time.
UNOXIDIZED CAFFEIC ACID

1 DAY OXIDATION

3 DAY OXIDATION

6 DAY OXIDATION

ABSORBANCE AT 260 NM

RETENTION TIME (MINUTES)
same period of time, which had been previously purged with nitrogen gas and stored in air-tight containers were not observed to develop any of the oxidation peaks observed above.

These oxidation products were chromatographically separated on a Waters preparative reversed phase column, rotary evaporated and tested for clastogenic activity in the CHO cell test system. To verify that these components had not been altered by the extraction procedure, samples were re-chromatographed on Vydac reversed-phase columns. No apparent degradation could be observed at an absorbance of 260 nm. Overlap of components in this procedure accounted for about 10% of the total peak recovered. Relative concentrations of components to be tested were obtained by calculating the percentage of the total oxidized solution chromatographed, and comparing the areas under the curve obtained for each component. None of the individual components or combinations of major components were observed to exhibit clastogenic activity even at dose levels 3 times greater than in a 1.0 mg/ml solution. Recombination of all components, also failed to elicit genotoxic effects in the CHO cell system at these dose levels.

Oxidation Intermediates

i) Generation of Peroxide:

Oxidation intermediates were measured in a number of ways. \text{H}_2\text{O}_2 was found to be present in oxidized solutions of caffeic acid, using a modified potassium iodide assay. The standard curve for this assay may be found in Figure 2. Here it can be observed that an increase in absorbance of the potassium iodide mixture correlated linearly with an increase in \text{H}_2\text{O}_2 content in the range of 1 to 100 \text{μM}.

Caffeic acid's production of \text{H}_2\text{O}_2 may be found graphically represented in Figure 6. During autooxidation a 1 mg/ml solution was
Figure 6:

The accumulation of H$_2$O$_2$ with time in a 1 mg/ml solution of caffeic acid. H$_2$O$_2$ content was assayed in the potassium iodide assay and plotted over a duration of 6 days. The caffeic acid solution was prepared in water and allowed to incubate at room temperature for the times specified.
found to contain approximately 60 μM of H$_2$O$_2$ after 1 day, 180 μM after 3 days, and 1200 μM after 6 days. The generation of H$_2$O$_2$ was not stoichiometrically related to the disappearance of caffeic acid as judged by HPLC separation at an absorbance of 260 nm.

Table 9 lists the amount of H$_2$O$_2$ present at concentrations of caffeic acid tested in the CHO cell assay. A pattern of increased clastogenic activity was found to parallel the early process of autooxidation in caffeic acid, however, this was followed by a decline in activity in the 3 day and 6 day solutions. That H$_2$O$_2$ must play some role in the observed activity is substantiated by a loss of activity in 3 day solutions upon the addition of a solution of 1.0 μg/ml catalase (Table 10). Table 11 documents the clastogenic activity of a stock H$_2$O$_2$ solution. Here it may be seen that a similar pattern of chromosome damage can be produced with concentrations of 10 to 50 μM H$_2$O$_2$, levels found in a 3 day solution of caffeic acid. H$_2$O$_2$ does not, however, appear to account for the ability of freshly prepared caffeic acid to damage chromosomes.

ii) Generation of Superoxide:

The production of another oxidation intermediate, the superoxide or O$_2^\cdot$ free radical, was measured using a ferricytochrome c assay. This assay measures the change in absorbance at 550 nm of a ferricytochrome c mixture as it changes from its oxidized to its reduced form. Superoxide dismutase was used to measure any background changes.

In Figure 7, the production of O$_2^\cdot$ can be found illustrated at pH values of 7.0, 8.0, and 9.0. The rate of production of O$_2^\cdot$ is pH
<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>$\text{H}_2\text{O}_2$ Content (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>freshly prepared</td>
</tr>
<tr>
<td>1.0</td>
<td>17.0</td>
</tr>
<tr>
<td>0.5</td>
<td>9.0</td>
</tr>
<tr>
<td>0.25</td>
<td>4.0</td>
</tr>
<tr>
<td>0.125</td>
<td>2.0</td>
</tr>
<tr>
<td>0.063</td>
<td>1.0</td>
</tr>
<tr>
<td>0.032</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>0.016</td>
<td>&lt;1.0</td>
</tr>
<tr>
<td>0.008</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

1 Hydrogen peroxide content was determined using a modified potassium iodide assay on the concentrations of caffeic acid listed. All values were obtained using the standard curve outlined in Figure 2. Autoxidized solutions were prepared by making up solutions of caffeic acid at pH 7.0 and allowing them to incubate at room temperature for a period of 3 days.
TABLE 10: The clastogenic activity of freshly prepared and autooxidized caffeic acid. ¹

<table>
<thead>
<tr>
<th>Concentration (mg/ml)</th>
<th>% Cells with Aberrations</th>
<th></th>
<th>3 day solution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>freshly prepared</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>no catalase</td>
<td>+catalase</td>
<td></td>
</tr>
<tr>
<td>1.0</td>
<td>T/MI</td>
<td>T/MI</td>
<td>T</td>
</tr>
<tr>
<td>0.5</td>
<td>12.3</td>
<td>8.2</td>
<td>T</td>
</tr>
<tr>
<td>0.25</td>
<td>3.8</td>
<td>1.0</td>
<td>MI</td>
</tr>
<tr>
<td>0.125</td>
<td>1.0</td>
<td>-</td>
<td>MI</td>
</tr>
<tr>
<td>0.063</td>
<td>0.5</td>
<td>-</td>
<td>18.4</td>
</tr>
<tr>
<td>0.032</td>
<td>-</td>
<td>-</td>
<td>1.0</td>
</tr>
</tbody>
</table>

¹ CHO cells were exposed to freshly prepared and 3-day oxidized solutions of caffeic acid, with or without catalase enzyme present, for 3 hours. They were then sampled 20 hours later for chromosome analysis. Catalase was used at a concentration of 1.0 µg/ml, a concentration found not to induce chromosome aberrations when present alone.
TABLE II: The clastogenic activity of commercially available hydrogen peroxide.1

<table>
<thead>
<tr>
<th>Peroxide Concentration (µM)</th>
<th>% Cells with Aberrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no catalase</td>
</tr>
<tr>
<td>500</td>
<td>T</td>
</tr>
<tr>
<td>250</td>
<td>T</td>
</tr>
<tr>
<td>125</td>
<td>MI</td>
</tr>
<tr>
<td>62</td>
<td>MI</td>
</tr>
<tr>
<td>31</td>
<td>18.4</td>
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<tr>
<td>16</td>
<td>1.0</td>
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<tr>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>-</td>
</tr>
</tbody>
</table>

1 CHO cells were exposed for 3 hours to commercially available hydrogen peroxide and sampled 3 hours later for chromosome aberrations. Catalase was present at a concentration of 1.0 µg/ml, a concentration found not to induce chromosome aberrations when present alone.
Figure 7:

The rate of production of $O_2^-$ in a 1 ml aliquot of a $10^{-4}$ M caffeic acid solution at various pH values. Curve "a" was generated by adding caffeic acid to a stock solution of ferricytochrome c at a final pH level of 8.97 and monitoring the change in absorbance at 550 nm. Curves "b" and "c" were prepared in an identical fashion but were added to buffered solutions of ferricytochrome c at final pH values of 7.98 and 6.99, respectively. The rate of production of $O_2^-$ was calculated at half maximum absorbance, using a molar extinction coefficient of $21 \text{ mM}^{-1}\text{cm}^{-1}$. 
dependent, being greatest at the higher pH values. Using a molar extinction coefficient of 21 mM$^{-1}$ cm$^{-1}$ (Van Gelder and Slater, 1962) the rate of production of $O_2^-$ can be calculated using the following formula:

$$\frac{\Delta \text{Abs.}}{t} \times \frac{1}{21} = \text{concentration of ferricytochrome c}_{\frac{1}{2}} \text{ at half maximum} = K_{0.5}$$

where \( \Delta \text{Abs.} \) = change in half maximum absorbance at 550 nm
\( t \) = time (in minutes) at which half maximum absorbance was reached.

\( K_{0.5} \) was calculated to be approximately 1.42 \( \mu \)M/min at pH 7.0, 2.86 \( \mu \)M/min at pH 8.0 and 3.81 \( \mu \)M/min at pH 9.0 for a $1 \times 10^{-4}$ M solution of caffeic acid. These values are expressed in terms of rate of production of $O_2^-$ at one half the maximum change in absorbance.

iii) Generation of Phenolic Free Radicals:

Aside from the production of intermediate $H_2O_2$, and $O_2^-$, the parent phenolic molecule was also found to undergo a transition to a free radical state. This could be most easily visualized by absorbance wavelength scans of caffeic and gallic acids at higher pH values (pH 9.0), or by oxidative degradation with selected enzymes.

Figure 8 demonstrates the change in the absorption spectrum of a $10^{-4}$ M solution caffeic acid at pH 9.0 over a 30 minute interval. Wavelength maximums at approximately 280 and 315 nm were found to degenerate with a new maximum forming at 415 nm. An almost identical
Figure 8:

The change in absorbance in caffeic acid at a pH of 9.0. The straight line indicates the absorbance spectrum of an aqueous solution of caffeic acid (pH 9.0) immediately after the preparation. The dashed line illustrates the spectrum of the same solution at pH 9.0, 30 minutes after preparation.
profile (Figure 9) was generated by the addition of 33 μg/ml horseradish peroxidase and $10^{-2}$ M $H_2O_2$. Note that no oxidation took place until both the enzyme and the peroxide were present.

Figures 10 and 11 show similar results for gallic acid. A wavelength maximum at 260 nm was observed to degenerate with subsequent production of a new peak at 415 nm at high pH (9.0) and by enzymatic oxidation with horseradish peroxidase.

To identify if phenolic free radical molecules were present, electron spin resonance (ESR) was performed on several of the phenolic molecules under a variety of conditions. As was expected, no ESR signal could be detected for the monohydroxy phenols, m- and p-coumaric, and ferulic acids even under conditions of high pH (pH greater than 12.0) and maximum input power (100 mWatts or mW) (Figure 12).

The phenolics possessing adjacent hydroxyl groups, however, generated distinct signals under the conditions tested. Figure 12 illustrates the primary phenolic free radical of protocatechuic acid, or 2,3-dihydroxybenzoic acid.

This signal was generated with 1 mW of microwave power and had a gain value of $1.25 \times 10^4$. It has 8 distinct hyperfine structures, 4 arranged on each side of a symmetry point. The pattern is characteristic of a molecule with 3 unequal interacting nuclei, each with a spin of 0.5. The $g$ value may be calculated to be $2.0042 \pm 0.0002$ based on the $g$-value of $2.0036 \pm 0.0002$ for DPPH (Kittel, 1971).

Values of hyperfine coupling constants for protocatechuic acid are given below. These were obtained by comparing the separation of peaks in terms of gauss distribution with published values. Complete overlap of published values with ones recorded in this study would indicate that hyperfine constants were completely analogous to the first free radical derived from oxidation of protocatechuic acid.
Figure 9:

The change in absorbance of caffeic acid when incubated with a solution of 33 μg/ml horseradish peroxidase and 10^{-2}M H_2O_2, at pH 7.0. The straight line indicates the absorbance spectrum of an aqueous solution of caffeic acid at pH 7.0. The dashed line is the curve generated 30 minutes after the addition of peroxide and peroxidase enzyme. Addition of peroxide or peroxidase enzyme alone was not found to change the absorbance spectrum from the one measured at pH 7.0 alone.
Absorbance (O.D. units)

Wave length (nm)
The change in absorbance of gallic acid at a pH of 9.0. The straight line indicates the absorbance spectrum of an aqueous solution of gallic acid, pH 9.0, immediately after preparation. The dashed line illustrates the spectrum of the same solution at pH 9.0, 30 minutes after preparation.
The change in absorbance of gallic acid when incubated with a solution of 33 μg/ml horseradish peroxidase and 10^{-2} M H_{2}O_{2}, at pH 7.0. The straight line indicates the absorbance spectrum of an aqueous solution of gallic acid at pH 7.0. The dashed line is the curve generated 30 minutes after the addition of peroxide and peroxidase enzyme. Addition of peroxide or peroxidase enzyme alone was found not to change the absorbance spectrum from the one measured at pH 7.0 alone.
Figure 12:

ESR spectra of $10^{-2}$ M solutions of "a" (m-coumaric acid) "b" (p-coumaric acid) "c" (ferulic acid) and "d" (protocatechuic acid) at pH 12.0. Measurements were made at 100 mW of power for "a", "b", and "c", and at 1 mW of power for "d". The gain setting was $1.25 \times 10^4$, with a modulation amplitude of 0.2 G, and a time constant of 0.25 seconds at a field setting of 3294 G, and microwave frequency of 9.45 GHz. All measurements were at room temperature and modulation frequency of 100 KHz. Signal for "a", "b", and "c" could be observed even at higher gain settings of $1.25 \times 10^4$. 
Hyperfine coupling constants of protocatechuic acid:

$$\begin{align*}
\text{Hyperfine Coupling (G)} \\
\text{pH} & \quad a_2^H & a_5^H & a_6^H \\
12.0 & 0.5 & 3.5 & 0.9
\end{align*}$$

from: Kalyanaraman and Sealy, 1982.

Figure 13 represents the phenolic free radical signal of gallic acid at pH 12.0. This signal was relatively strong and could be detected with a receiver gain setting of only $5.0 \times 10$ at 1 mW of microwave power. The pattern obtained is distinctive and may be referred to as a 1:2:1 hyperfine structure based on the ratio of its peak heights. The ESR hyperfine pattern of gallic acid is consistent for a molecule with 2 equal nuclei, each with a spin value of 0.5. The g-value calculated for gallic acid was $2.0049 \pm 0.0002$ based on the g-value of 2.0036 for DPPH (Kittel, 1971).

At pH 7.75, no signal could be detected (Figure 14) even at a receiver gain level of $1.25 \times 10^4$. This was true even in samples allowed to autooxidize at room temperature for several days. However, the addition of 50 µg/ml horseradish peroxidase and $10^{-2} \text{M } \text{H}_2\text{O}_2$ to freshly prepared gallic acid, led to the generation of the distinctive 1:2:1 hyperfine pattern. The signal generated in Figure 15 was considerably weaker than the one generated at high pH, and required
Figure 13:

ESR spectra of "a" ($10^{-2}$ M gallic acid at pH 12.0) and "b" (crystalline diphenylpicrylhydrazyl or DPPH). Measurements were made at 1 mW of microwave power, with a gain of 50, modulation amplitude of $3.2 \times 10^{-2}$ G, time constant of 1.0 second at a field setting of 3355 G and microwave frequency of 9.45 GHz. All measurements were made at room temperature and modulation frequency of 100 KHz.
Figure 14:

ESR spectra of 10^{-2} M solutions of "a" (freshly prepared gallic acid at pH 7.75) and "b" (gallic acid at pH 7.75 after 3 days). Measurements were made at 20 mW of microwave power, with a gain of 1.25 x 10^4, modulation amplitude of 4.0 x 10^{-1} G, time constant of 1.0 sec at a field setting of 3355 G and microwave frequency of 9.45 GHz. All measurements were made at room temperature and modulation frequency of 100 KHz.
Figure 15:

ESR spectra of $10^{-2}$ M solutions of gallic acid at pH 7.75. Figure "a" is the signal generated in the absence of enzyme and figure "b" is the signal obtained upon addition of 33 µg/ml horseradish peroxidase and $10^{-2}$ M $\text{H}_2\text{O}_2$. Measurements were made at 20 mW of microwave power, with a gain of $1.24 \times 10^4$, modulation amplitude of $4 \times 10^{-1}$ G, time constant of 1.0 sec at a field setting of 3355 G and microwave frequency of 9.45 GHz. All measurements were made at room temperature and a modulation frequency of 100 KHz.
receiver gain levels of $1.25 \times 10^4$. Some of the structure of the third peak appears lost in the background noise at this level. An analogous structure was derived by the addition of tyrosinase to a freshly prepared solution of gallic acid. It is represented in Figure 16.

In the case of gallic acid, values for hyperfine coupling constants were observed to be identical for signals generated by high pH and by the enzymes, peroxidase and tyrosinase. Values were assigned by comparing the separation of peaks observed in this study with those of published values. The hyperfine constants of gallic acid are given below:

<table>
<thead>
<tr>
<th></th>
<th>$a_2$</th>
<th>$a_6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.0</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>$7.5^a$</td>
<td>0.10</td>
<td>0.10</td>
</tr>
</tbody>
</table>

$a$: in the presence of peroxidase and tyrosinase as outlined in Materials and Methods.

from: coupling constants calculated by Dixon and Murphy, 1975.

Figure 17 represents the phenolic free radical signal of caffeic acid at pH 12.0. Again this signal was relatively strong and could be detected at a receiver gain level of $8 \times 10^3$ at 1 mW of microwave power. This pattern is exceedingly complex and is approximately what
Figure 16:
ESR spectra of $10^{-2}$ M solutions of gallic acid at pH 7.75. Figure "a" is the signal generated in the absence of enzyme and figure "b" is the signal obtained upon addition of 50 µg/ml tyrosinase (monophenol oxidase). Measurements were made at 20 mW of microwave power, with a gain of $1.24 \times 10^4$, modulation amplitude of $4 \times 10^{-1}$ G, time constant of 1.0 sec at a field setting of 3355 G and microwave frequency of 9.45 GHz. All measurements were made at room temperature and amodulation frequency of 100 KHz.
Figure 17:

ESR spectra of "a" (crystalline DPPH) and "b" (10^{-2} M caffeic acid at pH 12.0). Measurements were made with 1 mW of microwave power, with a gain of 8 x 10^7, modulation amplitude of 8 x 10^{-2} G, time constant of 3.0 sec at a field setting of 3355 G and microwave frequency of 9.45 GHz. All measurements were made at room temperature and a modulation frequency of 10 KHz.
one would expect for a molecule with 5 unequal nuclei, each with a spin value of 0.5. Twenty nine of the 32 expected peaks \((2^5 = 32)\) are resolved. If one alters the modulation amplitude to a setting of \(8 \times 10^{-1}\), and the time constant to 1.0 second, one is able to observe the main 7 peaks underlying its structure (Figure 18). This signal profile is important when comparing spectra of enzymatically-derived free radicals of caffeic acid.

At pH 7.5, no signal could be detected even at maximum receiver gain level. The addition of 50 \(\mu g/ml\) horseradish peroxidase and \(10^{-2} M\) \(H_2O_2\) led to the generation of the signal illustrated in Figure 19. This signal is considerably weaker and required maximum gain levels of \(2.5 \times 10^4\). Inherent in this spectra are the same 7 main peaks observed at alkaline pH. This was also observed to be the case when the enzyme, tyrosinase, was present (Figure 20).

Using peak separation diagrams of published values, hyperfine coupling constants were obtained and are listed below for caffeic acid.

\[
\begin{array}{cccccccc}
\text{pH} & a_2H & H & a_6H & H & a_\alpha & a_\beta \\
12.0 & 0.023 & 0.119 & 0.282 & 0.130 & 0.236 \\
7.5^a & 0.022 & 0.119 & 0.283 & 0.130 & 0.237 \\
\end{array}
\]

\(a\): in the presence of peroxidase and tyrosinase, as outlined in Materials and Methods.

coupling constants calculated from: Dixon et al., 1975; Ashworth, 1976.
Figure 18:

ESR spectra of a $10^{-2}$ M solution of caffeic acid at pH 12.0. The measurement was made at 1 mW of microwave power, with a gain of $2.5 \times 10^3$, modulation amplitude of $8 \times 10^{-4}$ G, time constant of 1.0 sec at a field setting of 3355 G and microwave frequency of 9.45 GHz. All measurements were made at room temperature and a modulation frequency of 100 KHz.
Figure 19:

ESR spectra of $10^{-2}$ M solutions of caffeic acid at pH 7.5. Figure "a" is the signal generated in the absence of enzyme and figure "b" is the signal obtained upon addition of 33 μg/ml horseradish peroxidase and $10^{-2}$ M H$_2$O$_2$. Measurements were made at 20 mW of microwave power, with a gain of $1.24 \times 10^4$, modulation amplitude of $4 \times 10^{-1}$ G, time constant of 1.0 sec at a field setting of 3355 G and microwave frequency of 9.45 GHz. All measurements were made at room temperature and a modulation frequency of 100 KHz.
Figure 20:

ESR spectra of $10^{-2}$ M solutions of caffeic acid at pH 7.5. Figure "a" is the signal generated in the absence of enzyme and "b" is the signal obtained upon addition of 50 µg/ml tyrosinase (monophenol oxidase). Measurements were made at 20 mW of microwave power, with a gain of $1.24 \times 10^3$, modulation amplitude of $4 \times 10^{-1}$ G, time constant of 1.0 sec at a field setting of 3355 G and microwave frequency of 9.45 GHz. All measurements were made at room temperature and a modulation frequency of 100 KHz.
Here we can observe the significant contribution of the cinnamic side chain in generating complexity of signal.
DISCUSSION

Phenolics and the Environment:

It has been estimated that most human cancers are of an environmental origin (for reviews see Higginson, 1968; Higginson and Muir, 1973; Armstrong and Doll, 1975; Wynder, 1976; Wynder and Gori, 1977). Although some of these cancers are due to occupational exposure to carcinogens and to smoking, a larger proportion can be correlated with dietary patterns. Table 12 lists proportions of cancer deaths attributable to various environmental factors, as reported by Doll & Peto in their report on "Avoidable Risks of Cancer in the U.S." commissioned by the U.S. Congress in 1981. Here it may be seen that diet plays a significant role.

The plant phenolics, which comprise the subject matter of this thesis, constitute a major portion of the components consumed in man's daily diet. Estimates vary but a generally accepted range is between 600 mg and several grams per day (Powell et al., 1974; Maga, 1978). Their biological activity is therefore of some interest in determining the role they may play in human carcinogenesis.

Because of the great diversity of phenolic substances and the wide range of experimental conditions used, conflicting reports have been published on their role in carcinogenesis. The synthetic antioxidants, butylated hydroxyanisole and butylated hydroxytoluene, have both been reported to inhibit carcinogen-induced neoplasia (Speier et al., 1978; Wattenberg, 1972; Wattenberg, 1973; Wattenberg et al., 1979). More recently, Wattenberg et al., 1980, have reported that the phenolic derivatives of cinnamic acid, such as O-hydroxycinnamic acid, 3,4-dihydroxy-cinnamic acid, were also able to inhibit benzo(a)pyrene-induced neoplasia in mice.
TABLE 12: Proportions of cancer cases attributed to various different factors by different authors.

<table>
<thead>
<tr>
<th>Factor or class of factors</th>
<th>England, Birmingham region, based on Higginson and Muir (1979)</th>
<th>United States, based on Wynder and Gori (1977)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Tobacco</td>
<td>30</td>
<td>7</td>
</tr>
<tr>
<td>Tobacco/alcohol</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Diet</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Life-style</td>
<td>30</td>
<td>63</td>
</tr>
<tr>
<td>Occupation</td>
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<td>2</td>
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<tr>
<td>Sunlight</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Ionizing radiations</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Iatrogenic</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Exogenous hormones</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>Congenital</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Unknown</td>
<td>15</td>
<td>11</td>
</tr>
</tbody>
</table>

* Deduced from histograms. Non-environmental factors equated with congenital and unknown.

In a review article by Stich and Rosin, 1983, phenolic derivatives were reported to inhibit the mutagenic activities of both direct-acting carcinogens and precarcinogens in the presence of mixed function oxidases. The report also goes on to demonstrate that they are able to reduce the in vitro formation of mutagenic and carcinogenic N-nitroso compounds. In man, phenolics were observed to reduce levels of nitrosoprine in urine, following administration of nitrate, proline and phenolic test substances.

Many deleterious effects have also been reported. Table 13 is a composite list of genotoxic events caused by phenolics under a variety of test conditions. Not included are the more obvious effects known to be brought about by the carcinogens, Aflatoxin B1 and safrole. For reviews of the more complex group of flavonoids see Nagao et al., 1981, and Brown, 1980.

The Induction of Chromosomal Abnormalities by Phenolics:

In Table 2 it may be observed that several of the phenolics tested caused chromosomal abnormalities in a CHO cell test system. These results parallel findings by Stich et al., 1981a. Two aspects are distinctive to this table and the results reported by Stich et al., 1981a. The first pattern is the more extreme effect of phenolics possessing adjacent hydroxyl groups, and the second pattern is that the presence of S9 diminished most clastogenic activity. Because molecules with adjacent hydroxyl groups are much more susceptible to oxidation, an investigation was begun on their ability to oxidize and form free radical by-products. It was believed that the oxidation products were responsible for the observed clastogenic activity and that the presence of S9 might serve to detoxify or modify such reactions at higher pH values.
<table>
<thead>
<tr>
<th>PHENOLIC GROUP</th>
<th>ASSAY</th>
<th>ORGANISM</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. SIMPLE PHENOLS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>phenol</td>
<td>sister chromatid exchange</td>
<td>human lymphocytes</td>
<td>Morimoto and Wolff (1980)</td>
</tr>
<tr>
<td>catechol</td>
<td>point mutation</td>
<td>S. cerevisiae</td>
<td>Kunz et al. (1980)</td>
</tr>
<tr>
<td></td>
<td>mitotic crossover</td>
<td>S. cerevisiae</td>
<td>Kunz et al. (1980)</td>
</tr>
<tr>
<td></td>
<td>sister chromatid exchange</td>
<td>human lymphocytes</td>
<td>Morimoto and Wolff (1980)</td>
</tr>
<tr>
<td></td>
<td>nephrotoxicity</td>
<td>rats</td>
<td>Calder et al (1975)</td>
</tr>
<tr>
<td></td>
<td>chromosome aberrations</td>
<td>CHO cells</td>
<td>Sitch et al (1981a)</td>
</tr>
<tr>
<td>pyrogallol</td>
<td>point mutation</td>
<td>S. typhimurium</td>
<td>Ben-Gurion (1979)</td>
</tr>
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<td>point mutation</td>
<td>E. coli</td>
<td>Yamaguchi (1981)</td>
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<td>mice</td>
<td>Billimoria (1975)</td>
</tr>
<tr>
<td></td>
<td>chromosome aberrations</td>
<td>CHO cells</td>
<td>Mitra and Manna (1977)</td>
</tr>
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</tr>
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<td>micronuclei</td>
<td>mice</td>
<td>Mitra and Manna (1977)</td>
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<td>2. BENZOIC ACIDS</td>
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<td>CHO cells</td>
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continued . . .
TABLE 13 continued:

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<th>S. cerevisiae</th>
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<td>CHO cells</td>
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The Oxidation of Phenolics:

The oxidation of phenolic molecules was observed to occur more rapidly at alkaline pH. Caffeic, chlorogenic and gallic acids were all observed to change dramatically in solubility and colour as their pH level was increased. Figure 4 illustrates the colour changes in caffeic acid. This effect is not surprising if we examine the reaction mechanisms outlined in Figure 21. The free radical resonance structures generated by high pH levels may be seen to form dimers with other radicals. This involves the formation of new C-C, C-O, or O-O bonds (in order of decreasing importance) which may be followed by further rearrangements. Continued oxidation produces polymeric material and many complex dark-coloured materials often synthesized by plants (Thomson, 1964).

Phenols with monohydroxy or hydroxyl groups in the meta or para positions are much slower to oxidize. This is because they lack the resonance stabilization process outlined in part A of Figure 21. Without this degree of resonance stabilization, the phenoxy free radical is less likely to be formed, and once formed, is much shorter lived. The change of collision and bond formation with another free radical is therefore much diminished.

HPLC separation of caffeic acid and its oxidation products using a reversed-phase Vydc column and a methanol gradient, demonstrated the presence of many oxidation products (Figure 5). On the basis of ultraviolet absorption at 260 nm, greater than 96% of a freshly prepared aqueous solution of caffeic acid was present as the parent compound. However, when the compound was prepared in its salt form at pH 7.00 for biological experiments, there was observed to be a 5 - 10% degradation of the compound. After a 6 day incubation period, only 45% of the original caffeic acid peak remained. The rest was present as oxidative
Figure 21:

Resonance stabilization and dimer formation in phenols with hydroxyl groups in the ortho position. Figure 21A illustrates some of the resonance stabilization structures available to the molecule.

From R.H. Thomson, 1964, p. 16.
by-products of various molecular weights.

The Characterization of Phenolic Oxidation Products:

If oxidation does play a major role in the genotoxicity of phenolics, it becomes important to isolate and characterize by-products which contribute to the activity of such a reaction. Employing a Waters reversed phase preparative column, high pressure liquid chromatography was used to separate out each of the component oxidation products of caffeic and chlorogenic acids. Samples were concentrated by rotary evaporation and tested in the CHO cell test system. None of the components tested could account for the clastogenic activity of the parent compound, even when tested at concentrations 3 times those found in a 1.0 mg/ml solution. Various combinations of peaks, and total recombination of peaks, also failed to induce clastogenic activity.

It would therefore appear that the source of clastogenic activity must either reside in the front peak of the chromatographic separation (not retained on a reversed-phase column) or was relatively unstable and did not survive the chromatographic separation and subsequent rotary evaporation. In an effort to resolve which was the case, tests were undertaken to identify and characterize oxidation products not retained on chromatographic columns.

Based on the chemical structure of these compounds, it was hypothesized that one such by-product could be hydrogen peroxide. Testing with a potassium iodide assay for the presence of peroxide, demonstrated significant levels of hydrogen peroxide in solutions of caffeic acid that had been allowed to autooxidize at room temperature over a period of days (Table 9). Elevated levels of hydrogen peroxide were also observed to occur in solutions raised in pH level for a period of at least one hour before returning to pH neutrality.

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The production of hydrogen peroxide was observed to occur at the same time as an increase in clastogenic activity of 1 day oxidized solutions. This activity appeared to taper off in solutions incubated for greater than 3 days. Tables 3 and 4 illustrate this effect for both caffeic and chlorogenic acids.

Hydrogen peroxide may be scavenged by two classes of related enzymes - the catalases and the peroxidases. These enzymes catalyze the divalent reduction of $H_2O_2$ to $2H_2O$, using $H_2O_2$ as the electron donor in the case of catalases, or using a variety of reductants in the case of peroxidases (Fridovich, 1976).

Commercially prepared catalase was observed to eliminate all clastogenic activity in both autooxidized solutions of caffeic acid and in hydrogen peroxide treated solutions (see Tables 10 and 11). That catalase had in fact removed all $H_2O_2$ was verified with the potassium iodide colourimetric assay. The hydrogen peroxide solutions were tested at concentrations found to be present in oxidized solutions of caffeic acid (Table 9). A similar degree of activity was observed in the 10 to 50 μM range for both cases. This would allow one to infer that some portion of the oxidized caffeic acid's activity was due to the presence of hydrogen peroxide.

In CHO cell cultures exposed to caffeic acid in the presence of horseradish peroxidase, the results were less dramatic (Table 6). Freshly prepared solutions did not appear to possess sufficient $H_2O_2$ to catalyze and carry on the reaction, while oxidized solutions appeared to have polymerized to a point that there was a general inability by the phenolics to act as reductants. Consequently, there was only a slight (if any) reduction in the clastogenic activity of caffeic acid in the presence of this peroxidase. This result has also been reported for gallic acid by Kamel et al., 1977.
Concurrent application of both catalase and peroxidase in freshly prepared solutions of caffeic acid produced no observed effect on the level of activity of caffeic acid on CHO cell chromosomes (Table 7). Presumably, the catalase destroyed any \( \text{H}_2\text{O}_2 \) present, thereby interfering with the initiation of the peroxidase-catalyzed reaction.

Further evidence of peroxide involvement comes from the measurement of enhanced activity in the presence of transition metals. Martell, 1980, has shown that compounds of a similar structure in the presence of transition metals, produce hydrogen peroxide. Figure 22 illustrates a possible mechanism of the catalytic effect of manganese (Mn) during the oxidation of caffeic acid (modified from Martell, 1980). Studies in this laboratory using HPLC (unpublished) have indicated that manganese does indeed stimulate the oxidative degradation of caffeic and chlorogenic acids at neutral pH. The generation of peroxide during this accelerated oxidation may account for the increased biological activity of some phenolic compounds in the presence of transition metals, such as Mn.

In Table 10 it may be observed that catalase was able to eliminate the clastogenic activity of oxidized solutions but not freshly prepared solutions of caffeic acid. This observation would lend support for the hypothesis of another oxidative by-product. One such product was measured to be superoxide or \( \text{O}_2^- \). Using a modified ferricytochrome c assay, elevated levels of \( \text{O}_2^- \) were measured at pH values of 7.0, 8.0 and 9.0 (Figure 7). These levels were completely eliminated by the addition of superoxide dismutase. The rate of production of \( \text{O}_2^- \) was observed to be pH-dependent.

Rates of production of superoxide anion radical were observed to be 1.42 \( \mu \text{M/min} \) at pH 7.0, 2.86 \( \mu \text{M/min} \) at pH 8.0, and 3.81 \( \mu \text{M/min} \) at pH 9.0 for a \( 1 \times 10^{-4} \) M solution of caffeic acid. These values are
**Figure 22:**

Manganese catalysis of peroxide formation during the oxidation of a dihydroxyphenol. This is a proposed mechanism to illustrate the enhancing effect of transition metals on the clastogenic activity of plant phenols.

Modified from A.E. Martell, 1980, p. 95.
Generation of $\text{H}_2\text{O}_2$:
higher than rates published by Aver'yanov, 1981. For a $1 \times 10^{-4}$ M solution of caffeic acid, they measured rates of 0.073 $\mu$M/min at pH 7.0 and 0.105 $\mu$M/min at pH 8.0. One reason for this discrepancy is that they use a different molar extinction coefficient for ferricytochrome c. Their value is listed as 18.5 mM$^{-1}$ cm$^{-1}$ from Margoliash and Frohwirt, 1959. The value used in this study was 21 mM$^{-1}$ cm$^{-1}$ (from Van Gelder and Slater, 1962), a difference of approximately 8%. It is not shown how the rates of production of superoxide are calculated in the Aver'yanov paper, but the overall change in absorbance during the reduction of ferricytochrome c appears to be similar. Perhaps it is sufficient to say that they were able to observe the production of superoxide anion radicals from caffeic acid.

A possible mechanism for the production of $O_2^-$ during the oxidation of caffeic acid is outlined in Figure 23. Because of its transitory nature, one would not expect superoxide free radicals to play a role in completely oxidized solutions of phenolics. It would seem plausible that the initial peak of clastogenic activity at 1 day of incubation, might be due to the presence of these short-lived free radicals. However with the subsequent formation of the polymerized quinone structures (Thomson, 1964) their electrophilic structure may act as scavengers of free radical products, and at least account in part for the slight decrease in activity of 3 day and 6 day samples.

The addition of superoxide dismutase to CHO cell cultures containing caffeic, chlorogenic or gallic acids appeared to increase the genotoxicity of these compounds. This result is not surprising if we examine the enzyme mechanism as outlined by McCord and Fridovich, 1969.

$$2 O_2^- + 2H^+ \rightarrow O_2 + H_2O_2$$
Figure 23:

Proposed mechanism for the production of superoxide anion free radicals from dihydroxyphenols in the presence of alkali. This is a reaction which can only take place under aerobic conditions.
Generation of $O_2^-$:

$$\text{alkali} \quad \rightarrow \quad \text{phenolate} \leftrightarrow \text{phenolate}$$

$$\text{phenolate} + O_2 \rightarrow \text{phenolate} + O_2^-$$
The net conversion of $O_2^-$ to $H_2O_2$ would therefore account for increased toxicity due to the accumulation of the more stable product, hydrogen peroxide. One would suspect concurrent applications of superoxide dismutase and catalase to decrease the genotoxicity of freshly prepared solutions of caffeic, chlorogenic and gallic acids. Table 7 illustrates that this is the case.

The effect, however, is not total and several reasons may be put forth to explain this. The first and most obvious explanation is that the enzymes were not in sufficient concentration to eliminate all the $H_2O_2$ and $O_2^-$ generated. Normally, this should not be the case for catalase, because the concentration used (5µg/ml) was shown to be sufficient to remove all clastogenic activity of 500 µM solutions of $H_2O_2$. The level of superoxide dismutase used (1.0 µg/ml) was found to be sufficient to catalyze the conversion of 100 µM of $O_2^-$/min to $H_2O_2$ in the xanthine/xanthine oxidase generating system. It seems doubtful that the rate of production of $O_2^-$ from the oxidation of caffeic acid would exceed this value.

Kono and Fridovich, 1982, have recently published a paper demonstrating that $O_2^-$ can actually reversibly inhibit the ability of catalase to breakdown $H_2O_2$. It may therefore be possible that active production of $O_2^-$ by the oxidizing caffeic acid molecules may be inhibiting catalase in its function. The transitory nature of the $O_2^-$ radical would suggest that this is only a temporary effect. This consideration would therefore only play a minor role in catalase mechanics after a 1 hour pre-incubation period.

Another possible explanation is that there are still other oxidation products present which are capable of inducing genetic damage. Several factors point to this conclusion. The enzyme tyrosinase is an oxygen and four electron transferring monophenol oxidase present in
almost all biological systems. It catalyzes the oxidation of several phenolics with the net production of \( \text{H}_2\text{O} \); but no \( \text{H}_2\text{O}_2 \) or \( \text{O}_2^- \).

This reaction as outlined in the Worthington Enzymes Catalogue and is given below.

\[
\text{HO} - \text{OH} + \text{O}_2 \rightarrow 2 \text{HO} - \text{O} + \text{H}_2\text{O}
\]

When tyrosinase was added to aqueous suspensions of caffeic, chlorogenic and gallic acids at neutral pH, they were observed to solubilize and change quite dramatically in colour and finally to a dark brown. No \( \text{H}_2\text{O}_2 \) could be detected using the potassium iodide assay. When CHO cells were incubated with caffeic, chlorogenic and gallic acids, in the presence of tyrosinase, the clastogenic activity of these compounds was noticeably increased (Table 6).

One possible explanation, then, for the activity observed in the superoxide dismutase/catalase experiment and in the tyrosinase treatment experiment, would be the presence of phenolic free radicals. These free radicals would act as intermediates in quinone formation and would be expected to be relatively short lived. One would therefore not anticipate such molecules to play a role in the clastogenic activity of 3 day old solutions of phenolics. Peroxide appears to be the genotoxic agent at that time. They would, however, be present in freshly prepared solutions which were actively taking part in the oxidation process.

Although there is no direct way of assaying their ability to induce genetic damage, their presence can be detected by the use of electron spin resonance measurements.
Electron Spin Resonance of Phenolic Free Radicals:

The presence of an odd electron generated during the oxidative process, makes electron spin resonance the method of choice in studying phenolic autooxidation. As expected, phenolic molecules which lacked clastogenic activity in the CHO cell chromosome aberration tests also failed to generate a signal even under conditions of high pH. Figure 12 illustrates that this is the case. These molecules could be classified as those having one or more hydroxyl groups in the meta or para positions with/or without a modified adjacent hydroxyl group (for example, M-coumaric, p-coumaric and ferulic acids). It should be pointed out that these phenolic acids are capable of autooxidation, but that the reaction rate was so slow as to make the generation of free radicals' signals too weak to be detected.

Phenolic acids which were observed to be active in the CHO cell test also appeared to generate strong ESR signals under oxidative conditions. The best example, gallic acid or 3,4,5,-trihydroxybenzoic acid illustrates this point. This molecule has three adjacent hydroxyl groups capable of forming semiquinone and quinone structures. With the presence of three hydroxyl groups adjacent to each other there occurs a fair degree of resonance stabilization and the signal generated by this molecule is relatively strong.

The odd electron which is generated during the oxidation process is then free to interact with the two remaining hydrogen atoms at positions 2 and 6 in a nuclear hyperfine coupling reaction. The modification of the spin energy of this extra electron by the magnetic fields of these two hydrogens would be expected to split the signal into a number of hyperfine structures. The number of structures can be calculated in the following way.
In a magnetic field the Z component of the magnetic moment of the proton can have either of two possible values $+\frac{1}{2}$ and $-\frac{1}{2}$. Since this spin can interact with two hydrogens, in the case of gallic acid, one would assume that there are 4 possible combinations, each of equal intensity. However, since these protons are of an equivalent nature (positions 2 and 6 are equivalent in gallic acid) the $+\frac{1}{2}$, $-\frac{1}{2}$ and $-\frac{1}{2}$, $+\frac{1}{2}$ situation cannot be distinguished and the result is 3 peaks with an intensity of 1:2:1. This is observed in our measurements of gallic acid and are diagrammatically represented in Figures 13, 15 and 16. The hyperfine coupling constants (the distance between peaks measured in gauss) would also be expected to be identical and this was also observed to be the case. The constants measured for gallic acid agree with those published by Dixon and Murphy, 1975.

ESR signals were measured for gallic acid under two forms of oxidizing conditions. At high pH the spectra generated were of a very high intensity. This would reflect a very large free radical concentration. Enzymatic oxidation produced an identical signal with identical hyperfine splitting constants. However, the intensity was greatly diminished and required maximum gain settings. Increasing the concentration of enzyme only marginally increased the intensity of the signal. Enzyme concentrations of greater than 50 µg/ml were found to enhance quinone polymerization with subsequent loss of signal. What is significant in these measurements is the fact that peroxidase and tyrosinase, two enzymes which are prevalent in the body, were capable of simulating oxidative conditions and led to free radical formation under simulated biological conditions.

Protocatechuic acid, or 3,4-dihydroxybenzoic acid is completely analogous in structure to gallic acid, but is missing a hydroxyl group at position 5. Because of this it lacks some degree of resonance.
stabilization and produces a slightly weaker signal. The extra electron generated during oxidation is now able to interact with 3 protons at position 2, 5 and 6. We would therefore expect the hyperfine splitting to occur as 8 separate peaks. This is illustrated in Figure 12. However, since none of the protons are equivalent in nature, there would be no overlap as was seen in gallic acid and the coupling constants would be expected to differ from proton to proton. This is what was observed. The constants measured are in agreement with those published by Kalyanaraman and Sealy, 1982.

Caffeic acid or 3,4-dihydroxycinnamic acid is analogous to protocatechuic acid in structure, but possess the cinnamic acid structure instead of the benzoid acid group. Its resonance stabilization structure is also similar to protocatechuic acid and it is therefore not surprising that the signal intensities were also very similar. However, the two additional protons on the cinnamic acid group, increased the complexity of signal considerably. Under ideal conditions 5 protons would result in 32 hyperfine coupling interactions. At high pH with a low time constant we were able to resolve 29 of these peaks (Figure 17). Because of overlap of these peaks, 3 appeared obscured at this level of measurement. The hyperfine coupling constants measured under these conditions are the same as those measured by Dixon et al., 1975.

When the time constant was increased and some of the resolution lost, 7 major groups of peaks could be observed (Figure 18). These peaks correspond to the peaks resolved for the much weaker enzymatic oxidation state of caffeic acid. Because of the weakness of this signal, it was not possible to resolve out the full 32 hypothetically present. It is possible to say, however, that peroxidase, tyrosinase and alkaline conditions all produced the same parent phenolic free radical (Figure 18, 19 and 20).
The Importance of Phenolic Oxidative By-Products:

The importance of such oxidative by-products has been recorded extensively in the literature (reviewed by Troll, 1982). Hydrogen peroxide, the first oxidative by-product to be examined in this thesis, has been found to induce genetic damage in a variety of ways. It is an intermediate product in many radical reactions and it can degrade spontaneously into OH' radicals or continue to react by way of radical intermediates. In DNA, H₂O₂ produces base changes, single-strand breaks and inhibits replication, but produces virtually no point mutations (Freese, 1971; Stich et al., 1978; Bradley et al., 1979). It has been found to be toxic to cell cultures (Peterkofsky and Prather, 1977) and has been found to induce chromosome aberrations in both ascites tumours in mice (Schoneich, 1967) and CHO cells (Stich et al., 1978). Recently, Speit et al., 1982, has shown it to induce sister chromatid exchanges in V-79 Chinese hamster cells.

Traditional Ames test strains are not sensitive to the effects of H₂O₂ in solution. This has led to the development of a new series of strains known as the TA102 tester strains. These strains possess A'T base pairs at the site of mutation which are sensitive to oxidative mutagens. They are therefore able to demonstrate considerable genetic damage in the presence of hydrogen peroxide (Levin et al., 1982). Carcinogenic potential has been demonstrated by Ito et al., 1981. Here, they were able to show a prevalence of gastro-duodenal nodules in mice treated with H₂O₂.

Because of the low concentrations of H₂O₂ attainable in vivo, it seems dubious that its toxicity is due to direct attack. More likely its reactivity with another oxidation product, the superoxide or O₂⁻ radical, to form hydroxyl radicals (OH') is responsible for its in vivo effects.
Below are listed possible reaction mechanisms for the production of OH radicals. The reactions outlined do not represent a complete list, but do illustrate the interdependence of free radical by-products in the development of DNA damage.

\[
\begin{align*}
e^- + O_2 & \leftrightarrow O_2^- \quad (1) \\
e^- + O_2^- + 2H^+ & \leftrightarrow H_2O_2 \quad (2) \\
e^- + H_2O_2 + H^+ & \leftrightarrow HO^- + H_2O \quad (3) \\
e^- + HO^- + H^+ & \leftrightarrow H_2O \quad (4)
\end{align*}
\]

from Forman and Boveris, 1982.

The OH radical is the most potent oxidant known. Because of its instability or high reactivity, it is difficult to measure. Some success has been reported using ESR - spin trapping techniques, but the complexity of the spin adduct spectra make this technique a difficult one to pursue. What is more easily measured are the reaction products of hydroxyl radical reactions.

Chemical physical measurements have demonstrate that the hydroxyl radical produces single strand breaks in DNA and has also been implicated in the formation of several types of base and nucleoside damage. In the case of strand breakage, it appears that cleavage occurs on the C₅' or C₃' H's with production of a DNA radical leading to phosphodiester bond breaks. Base nucleoside damage is most frequently in the form of OH adducts at the C₅ - C₆ bond of pyrimidines and imidazole ring breakage in the case of purines (Scholes, 1978). This form of damage can lead to sequence miscoding and local denaturation of the double strands.

Superoxide, a free radical found to be present in solutions of phenolic acids, is also a potent oxidant. Its significance in the
induction of chromosomal damage appears to be considerable. Three pathways which are of particular interest are outlined in Figure 24. Here it may be seen that superoxide can: oxidize SH groups to S-S bridged groups (Pathway 1); dismutate to form $\text{H}_2\text{O}_2$ plus ground-state oxygen (Pathway 2) or react with ferric ions to form ferrous ions (Pathway 3). Each pathway may lead to significant changes in cellular metabolism (from Oberley & Buettner, 1979).

In Pathway 1, the formation of disulfides can result in conformational changes of essential proteins. This could result in the activation or inactivation of key enzymes required in cellular metabolism and division. The role of sulfhydryl groups in carcinogenesis has been reviewed by Harington, 1967. Pathway 2 illustrates how superoxide can dismutate to form $\text{H}_2\text{O}_2$ and ground-state oxygen. Dismutation occurs much more rapidly in the presence of superoxide dismutase and the consequence of the production of $\text{H}_2\text{O}_2$ has already been discussed. The last pathway, in which superoxide donates electrons to metals may also have serious consequences for cellular metabolism. By altering the oxidation states of essential transition metals, it is possible to also alter the overall oxidation-reduction potential of a cell. Transformed cells appear to be particularly sensitive to such changes (Fernandez-Pol et al., 1977).

Emerit et al, 1982, have recently demonstrated that superoxide radicals are capable of inducing chromosome breakage and sister-chromatic exchange. It is this form of activity which becomes evidence in the induction of chromosomal abnormalities in CHO cells.

Perhaps one of the most outstanding features of the ability of superoxide radicals to cause damage, is their ability to traverse cellular barriers. Superoxide anion radicals have been shown to cross membranes of erythrocytes (Lynch and Fridovich, 1978) and granulocytes.
Figure 24:

Major pathways for the induction of cellular damage by the superoxide anion free radical.

From Oberley and Buettner, 1979, p. 1144.
(Gennaro and Romeo, 1979) through anion channels. Superoxide anion radicals have also been shown to cross artificial lipid bilayer membranes at temperatures above the lipid phase-transition (Rumyantseva et al., 1979). It is therefore possible that intracellularly generated superoxide can pass directly through lipid membranes which are fluid at 37° (Powis et al., 1981). This evidence would suggest that although phenolics may be localized in their effects, their generation of superoxide radicals may be less so.

Other Dietary Sources of Free Radical Oxidation Products:

The ability of plant phenolics to generate hydrogen peroxide and free radical oxidation products does not appear to be unique. Several other components of diet have been reported to share this activity.

Vitamin C, or ascorbic acid, is a required cofactor in some oxidation reactions requiring molecular oxygen. It has been reported to cleave DNA and this cleavage has been found to be oxygen dependent (Stich et al., 1976). Sodium ascorbate has been shown to increase the frequency of sister chromatid exchanges (a sensitive indicator of DNA damage) (Speit et al., 1980) and has been shown to induce mutations in *S. typhimurium* (Stich et al., 1978). The induction of DNA damage appears to be catalase sensitive (Stich et al., 1979; Peterbofsky and Prather, 1976) suggesting that hydrogen peroxide may be the cause of the damage. Superoxide dismutase has also been shown to reduce DNA damage, implicating superoxide radicals as an alternative cytotoxic agent (Morgan et al., 1976). In a paper by Yamaguchi, 1981, ascorbic acid, as well as epinephrine, cytochrome c, pyrogallol, pyrocatechol and menadione, were all found to be sensitive to these enzymes.

Pyrolysates of tryptophan, glutamic acid and globulin were also
found to be mutagenic in *S. typhimurium* strains, TA98 and TA100 and this activity could be reduced by the presence of myeloperoxidase (Yamada et al., 1979). This would suggest again, that peroxides are significant factors in the mutagenic activity of amino acid and protein pyrolysates. They also appear to be the active components of autooxidized fatty acids (Yamaguchi et al., 1980).

**Biological Protection Mechanisms:**

Obviously, organisms have evolved mechanisms to protect themselves against the genotoxic effects of such oxidation by-products. In fact, an early explanation for the different oxygen tolerances of aerobes and obligate anaerobes was based on H$_2$O$_2$ tolerance. Thus, aerobes were thought to contain catalase as a defence mechanism against H$_2$O$_2$, while anaerobes lacked this enzyme and were subsequently killed by H$_2$O$_2$ when exposed to oxygen levels present in air. With the discovery of the more short-lived oxygen free radicals, it became obvious that other enzymes must exist which act in a protective fashion toward free radical damage. Thus, superoxide dismutase was discovered and extensively characterized by McCord and Fridovich (1969). The evolutionary development of such enzymes can only serve to illustrate the importance of oxidative reactions to higher animals.

One would expect, then, that susceptibility to oxidative by-products might depend on the presence of such enzymes to act in a protective role. This appears to be the case. In such tumor cell lines as Ehrlich ascites tumor cells and Morris hepatoma cells, reduced levels of superoxide dismutase have been observed (Dionisi et al., 1975; Sahu et al., 1977). These cells are particularly sensitive to the effects of free radical producing drugs such as bleomycin and streptonigrin. In both cases it was concluded that superoxide radical was one of the
mediators for the enhancement of DNA chain breakage (Sausville et al., 1978; Cohen et al., 1963). Preferential killing of these tumor lines is thought to explain their effectiveness.

Oxidation Reactions in Man:

There seems little doubt that the ingestion of phenolics in the diet of man has far-reaching consequences in the carcinogenic process. Oxidation of these phenolics has been shown to lead to polymerization of quinone products with concurrent production of activated oxygen species. Factors which appear to favour the oxidative process include time, increased oxygen-pressure, high pH and the presence of transition metals.

One must consider, then, whether such situations arise in vivo. In man, the average transit time for food to pass through the body is about 8 hours (Mariella and Blau, 1968). It might, therefore be expected that a higher percentage of the finalized oxidation products would be found in the large intestine and colon. Since man is an aerobic organism, the oxygen pressure of blood hemoglobin is about 100 mm Hg in arterial blood dropping to approximately 35 mm Hg in the veins (Clinical Hematology, 1981a). These levels are well within the ranges used to measure the oxidation of phenolics reported in this thesis.

The pH values of various organs in man tend to vary from acidic levels in the stomach of between 1.0 and 3.0 (CRC Handbook, 1982) and the relatively alkaline areas of the pancreas and duodenum at pH 7.5 - 8.6 (Mariella and Blau, 1968). We would therefore expect rates of oxidation to be greatest in areas of highest pH. Levels of transition metals are more difficult to measure. Blood tends to be relatively high in iron (approximately 18 μmol/l in serum) and copper (13 - 23 μmol/l of
whole blood) (Clinical Hematology, 1981b) but these values are largely
determined by the health and diet of the individual.

We might therefore conclude that the oxidative process of phenolics
is possible in man. There are, however, several mitigating factors.
The first and primary one is that phenolics may be metabolized in man to
glycosides and glucuronate derivatives. The metabolic fate of
protocatechuic acid has been documented by Scheline, 1966, who found
that it was excreted largely unchanged, but partly combined with
glucuronic and sulphuric acids in rabbits. DeEds et. al., 1957, has
shown that it may also be methylated in rats and rabbits to vanillic
acid (4-hydroxy-3-methoxybenzoic acid). The metabolism of gallic acid
after oral or intraperitoneal administration to the rat and rabbit has
been studied by Booth et al., 1959. This group found that the major
urinary metabolite besides gallic acid itself, was 3,5-dihydroxy-4-
methoxybenzoic acid.

Intestinal microflora appear to have a marked effect on the
metabolism of these compounds. The hydrolysis of glucuronides appears
to occur in the presence of these organisms. This may be an important
feature in the metabolism of compounds excreted in the bile as
glucuronide conjugates (Scheline, 1966). There is no information to
date, as to whether these metabolically altered phenolics are capable of
oxidizing and forming peroxides and free radical metabolites.

The body also possesses several enzyme systems for the detoxifi-
cation of activated oxygen species. Catalases, peroxidases and
superoxide dismutases have all been discussed in some detail as to their
role in protecting organisms from the toxic effects of oxidative
by-products. The role of such enzymes cannot be underestimated in the
body. As was shown in Table 2, the liver homogenate system known as S9
was able to reduce the clastogenic activity of all phenolic substances
measured.
S9 is a term used to refer to the 9000 x g supernatant fraction of rat-liver homogenate prepared from male rats pretreated with a single 0.5 g/Kg intraperitoneal dose of Aroclor 1254 (a commercial mixture of polychlorinated biphenyls). Although this is largely a chemically undefined mixture, inherent to this particular fraction are the liver microsomes. The biochemical composition of the microsomal fraction has been extensively characterized and has been found to contain a wide variety of enzymes, including cytochrome oxidases, catalases, hydroxylases, and various lytic enzymes (Amar-Costesec et al., 1974). It is therefore not surprising that S9 was capable of altering the clastogenic activity of the phenolic substances tested.

We are faced therefore, with an overwhelming variety of conditions by which ingested phenolics might play a role in the process of carcinogenesis.

**Phenolics in the Process of Carcinogenesis:**

The process of carcinogenesis is believed to occur as a prolonged, multistep development which is usually divided into two stages. The first stage, is considered to be an initiation phase where a low dose of a true carcinogen is applied to a target tissue. This is believed to be followed by a promotion phase where repeated applications of such substances act to enhance the production of a malignant tumour. Evidence for this process is reviewed by Berenblum and Shubik, 1979, for a model system of skin carcinogenesis in mice.

**Phenolics in the Initiation of Carcinogenesis:**

The "initiation" of a tumour requires by definition an irreversible change in one or more of the cells of the target tissue. This is believed to be the result of a mutation or alteration in the genetic
material of the cell. As has been discussed previously, the oxidative by-products, $H_2O_2$ and $O_2^-$, are capable of inducing strand breaks and mutations in exposed cells. One might hypothesize, then, that organ specificity might arise in areas which are conducive to the oxidation of phenolics.

Safrole, a carcinogenic plant phenolic isolated from the genus Heterotropa, appears to be an example of this phenomenon. This compound is classified as a weak hepatocarcinogen and is metabolized by the liver to its 1'-hydroxy derivative. The 1'-hydroxy metabolite is a stronger hepatocarcinogen than the parent compound (Miller et al., 1983; Miller et al., 1979). It is possible that the oxidation of safrole in the liver might lend itself to the production of activated oxygen species and the initiation of a neoplastic process.

Phenolics as Promoters of Carcinogenesis:

Recent studies have demonstrated that tumour promoters can damage DNA without reacting with it directly. One of the best known promoters, TPA (a phorbol ester 12-0-tetradecanoylphorbol-13-acetate) has been found to stimulate cells by producing activated forms of oxygen, including superoxide anion radicals and peroxides, agents which are free radicals or generators of free radicals (Marx, 1983).

The clastogenic activity of these free radical products has already been discussed. However, many of the mechanisms of how tumour promotion occurs, remains little understood. Free radical-induced chromosomal rearrangement of cellular oncogenes might explain how genes enter a "de-regulated" state. If separated from adjacent repressor genes, these oncogenes might find themselves able to be expressed. This would eventually lead to a transformed state.
Recent evidence has shown that this may be the case. Birnboim, 1982 has published a paper in which a correlation is found between the tumour-promoting ability of a compound and the amount of DNA damage induced. They have found that inhibitors of TPA-induced promotional activity, also reduce the number of DNA strand breaks. Moreover, DNA damage was also found to be prevented by enzymes which block formation of superoxide and peroxide, such as superoxide dismutase and catalase. Inhibitors of these enzymes were found to restore the tumour-promoting activity of the compounds tested.

Amplification of these effects may occur in the white blood cells. Polymorphonuclear leukocytes or PMN's are a type of phagocytic immune cell. When stimulated, they undergo an "oxidative burst" with marked increases in oxygen consumption as well as production of superoxide radicals and hydrogen peroxide. It is believed that it is the production of these activated oxygen species which allows them to kill and scavenge antigenic material.

When PMN's or other phagocytes are treated with such well known promotors as TPA, they are found to undergo an oxidative burst seconds after application of the compound (Goldstein et al., 1981). The subsequent production of superoxide and peroxide may therefore act to amplify the promotor's initial effect. This observation may explain why cells are so sensitive to the application of promotor substances.

In the previous discussion, evidence has been presented which confirms that during the process of oxidation, plant phenolics are capable of producing peroxide and free radical intermediates. Under conditions favouring oxidation, then, we might expect phenolics to act as promotors of carcinogenesis.
At present, evidence for such a case in humans is only speculative. One example may be found in the correlation between betel nut (Areca betle) chewing and the incidence of oral, pharyngeal and esophageal tumours (Ranadive et al., 1979). A common practice among betel nut chewers is to mix lime with the betel nut and/or tobacco to form a "quid" which is chewed continuously. The high polyphenolic content of betel nut might then be oxidized at the higher pH levels obtained with the use of lime, and a chain of reactions begun leading to circumstances favourable for the production of a tumour. This hypothesis has been reviewed by Stich and Rosin, 1983.

The ability of plant phenolics to act as promotors of carcinogenesis may also be modulated by other components of diet. The antioxidant, vitamin E, or α-tocopherol, has received renewed interest as an essential nutrient for humans. A major function of vitamin E, in animals, appears to be that of an antioxidant, inhibiting both enzymatic and nonenzymatic lipid peroxidation (Lehninger, 1970). Vitamin E and several other antioxidants act by competing for reaction with peroxy free radicals, RO₂⁻. In this action, free radicals are removed from the system via formation of tocopherol quinones or dimers. In the presence of an antioxidant, the predominant termination reaction will be that involving the antioxidant, and the termination rate will be largely determined by the structure of the antioxidant (Witting, 1980).

The similarity of structure (tocopherols and phenols) would suggest that under the proper reaction conditions, plant phenolics might also be able to act as antioxidants. This appears to be the case and may be seen illustrated as inhibitors of carcinogenesis.
Phenolics as Inhibitors of Carcinogenesis:

Any discussion of carcinogenic potential must include recent findings that phenolics can also act in an inhibiting role. Since carcinogenesis appears to be a multistep process, this can occur in several ways. In addition, phenolics, being relatively reactive molecules also appear to be able to interact in this process in several ways.

The first and most obvious way is for the phenolics to bind directly with the carcinogenic substance. Ferulic, caffeic, chlorogenic and ellagic acids appear to inhibit the mutagenicity of the reactive species of benzo(a)pyrene. They are believed to do this by direct interaction resulting in the formation of mutagenically inactive complexes (Woods et al., 1982).

Several phenolics have also been observed to bind one of the metabolites of the carcinogen and mutagen, N-Methyl-N-Nitro-N-Nitrosoguanidine or MNNG. In a study using mutagenicity in *Salmonella typhimurium*, Stich and Rosin, 1983, were able to demonstrate that addition of phenolics such as gallic acid, caffeic acid, chlorogenic acid and commercially prepared tannins were able to inhibit mutagenicity of MNNG only when applied concurrently with the carcinogen.

Phenolics also appear to interact and thereby interfere with the activation of precarcinogens. Aflatoxin B1 or AFBl requires activation by microsomal mixed function oxidases to become the reactive AFBl-2,3-oxide which is carcinogenic (Lin et al., 1978; Neal and Colley, 1978). HPLC analysis of the metabolites of AFBl would suggest that phenolics suppressed mutagenicity in *Salmonella typhimurium* by interfering with its metabolic activation (Chan, 1982).
Phenolics also appear to inhibit carcinogenesis by acting as traps for nitrosation reactions. They accomplish this by reacting with nitrite to form C-nitroso phenolic compounds (Mirvish, 1981; Walker et al., 1982). The reactivity of the phenolics in such a reaction appears to depend on the number of hydroxy groups and their position. Phenolics also react with nitrite to produce quinones and nitrous oxide. This pathway involves free radical production, and may lend itself to promotional activity.

SUMMARY

In summary, this thesis has attempted to provide evidence of how phenolic molecules may play a key role in the process of carcinogenesis. Under oxidative conditions they appear to produce significant levels of hydrogen peroxide, superoxide and parent phenolic free radicals. These oxidative products are all capable of inducing damage to DNA and may account for the clastogenic activity of the compounds tested at pH 7.0. The presence of transition metals and high pH appeared to increase the rate of production of these products. Activated oxygen species have been implicated as a mechanism for both the initiation and promotion of carcinogenic activity. Phenolics, may therefore play a significant role as both initiators and promoters of carcinogenic activity by serving as sources of these activated species. Under less oxidative conditions, however, phenolics appear to act as inhibitors of chemical carcinogenesis. Which activity occurs most likely will depend on the number and position of the hydroxyl groups and the biological conditions inherent to the reaction.
REFERENCES


Dixon, W.T., and Murphy, D., Triple oxidations of some polyhydric phenols by cerium (IV) in acid solutions as observed by electron-spin resonance spectroscopy. J. Chem. S. P2, 8: 850, 1975.


APPENDIX 1:

Molecular structures of simple phenols.

Phenol

Catechol

Resorcinol

Hydroquinone

Phloroglucinol

Pyrogallol

APPENDIX 2:

The molecular structure of i) cinnamic acids and

\[ R' OH R \]
\[ CH=CHCOOH \]

8) \( R=R'=H \): p-coumaric Acid
9) \( R=OH, R'=H \): caffeic Acid
10) \( R=OCH_3, R'=H \): ferulic Acid
11) \( R=R'=OCH_3 \): sinapic Acid

ii) benzoic acids

\[ R' OH R \]
\[ COOH \]

1) \( R=R'=H \): p-hydroxybenzoic Acid
2) \( R=OH, R'=H \): protocatechuic Acid
3) \( R=OCH_3, R'=H \): vanillic Acid
4) \( R=R'=OH \): gallic Acid
5) \( R=R'=OCH_3 \): syringic Acid
6) \( R=H \): salicylic Acid
7) \( R=OH \): gentisic Acid

From Ribéreau-Gayon, 1972, p. 82.
APPENDIX 3:

The molecular structures of commonly occurring flavonoids.

Chalcone
Naringenin chalcone

Flavanone
Naringenin

Flavone
Apigenin

Flavonol
Kaempferol

Anthocyanidin
Pelargonidin

Isoflavone
Genistein