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EVALUATION OF OXIDATIVE STABILITY OF LIPIDS BY FLUORESCENCE SPECTROSCOPY AND CHARACTERIZATION OF FLAVONOID ANTIOXIDANT CHEMISTRY

By

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ABSTRACT

EVALUATION OF OXIDATIVE STABILITY OF LIPIDS BY FLUORESCENCE SPECTROSCOPY AND CHARACTERIZATION OF FLAVONOID ANTIOXIDANT CHEMISTRY

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Fluorescence spectroscopy is a sensitive and rapid technique that can be exploited for evaluation of the oxidative stability of lipids. In this study, two assays based on quenching of the fluorescence intensity of an extrinsic probe and an increase in its fluorescence anisotropy due to the free radicals generated during lipid peroxidation were developed for evaluation of antioxidant efficacy and for monitoring the progress of lipid peroxidation. The assays were used to evaluate the efficacies of metal chelating and free radical scavenging antioxidants.

By use of the assays, the effects of chelators on metal-ion-induced peroxidations were found to be dependent on the type of metal ion used to initiate peroxidation and on the mole ratio of chelator relative to lipid. Flavonoids and isoflavonoids were the class of compounds selected to study prototypic, phenolic free radical scavenging antioxidants. These compounds were more effective at suppressing metal-ion-induced peroxidations than peroxidations induced by free radicals, an indication that metal chelation plays a larger role towards their antioxidant mechanism than has previously been believed. Additionally, fluorescence anisotropy measurements were conducted using a series of probes with the chromophore at varying levels of penetration into the membrane. The results suggest that the flavonoids and isoflavonoids partition preferentially into the hydrophobic interiors of membranes and

decrease membrane fluidity in this region. The reduced fluidity could limit accessibility of free radical species generated during lipid peroxidation and stabilize the membrane, a structural mechanism of antioxidant action for these compounds.

Distinct structure-activity relationships were revealed for the antioxidant activities of flavonoids and isoflavonoids. Presence of electron-withdrawing groups enhanced activity whereas electron-donating groups decreased activity, with the substitution pattern on the B-ring of these compounds being an especially important determinant of activity. Among the isoflavonoids, the biological metabolites of these compounds had similar or superior antioxidant activities in comparison to the parent compounds, a finding that suggests that these compounds may retain their activity under *in vivo* conditions. An oxidation product of the reaction between the isoflavonoid genistein and peroxyl radicals was characterized and could serve as a marker for genistein antioxidant chemistry.

To my parents

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AAPH	2,2'-Azobis(2-Amidinopropane) Dihydrochloride
ADP	Adenosine Diphosphate
AIBN	2,2'-Azobis(isobutyronitrile)
16-AP	16-(9-Anthroyloxy)Palmitic Acid
6-AS	6-(9-Anthroyloxy)Stearic Acid
12-AS	12-(9-Anthroyloxy)Stearic Acid
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
cis-PA	*cis*-Parinaric Acid
DMSO	Dimethyl Sulfoxide
DPH	1,6-Diphenyl-1,3,5-Hexatriene
DPH-PA	3-(*p*-(6-Phenyl)-1,3,5-Hexatrienyl)Phenylpropionic Acid
DPPC	Dipalmitoylphosphatidylcholine
EDTA	Ethylenediaminetetraacetic Acid
HEPES	4-[2-Hydroxyethyl]-1-Piperazine Ethanesulfonic Acid
HPLC	High Performance Liquid Chromatography
LUV	Large Unilamellar Vesicle
MDA	Malondialdehyde
MLV	Multilamellar Vesicle
MOPS	3-[N-Morpholino] Propanesulfonic Acid
NMR	Nuclear Magnetic Resonance
NTA	Nitrilotriacetic Acid
O-Dma	*O*-Desmethylangolensin
PCOOH	Phosphatidylcholine Hydroperoxide
PDA	Photodiode Array
SLPC	1-Stearoyl-2-Linoleoyl-*sn*-Glycero-3-Phosphocholine
SUV	Small Unilamellar Vesicle
TBA	2-Thiobarbituric Acid
TBARS	2-Thiobarbituric Acid Reactive Substances
TBHQ	*tert*-Butylhydroquinone
UV	Ultraviolet

INTRODUCTION

Lipid peroxidation is a very undesirable reaction in foods and is generally recognized as the primary factor limiting the shelf-life of most processed food products (Loliger, 1992). To mitigate the detrimental effects of lipid peroxidation on food quality, many highly effective synthetic antioxidants have been developed. In recent years, however, considerable interest in the use of plant derived antioxidants has developed, fueled in part by consumer concerns about the safety of synthetic compounds in the food supply. This interest has been stirred further by observations that many of these plant compounds, which are potent antioxidants, also display antiatherogenic, antimutagenic, and anticarcinogenic activities (Duthie, 1991; Kinsella *et al.*, 1993). Hence, introduction of such compounds in food products may impart health benefits in addition to stabilizing the product.

The screening of a large number of compounds or plant extracts for possible antioxidant activity requires a rapid and sensitive assay for evaluation of oxidative stability of lipids. The current methods for antioxidant evaluation, however, suffer from serious limitations. Most of the assays are time-consuming, have limited sensitivity and specificity, and require large amounts of test material. Often the methods used (such as high temperatures to accelerate peroxidation) introduce artifacts into the sample which complicates interpretation of the antioxidant efficacy. Additionally, the model systems employed in many

of these assays are not representative of the structural and functional characteristics of the substrate in the food product.

The primary aim of this research was to develop a rapid and sensitive assay for evaluation of the oxidative stability of lipids that employed a model system which addressed many of the concerns about the experimental systems used by other researchers. The model system selected for the study was a chemically, well-defined liposomal system that was representative of biological membranes.

Fluorescence spectroscopy was chosen as the technique for use in development of an assay for routine evaluation of the efficacy of antioxidants due to its inherent advantages of speed, simplicity and sensitivity. The overall hypothesis of this research was that fluorescence spectroscopy could be utilized for the evaluation of the antioxidant potencies of metal chelators and free radical scavengers, and that the technique had the required sensitivity to distinguish between the antioxidant activities of structurally related compounds.

Using fluorescence, the susceptibility of a probe to peroxidative damage with accompanying loss of its fluorescent character can be used to monitor the progress of lipid peroxidation directly and sensitively. In addition, the anisotropy parameter of these probes provides sensitive responses to changes in fluidity of the environment surrounding the probe. This can be used to follow the decrease in membrane fluidity that accompanies a rise in peroxidation.

Upon validation of the fluorescence spectroscopic assay using direct measures of lipid peroxidation, the second objective of this research was to test the ability of the method to discriminate between antioxidant activities of compounds within a structurally related class.

Flavonoids and isoflavonoids were the classes of compounds selected for evaluation in this study because they display excellent potential for use as antioxidants. These compounds exhibit a broad spectrum of positive pharmacological properties, including vasoprotective, antiinflammatory, antiviral, and antitumor activities. Many of these pharmacological properties are believed to be related to the antioxidant activity of these compounds (Rapta *et al.*, 1995).

Having established the ability of this method to distinguish differences in antioxidant activities among these compounds, the third objective of the study was to define the substitution patterns on the flavonoids and isoflavonoids that are necessary for a high antioxidant activity. Establishment of a structure activity relationship for flavonoids and isoflavonoids will allow for their selection as antioxidants based on structure alone, without having to screen every possible compound for activity.

For successful use of any compound to enhance oxidative stability of lipids, it is imperative that their mechanism of antioxidant action be completely understood. The antioxidant mechanisms for flavonoids and isoflavonoids have not been clearly established. The polyphenolic structure of flavonoids and isoflavonoids confers them with the ability to scavenge free radicals and also to chelate transition metals, two possible mechanisms of action. Additionally, these compounds may act as membrane stabilizers by decreasing fluidity in the lipid bilayer. The next aim of this study was to investigate the mechanisms of antioxidant action for flavonoids and isoflavonoids.

Prior to incorporating the pharmacologically relevant doses of these compounds in foods, their oxidation products need to be characterized to ascertain the safety of these

breakdown products. Profiling the non-radical products formed during the antioxidant reactions of a representative flavonoid or isoflavonoid would be the final aim of this study.

This dissertation was organized into a series of chapters. Each chapter covered a specific aim of the study and was prepared as a manuscript with its specific abstract, introduction, experimental procedures, results and discussion sections. Sections that were common to the entire dissertation included the initial abstract, introduction and literature review sections, and the final conclusion, future research and list of references sections.

CHAPTER 1

LITERATURE REVIEW

1.1 LIPID PEROXIDATION

Lipid peroxidation, the oxidative degradation of polyunsaturated lipids, is well-recognized as a major concern to food scientists since it results in the development of undesirable 'off-flavors' and potentially toxic reaction products (Coupeland and McClements, 1996). In recent decades, its relevance to the field of biology and medicine has also received increasing attention. Lipid peroxidation is a major contributor to membrane damage in cells, disrupting their important structural and protective functions (Barclay and Vinquist, 1994). It has also been implicated in a variety of pathological processes, such as inflammation, muscular dystrophy, ischemia / reperfusion and aging (Choe and Yu, 1995; Dix and Aikens, 1993; Yu *et al.* 1992).

1.1.1 Mechanism of Lipid Peroxidation

Lipid peroxidation proceeds by a classic free-radical chain mechanism involving three discrete phases: (i). Initiation, the formation of a carbon-centered lipid radical ($L^{\cdot}$) by abstraction of a hydrogen atom from an unsaturated fatty acid (LH) in the presence of an

initiator (Equation 1); (ii). Propagation, further reactions of L· with molecular oxygen and lipid to yield peroxyl radicals (LOO·) and lipid hydroperoxides (LOOH) (Equations 2 and 3); and, (iii). Termination, the formation of non-radical final products upon combination of two radicals (Equation 4) (Gutteridge, 1988).

Initiation	LH	+	Initiator	→	L·				(1)
Propagation	L·	+	O_2	→	LOO·				(2)
	LOO·	+	LH	→	LOOH	+	L·		(3)
Termination	L·	+	L·	→	LL				(4)

1.1.2 Role of Transition Metals

The direct reaction of LH with oxygen is spin-forbidden since the ground-state of lipids is of singlet multiplicity whereas that of oxygen is of triplet multiplicity (Miller *et al.*, 1990). Lipid peroxidation must, therefore, occur via reactions that relieve this spin-restriction between lipids and oxygen (Minotti and Aust, 1992). Transition metals such as iron and copper are efficient catalysts of lipid peroxidation because their reactions with dioxygen are not spin-restricted (Buettner and Jurkiewicz, 1996) and also because they can oscillate between reduced and oxidized states with great ease (Minotti, 1993).

Of the transition metals, iron plays the most significant role in lipid peroxidation. Two basic mechanisms have been established for understanding the role of iron in catalyzing lipid peroxidation. According to the first mechanism, Fe(II) catalyzes the decomposition of trace

amounts of preformed LOOHs to form the highly reactive alkoxyl radicals ($LO^{\cdot}$) which abstract hydrogen from a neighboring allylic bond (Minotti, 1992) (Equations 5 and 6). This is referred to as the "LOOH-dependent" lipid peroxidation (Minotti and Aust, 1987).

$$Fe(II) + LOOH \rightarrow Fe(III) + OH^{-} + LO^{\cdot} \quad (5)$$

$$LO^{\cdot} + LH \rightarrow L^{\cdot} + LOH \quad (6)$$

According to the second mechanism, Fe(II) reacts with oxygen to yield the superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2) (Equations 7 and 8). This is called the "LOOH-independent" lipid peroxidation since the reaction of Fe(II) with H_2O_2 eventually liberates hydroxyl radicals ($^{\cdot}OH$) that can abstract hydrogen from LH irrespective of any preformed LOOH (Equations 9 and 10) (Minotti, 1992).

$$Fe(II) + O_2 \rightarrow Fe(III) + O_2^{\cdot -} \quad (7)$$

$$2\,O_2^{\cdot -} + 2\,H^{+} \rightarrow H_2O_2 + O_2 \quad (8)$$

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + OH^{-} + {}^{\cdot}OH \quad (9)$$

$$^{\cdot}OH + LH \rightarrow H_2O + L^{\cdot} \quad (10)$$

1.1.3 Role of Antioxidants

The ubiquity of oxygen and transition metal catalysts in food products poses a serious challenge to the food technologist to minimize the undesirable peroxidation of the lipid

constituents (Graf, 1994). An antioxidant is defined as "any substance that, when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate" (Halliwell, 1990).

Based on the mode of action, antioxidants can be primarily divided into three categories: Type I, Type II and Type III (Labuza, 1971). The Type I antioxidants, such as tocopherols, work by scavenging free radicals and suppressing free radical oxidation by molecular oxygen. The Type II antioxidants, including metal chelating agents like ethylenediaminetetraacetic acid (EDTA),citric acid and various forms of ascorbic acid, prevent the production of free radicals. By sequestering the metal ions catalysts, these compounds suppress the generation of free radicals and reduce the overall rate of oxidation. The Type III antioxidants include environmental factors such as the physical removal of headspace oxygen through vacuum processing or modified atmosphere packaging.

1.1.3.1 Free Radical Scavenging Antioxidants

Chain-breaking antioxidants (AH) can interfere with the lipid peroxidation chain reaction by providing an easily-donatable hydrogen atom for abstraction by lipid radicals. These reactions compete with the chain propagating reactions of lipid peroxidation (Equations 11 and 12). The antioxidant-derived radical ($A^{\cdot}$) may further react with another peroxyl radical, dimerize to A_2, or get converted back to AH by reaction with another molecule (Equations 13, 14 and 15) (Gutteridge and Halliwell, 1990).

$$AH + LOO\cdot \rightarrow LOOH + A\cdot \quad (11)$$

$$LO\cdot + AH \rightarrow LOH + A\cdot \quad (12)$$

$$LOO\cdot + A\cdot \rightarrow LOOA \quad (13)$$

$$LO\cdot + A\cdot \rightarrow LOA \quad (14)$$

$$A\cdot + A\cdot \rightarrow A_2 \quad (15)$$

Many of the food antioxidants, such as α-tocopherol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate, act by a mechanism of free radical scavenging. They reduce the primary radical by a one-electron reduction to a non-radical chemical species, and in the process, get transformed into oxidized antioxidant radicals themselves. The two basic conditions that must be satisfied for a molecule to be defined as a chain-breaking antioxidant are: (i) when present in low concentrations, relative to the substrate to be oxidized, it can delay, retard or prevent the free-radical mediated oxidation; and (ii) the resulting radical formed after scavenging must be of such low reactivity that no further reactions with lipids can occur (Halliwell, 1990).

These chain-breaking, free radical scavenging antioxidants are generally phenolic compounds with one or more hindered phenolic hydroxyl groups. These phenolic compounds are excellent hydrogen atom or electron donors; in addition, their radical intermediates are relatively stable due to resonance delocalization and lack of suitable sites for attack by molecular oxygen (Shahidi and Wanasundara, 1992). The efficacy of these compounds as antioxidants is related to their chemical structure. In addition to modulating the radical

scavenging activity of the hydroxyl group, the chemical structure of these antioxidants also influences their physical properties such as volatility, lipid solubility and thermal stability (Halliwell, 1994).

1.1.3.2 Metal Chelators

In certain cases, chelators form thermodynamically stable complexes that can remove metals that are complexed to biomolecules, thereby protecting them from site-specific oxidation. Complexation of metals may also hinder their ability to participate in redox reactions (Buettner and Jurkiewicz, 1996). However, the use of chelators cannot guarantee the removal of metal ions as catalysts of lipid peroxidation.

Various chelators like adenosine diphosphate (ADP), EDTA, citrate and histidine have been shown to have a prooxidant, antioxidant or no effect on the rates of metal-catalyzed lipid peroxidation (Ahn *et al.* 1993; Tampo *et al.* 1994; Ursini *et al.* 1989; Yoshida *et al.* 1993). The information available in the literature on the effects of chelators on metal-catalyzed lipid peroxidation is contradictory and not well understood due to the lack of standardized test conditions used in these studies. This is because the results obtained with metal chelators are greatly dependent on the source and preparation of tissues, cells, membranes or purified lipids, the solvents used, the concentrations of metal complexes, the presence or absence of oxygen source, and the methods used for measurements of these effects (Schaich, 1992).

Many studies have demonstrated the complexity imparted by chelators on the rates of metal-catalyzed lipid peroxidation. Chelators are critical determinants of the catalytic mode and effectiveness of metals (Schaich, 1992). Kinetics, mechanisms, and products of

metal-catalyzed lipid peroxidation can be altered by chelation. The presence and nature of chelators and the chelator to iron ratio is known to have a strong effect on iron autoxidation (Minotti, 1993). Chelators alter the redox potential of metals and may also render steric effects which may influence the rate or efficiency of coordination or binding to the target molecule and/or hydroperoxide (Chevion, 1988).

The effects of chelators on rates of transition metal catalyzed lipid peroxidation are dependent on the metal affinities and charges of the chelators, and also on their partitioning properties between lipid and aqueous phases. The valence state of metal they stabilize, the metal coordination sites they occupy, the type of electron transfer reactions they mediate and the redox potential of their complexes all influence the rates of lipid peroxidation (Schaich, 1992).

While there is some information available on the effects of other chelators, most studies have dealt with EDTA and its effects on metal-catalyzed lipid peroxidation. EDTA is known to remove the "free" or loosely bound complexed iron from solution, thereby making it unavailable for participation in peroxidation reactions. Additionally, it lowers the Fe(II) / Fe(III) redox potential and thus, limits the capability of iron to act as an oxidizing agent. However, the lower redox potential makes EDTA-iron a better reductant, so EDTA-Fe(II) chelates reduce lipid hydroperoxides faster than uncomplexed iron. In the presence of reducing agents that can recycle the iron, EDTA complexation may even result in marked acceleration of chain propagation and branching reactions (Tien *et al.*, 1982). The net effect of EDTA results from the balance between these actions in individual systems, and has led to

apparently contradictory reports and interpretations of effects of EDTA on lipid peroxidation (Schaich, 1992).

The ratio of chelator to metal cation can also markedly affect the mechanism of initiation of lipid peroxidation (Kanner *et al.*, 1987). EDTA is known to accelerate lipid peroxidation at chelator to metal ratios of less than one, whereas it inhibits peroxidation at chelator to metal ratios of greater than one. This is because EDTA, in stoichiometric excess, deactivates the iron ion by surrounding it with tightly bound ligands that cannot be replaced with reagents such as hydroperoxides (Waters, 1971).

In addition to chelator to metal ratio, lipid configuration also alters the effects of the chelator-metal complex on the rate of lipid peroxidation. In liposomes and microsomes, EDTA appears to prevent iron penetration through the membrane and the formation of a site-specific attack of ·OH radical towards the unsaturated fatty acids. This effect is eliminated upon dispersion of the liposomes or microsomes with detergents (Kanner *et al.*, 1986; Girotti and Thomas, 1984). Surface charge on membranes and chelators also affects molecular access and binding as well as the dynamics of electron transfer reactions through lipid phases, thereby altering membrane peroxidation rates (Schaich, 1992).

1.1.4 Methodology for Measuring Oxidative Stability of Lipids

1.1.4.1 Need for a rapid and sensitive assay

Extremely potent synthetic antioxidants such as BHA, BHT, *tert*-butylhydroquinone (TBHQ) and various esters of gallic acid have been developed and proven to be extremely

effective in increasing the oxidative stability of foods (Namiki, 1990). Though these synthetic antioxidants have been very thoroughly tested for their toxicological behavior, some of them are now coming into disfavor as new toxicological data impose some caution on their use (Haigh, 1986). This has led to increased interest in the use of plant-derived compounds as antioxidants to satisfy consumer concerns regarding synthetic compounds. In order to screen a large number of compounds for possible antioxidant activity, it is necessary to have a simple, sensitive and rapid assay for evaluating the oxidative stability of lipids.

1.1.4.2 Current Methodology for Evaluation of Oxidative Stability of Lipids

The current methods for antioxidant evaluation and for measurement of oxidative stability of lipids suffer from serious limitations. Accelerated stability tests are most commonly used to estimate the shelf-life of a food. To speed up the oxidation process in these tests, several parameters such as temperature, metal catalysts and oxygen pressure are manipulated. Heating is most commonly used to accelerate oxidation since the rate of reaction increases exponentially with the absolute temperature (Ragnarrson and Labuza, 1977).

However, the higher the temperatures, the greater the number of limitations associated with these tests. Results obtained with accelerated tests like the Rancimat (produced by Metrohm Ltd., CH-9100, Herisau, Switzerland) and the Active Oxygen Method are not representative of real-life storage conditions since the mechanism of lipid peroxidation changes significantly at the elevated temperatures (100-140^{0}C and 98^{0}C respectively) used in these tests (Frankel, 1993).

The effects of temperature on multi-component food systems can be very complex. The physical and chemical changes occurring at these higher temperatures could drastically affect the chemical reactivity through their effect on the distribution of reactants, metal binding properties, viscosity, metal prooxidant effectiveness, water activity and other variables. Other limitations associated with these high temperatures are that volatile antioxidants such as BHA and BHT undergo significant losses at the elevated temperatures, decomposition of phenolic antioxidants in natural extracts occurs, and the rate of oxidation becomes dependent on oxygen concentration because solubility of oxygen decreases at elevated temperatures. Additionally, Maillard reaction products that possess proven antioxidant activities may be formed at the higher temperatures (Ragnarsson and Labuza, 1977).

In the accelerated stability tests, the induction period or the onset of oxidation is determined using standard methodology for measurement of lipid peroxidation. The 2-thiobarbituric acid (TBA) test is the most frequently used method for assessing peroxidative damage to lipids. This spectrophotometric test measures the amount of pink chromagen formed by reaction of two molecules of TBA with one molecule of malondialdehyde (MDA), one of the many decomposition products of peroxidation of polyunsaturated fatty acids. As lipids are peroxidized, the amount of TBA-reactive substances (TBARS) increases in the product, and as such, the measurement of TBARS provides a useful index of lipid peroxidation (Gray and Monahan, 1992).

However, the interference by other aldehydes and a large number of biological molecules (such as amino acids, bile pigments, and some carbohydrates) and the generation

of MDA during the assay itself make the TBA test somewhat non-specific and unsuitable for certain test substrates (Gray and Monahan, 1992). In addition, the formation of MDA occurs so far downstream from the initial production of lipid hydroperoxides that it is difficult to translate rates of MDA formation into direct rate constants for expressing antioxidant activity of test compounds.

Peroxide value determination is often used for measurement of hydroperoxides, one of the initial products formed upon peroxidation of lipids. Peroxides may be measured by a variety of methods. The most commonly used methods utilize iodometric techniques similar to the 1993 Association of Official Analytical Chemists (AOAC) method, in which peroxide value is reported as milliequivalents (meq) of iodine per kilogram of fat. Determination of hydroperoxides may not be a useful measure of lipid peroxidation in foods during prolonged storage, as breakdown to secondary products such as aldehydes may occur, leading to an underestimation of the degree of oxidation (Melton, 1983).

Another method for assessing the extent of lipid peroxidation involves measurement of conjugated dienes structures which absorb ultraviolet (UV) light in the range of 230-235 nm. Conjugated dienes reflect the initial damage to the unsaturated fatty acids upon exposure to oxygen, and as such, are useful in assessing the early stages of the peroxidation process in studies of pure lipids (Halliwell and Chirico, 1993). However, application of this simple absorbance technique to more complex biological and food systems can lead to serious misinterpretation problems because changes in absorbance in this region of the spectrum cannot be uniquely attributed to conjugated dienes. It has been shown that human body fluids contain a non-oxygen-containing isomer of linoleic acid, octadeca-9-*cis*-11-*trans*-dienoic acid,

that absorbs UV radiation at the conjugated diene wavelength (Dormandy and Wickens, 1987). Though this compound was initially proposed to result from hydrogen abstraction of linoleic acid and reaction of the resulting carbon-centered radicals with protein, it was later identified as a product of bacterial fatty acid metabolism (Jack *et al.*, 1991). Since conjugated diene products may also be present in animal diets, use of this technique can also lead to erroneous results in attempts to measure lipid peroxidation in tissues (Holley and Slater, 1991).

1.1.5 Model Systems for Measurement of Lipid Peroxidation

The inconsistent reports in the literature on the effects of metals on rates of lipid peroxidation in biological systems result from the lack of precisely controlled conditions and defined composition of laboratory test tube conditions in the cells, tissues, membranes and organelles used in these studies (Schaich, 1992).

1.1.5.1 Biological Membranes as Substrates for Lipid Peroxidation

For many food products, the membrane constituents of the cells comprising the food are the primary sites of peroxidative damage. This is likely related to the large surface exposure of membrane phospholipid molecules to the aqueous phase containing peroxidation-initiating species, in contrast to neutral lipid molecules in fat droplets which have a much lower surface to volume ratio. Sarcoplasmic reticulum microsomes (Monahan *et al.*, 1994; Dinis *et al.*, 1993) and mammalian erythrocyte membranes (McKenna *et al.*, 1991; Thomas *et al.*, 1990) are useful models in membrane peroxidation studies. However, use of biological

membranes as lipid substrates to evaluate antioxidant activity of compounds is complicated by the presence of numerous endogenous prooxidative and antioxidative factors, including transition metals, heme proteins, catalases, glutathione reductase, and superoxide dismutase (Kanner *et al.*, 1988; Kellogg and Fridovich, 1975; Girotti and Thomas, 1984).

1.1.5.2 Liposomes as Models for Membrane Lipid Peroxidation

Liposomes - self-assembling colloidal particles in which a lipid bilayer encapsulates a fraction of the surrounding aqueous medium (Lasic and Papahadjopoulos, 1995) - constitute a simple and convenient system for studying membrane lipid peroxidation and its inhibition by antioxidants without the ambiguities introduced by enzymes or scavengers that may be present in more complex biological systems (Vigo-Pelfrey and Nguyen, 1991). Using liposomes as the substrate, the oxidation process can be reproduced with different levels of complexity.

On the basis of size and number of bilayers, liposomes are primarily categorized into three types - multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs) and large unilamellar vesicles (LUVs). MLVs, which are typically greater than 400 nm in diameter, consist of numerous concentric bilayers separated by narrow aqueous channels (Hope *et al.*, 1986). Liposomes comprising a single, bimolecular layer of lipids are divided into two classes based on size: vesicles that range in diameter from 20 to 50 nm are considered to be SUVs, whereas those with a diameter of 60 nm or greater are classified as LUVs (Chapman, 1984). MLVs are formed spontaneously when dry phospholipid films swell in excess water or buffer,

whereas some energy must be dissipated into the system in order to produce SUVs and LUVs, since they possess higher free energies (Lasic, 1988).

Despite their ease of preparation, MLVs represent a poor choice as a model for lipid peroxidation studies due to their heterogeneity in size, the relatively small proportion of lipid that is exposed to the aqueous phase and the different degrees of accessibility from the external aqueous medium into the internal bilayers (Hope *et al.*, 1986). SUVs also inadequately mimic the properties of natural biomembranes. In SUVs, a disproportionate amount (approximately 70%) of the total lipid is located in the outer leaflet of the vesicle. In addition, the small radius of curvature in SUVs imposes strains in packing of the lipid molecules that are not associated with biological membranes (Chapman, 1984). LUVs offer the combined advantages of MLVs and SUVs. Their single bilayer, uniform size and large radius of curvature makes LUVs an excellent model for lipid peroxidation studies.

1.2 FLUORESCENCE SPECTROSCOPY

Fluorescence spectroscopy is a method that can easily provide extensive information regarding the structure and interactions of macromolecules that may only be obtainable by other approaches with difficulty (Bentley *et al.*, 1985). Despite the inherent simplicity and reliability of this technique, the utility and potential of fluorescence methods has yet to be fully recognized.

1.2.1 Advantages of Fluorescence Spectroscopy

Fluorescence spectroscopy offers several advantages for characterization of molecular reactions and interactions. First, it is approximately 1000 times more sensitive than spectrophotometric techniques, an attribute that becomes important when characterizing mechanisms and identifying small amounts of unique reaction products. Second, fluorescent compounds are exquisitely sensitive to their environment. Lipophilic and amphipathic membrane probes are virtually non-fluorescent in the aqueous phase, but when embedded in the hydrophobic environment of the lipid bilayer, they are highly fluorescent. This environmental sensitivity allows characterization of phenomena such as changes in membrane fluidity and cross-linking reactions that may occur during lipid peroxidation. Third, fluorescence methods once established are relatively rapid; thus, a substantial amount of information can be quickly obtained (Strasburg and Ludescher, 1995).

1.2.2 Basis of the Fluorescence Assay

The underlying principles which make fluorescence a useful method for peroxidation studies are derived in part from the molecular characteristics of the fluorescent probes selected for these studies. Typically, these probes possess a conjugated double bond structure which gives rise to their fluorescent properties, as well as to a susceptibility to reaction with free radical intermediates of lipid peroxidation produced by added initiators such as Fe(II) metal ions, resulting in the formation of non-fluorescent products. The rate of decay of fluorescence intensity, therefore, reflects the rate of peroxidation. Addition of free-radical scavenging antioxidants (which compete with the fluorescent probe for reaction), or of metal

chelators (which alter the reactivity of the metal ion initiator species), will alter the rate of fluorescence intensity decay. The extent to which fluorescence decay is inhibited by the added compound is an indicator of its antioxidant efficacy. These properties combine to make fluorescence spectroscopy a useful tool for rapid evaluation of antioxidant candidates and for identifying structural characteristics which define the antioxidant activities of structurally related molecules.

1.2.3 Selection of Probe

Today, many well-characterized membrane probes are commercially available, and selection of a probe must be made after careful consideration of the factors outlined below.

1.2.3.1 Perturbation of membrane

The insertion of extrinsic probes into the membrane inevitably results in some perturbation of the system (Lentz, 1993) which may, in turn, alter a membrane's susceptibility to peroxidation. Use of fluorescent reporter molecules, which are structurally similar in chain length and bulk to the membrane lipids, can reduce the amount of structural perturbation of the lipid bilayer. Further reductions in the levels of macroscopic perturbation can be achieved by use of a low concentration of probe molecules relative to the lipid molecules. A ratio of less than one probe molecule to one hundred lipid molecules is usually desirable in membrane studies.

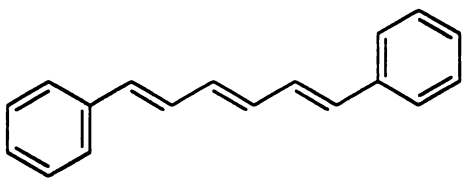
1.2.3.2 High quantum yield

Minimization of fluorescent probe concentration results in reduction of fluorescent intensity. To partially offset the reduced signal, the fluorophore of choice should have a high quantum yield; i.e., a high ratio of photons emitted to photons absorbed. Probes with high quantum yield can be used at low concentrations, thereby minimizing the structural perturbations to the membrane while maintaining adequate signal intensity.

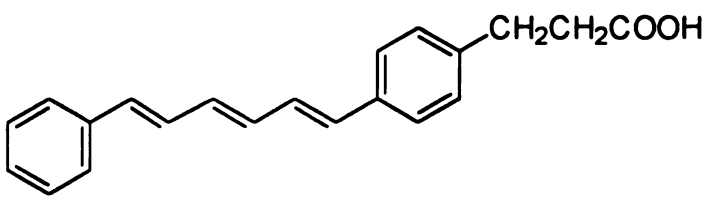
1.2.3.3 Location of the extrinsic fluorescent probe

The precise orientation and location of a fluorophore in the lipid bilayer are also of great importance for the interpretation of experimental observations, and hence need to be clearly established. The partitioning of the fluorophore in the lipid bilayer can be predicted from the geometry of the fluorophore as well as of the whole probe molecule (Borenstain and Barenholz, 1993). In membranes, symmetric, non-polar molecules such as 1,6-diphenyl-1,3,5-hexatriene (DPH, Figure 1.1) diffuse rapidly into the hydrophobic interior of the bilayer with a lack of orientational specificity, thereby providing only bulk-averaged information (Beck *et al.*, 1990). By appropriate chemical modification, such probes can be localized in specific regions of the membrane. The fluorescent probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA, Figure 1.1), an anionic derivative of the parent DPH molecule, has a more restricted orientation in the membrane. The polar substituent of DPH-PA provides a surface anchor that ensures that the charged substituent on the probe is localized at the lipid-water interface of the membrane, whereas the lipophilic tail of the probe

(a)



(b)



(c)

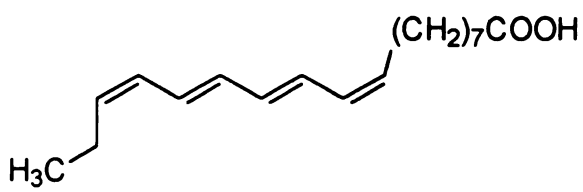


Figure 1.1 Structures of some extrinsic probes commonly used for membrane studies: (a), 1,6-diphenyl-1,3,5-hexatriene (DPH); (b), 3-(*p*-(6-phenyl)1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA); and (c), *cis-trans-trans-cis*-9,11,13,15-octadecatetraenoic acid (*cis*-PA).

is embedded in the hydrophobic interior of the membrane, lying parallel to the acyl chains (Beck *et al.*, 1990).

1.2.3.4 Photodamage

Fluorescent molecules are, to varying degrees, subject to photooxidation during the course of an experiment. Photochemically-induced damage to the fluorescent probe usually results in loss of fluorescence which may confound interpretation of chemical peroxidation reaction kinetics. For example, the fluorescent probe *cis-trans-trans-cis*-9,11,13,15-octadecatetraenoic acid, commonly referred to as *cis*-parinaric acid (*cis*-PA, Figure 1.1), has been used by various researchers to monitor lipid peroxidation in membranes because of its close structural similarity to intrinsic membrane lipids (de Hingh *et al.*, 1995; Dinis *et al.*, 1993; Kuypers *et al.*, 1987; Laranjinha *et al.*, 1992; McKenna *et al.*, 1991; Van den Berg *et al.*, 1988). However, it is very photolabile and undergoes light-induced dimerization (Morgan *et al.*, 1980). This necessitates special handling requirements for the probe, and the use of excitation light of the lowest possible intensity for short periods of time (Lentz, 1993). This disadvantage may preclude the use of *cis*-PA in experiments requiring long exposure of sample to light. In such cases, one may select alternative probes like DPH and DPH-PA that exhibit greater photostability.

1.2.3.5 Labeling the lipid assembly by the fluorescent probe

Another important point to consider during probe selection for the study of membranes is the requirement for a high lipid to aqueous phase partition coefficient ($\geq 1 \times$

10^8). Probes that are hydrophobic (DPH) or amphipathic (DPH-PA and *cis*-PA) have very low fluorescence in the aqueous phase and partition spontaneously into the lipid environment of the membranes. This ensures that the level of fluorophore remaining in the aqueous phase during the experiment is minimal (Borenstain and Barenholz, 1993), and that the observed fluorescence is reporting molecular reactions occurring in the lipidic regions alone.

1.2.3.6 Probe concentration

For unambiguous interpretation of results, the optimal concentration of fluorescent probe and the ratio of probe molecules to that of lipid molecules needs to be determined. The fluorescent intensity of the probe must be a linear function of its concentration; i.e., as fluorescent molecules react with free radicals during peroxidation, the loss of fluorescence intensity should be directly proportional to the reaction progress. This linearity is generally valid only at low concentrations. As membranes become concentrated with probes, the fluorescent intensity dependence on the concentration of the probe is reduced and may even decrease because of the inner filter effect or quenching of fluorescence by the probe molecules themselves (Borenstain and Barenholz, 1993).

1.3 FLAVONOIDS AND ISOFLAVONOIDS

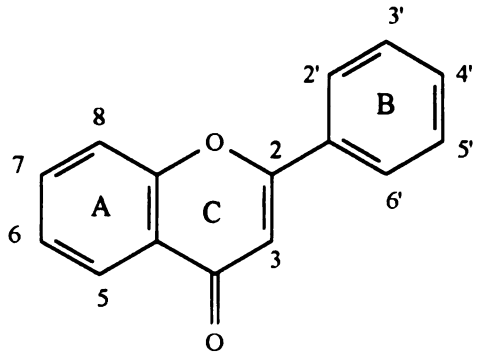
1.3.1 General Structure and Major Classifications

Flavonoids are ubiquitous, naturally occurring, low molecular weight aryl compounds found in photosynthesizing cells, seeds, fruits and flowers (Das, 1994). Over 4,000

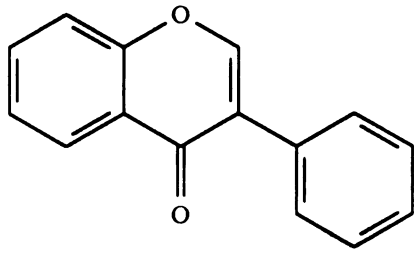
flavonoids have been identified from both higher and lower plants, and the list is constantly expanding (Cook and Samman, 1996).

Flavonoids exhibit a wide diversity in structure. Based on a few backbone structures, various hydroxylation, methoxylation, sulfation and/or glycosylation patterns exist (Bors *et al.*, 1990). The basic feature common to all flavonoids is the flavone nucleus made up of two benzene rings (A and B) linked through a heterocyclic pyrane C ring. The position of the benzenoid B ring divides the flavonoid class into the two subclasses of flavonoids (2-position) and isoflavonoids (3-position) (Figure 1.2). The six-membered C ring is either a γ-pyrone, i.e., has a C_2-C_3 double bond (flavonols and flavones) or its dihydro derivative (flavanols and flavanones) (Havsteen, 1983).

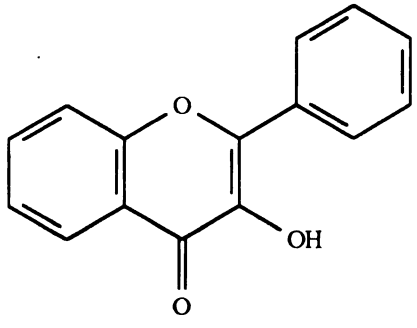
These compounds are often hydroxylated at positions 3, 5, 7, 3', 4' and 5'. The presence or absence of a hydroxyl group at position 3 further results in the generation of two main subgroups for the flavonoids and isoflavonoids: 3-hydroxy flavonoids and isoflavonoids (flavonols, flavanols, isoflavonols, isoflavanols) and 3-deoxy flavonoids and isoflavonoids (flavones, flavanones, isoflavones, isoflavanones). These compounds occasionally occur naturally in plants as their free aglycone forms; however, the most frequently encountered forms are the glycoside derivatives. For the flavonoid glycosides, the glycosidic linkage is normally located at positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose or arabinose (Brandi, 1992).



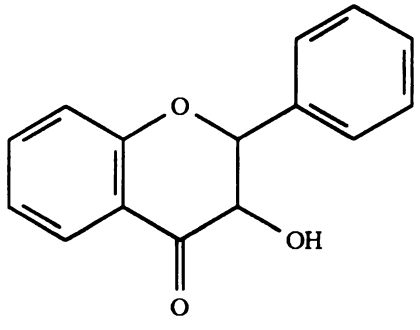
Flavone



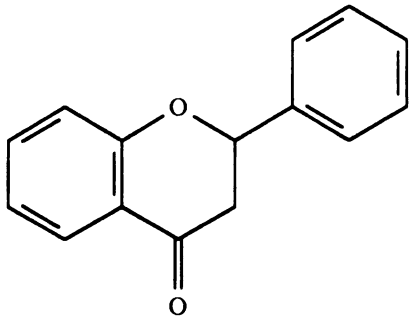
Isoflavone



Flavonol



Flavanol



Flavanone

Figure 1.2 Main classes of the flavonoid family.

1.3.2 Dietary Intake and Food Sources

1.3.2.1 Flavonoids

Flavonoids are widespread among plant and plant products (i.e., fermented and processed foods) (Formica and Regelson, 1995). Their daily consumption in the diet is difficult to determine since intake is strongly dependent on feeding habits and, in this field, exhaustive tables on food composition are not always available (Manach *et al.*, 1996). Until recently, data on human flavonoid intake were obtained from Kühnau (1976) who estimated the average intake of all dietary flavonoids in the Western diet to be approximately 1 g/day (expressed as glycosides) of which about 170 mg (expressed as aglycones) consisted of flavonols, flavanones and flavones. These values have been widely cited in the literature; however, they were based mainly on food analysis techniques of doubtful accuracy (Hertog *et al.*, 1993a). Recently, the consumption of the flavonols quercetin, luteolin, kaempferol, apigenin, and myricetin (and their glycoside derivatives) was documented in The Netherlands using more advanced technologies (Hertog *et al.*, 1993a; Hertog *et al.*, 1993b; Hertog *et al.*, 1992). Based on these analyses, the average dietary intake of flavonoids in The Netherlands was estimated to be approximately 23 mg/day (expressed as aglycones); with quercetin being the major dietary flavonoid (16 mg/ day) (Hertog *et al.*, 1993b).

Tea, coffee, cocoa, fruit juices, red wines, beer and vinegar are some of the important dietary sources of flavonoids, accounting for approximately 25-30% of total flavonoid intake (Kühnau, 1976). The flavonoid glycosides, particularly quercetin and kaempferol glycosides, are found in the edible portions of a majority of food plants, e.g. citrus and other fruits;

berries, leafy vegetables; roots, tubers and bulbs; herbs and spices; legumes; cereal grains; and tea and cocoa (Brown, 1980).

1.3.2.2 Isoflavonoids

In marked contrast to the flavonoids, isoflavonoids have a very limited distribution in the plant kingdom. Over 90% of the fully characterized isoflavonoids (aglycones and glycosides) are produced by species belonging to the large and taxonomically-advanced subfamily Papilionoideae of the Leguminosae (Ingham, 1983). Even within the two other smaller and more primitive subfamilies Caesalpinioideae and Mimosoideae of the Leguminosae, only one or two plants have been reported to contain isoflavonoids (Dewick, 1988). As a consequence of their limited distribution, restricted primarily to tropical legumes, isoflavonoids represent a very minor part of Western diets (Coward *et al.*, 1993).

Soybeans are a rich dietary source of isoflavonoids. In soybeans and foods derived from soy, isoflavones are found in concentrations ranging from 0.1 to 5 mg/g (Coward *et al.*, 1993). Genistein (4',5,7-trihydroxyisoflavone) and daidzein (4',7-dihydroxyisoflavone) are the two most abundant isoflavones found in soy (Wang and Murphy, 1994a). These two isoflavones occur principally as their 7-*O*-glucosides, genistin and daidzin (Wang and Murphy, 1994b).

1.3.3 Absorption and Metabolism

1.3.3.1 General Pathway

Most of the flavonoids enter the diet as glycosides, with quercetrin and rutin (glycosides of quercetin) being the most commonly consumed flavonoids (Bokkenheuser and Winter, 1988). The hydrophilicity and relatively high molecular weight of the glycosides preclude their absorption in the small intestine. Furthermore, these flavonoid β-glycosides are resistant to hydrolysis by the intestinal digestive enzymes and pass largely unaltered into the large intestine (Brown, 1980). The microflora of the mammalian lower bowel produce glycosidases capable of hydrolyzing the flavonoid glycosides to their constituent aglycones and sugars (Hawksworth *et al.*, 1971; Prizont *et al.*, 1976; Scheline, 1968). In addition to glycoside hydrolysis, the resident microflora can cleave the pyrone ring (ring C) to yield a variety of phenolic acids, such as phenylpropionic and phenylacetic acid derivatives, and other derivatives (Brown, 1980; Hackett, 1986). However, as glycosidase activity proceeds at a faster rate than ring cleavage, the intact flavonoid aglycone can persist in the large intestine with the clear potential for absorption (Formica and Regelson, 1995).

The major metabolic reactions occurring in the lower gut appear to be hydrolysis and reductions. Liver is the site for most conjugation and oxidation reactions of these compounds. A conjugation reaction with glucuronic acid or sulfate in the liver is perhaps the most common final step in the metabolic pathways of intact flavonoids. The polar, water soluble flavonoid glucuronides and sulfates appear to be readily excreted by mammals. These polar conjugates are either excreted into the urine or into the duodenum with bile salts, where

they come into contact with the enzymes of intestinal micro-organisms. It appears likely that in case of the flavonoids preferentially excreted in the bile, ring-fission products and flavonoid aglycones are the metabolic products not only of unabsorbed orally administered flavonoids, but also of their biliary metabolites. The probable fate of these biliary flavonoid glucuronides is metabolic hydrolysis in the lumen of the intestine. The liberated flavonoids may undergo further metabolism by intestinal micro-organisms or be reabsorbed from the intestine, transferred via the hepatic portal vein to the liver and metabolized and re-excreted in bile creating an enterohepatic circulation (Hackett, 1986).

Despite the potentially significant effects of these compounds *in vivo*, there is a paucity of information about the absorption, metabolism and excretion of individual flavonoids and isoflavonoids in humans. Most studies of individual flavonoid metabolism in humans have used pharmacological doses of these compounds rather than the estimated dietary intake levels of 23 to 170 mg/day; thereby, making it difficult to extrapolate results of these studies to explain the absorption and metabolism of dietary flavonoids (Cook and Samman, 1996).

1.3.3.2 Flavonoids

The metabolism and absorption of quercetin, the major representative of the flavonol subclass, has been examined in some studies. Gugler *et al.* (1975) investigated the metabolism of quercetin in six volunteers after the administration of single intravenous (100 mg) or oral (4 g) doses. Of the intravenous dose, approximately 7% was excreted in the urine as a conjugated metabolite, and less than 1% was excreted unchanged. In contrast, after the

oral administration of quercetin, no measurable plasma concentrations could be detected and nor was any quercetin found in the urine, either unchanged or in a metabolized form. The fecal recovery for quercetin after the oral dose was around 53%, indicating extensive degradation by gut microflora. This led the researchers to conclude that oral administration of flavonoids may be of questionable value. An earlier study with quercetin and rutin also failed to detect any of the two compounds in urine after their oral administration (Clark and MacKay, 1950).

Recent studies, however, appear to dispute these findings. In a human study with ileostomy subjects, absorption of quercetin aglycone was found to be 24% (Hollman *et al.*, 1995). The absorption of quercetin glucosides from onions was found to higher, at 52%. The data from this study suggest that humans absorb appreciable amounts of quercetin and that the absorption of glycosides in the small intestine is possible.

A later study by the same group of researchers extended these findings. Hollman *et al.* (1996) examined the time course of plasma quercetin concentration in two subjects after ingestion of fried onions containing quercetin glucosides equivalent to 64 mg of quercetin aglycone. The mean peak plasma level of quercetin was 200 ng/ml and was reached 2.9 h after ingestion of the onions, with an average half-life of absorption of 0.87 h. The half-life of the elimination phase was 17 h, suggesting that repeated intake of quercetin glucosides would lead to a build-up of the concentration in plasma. Quercetin was still detectable in the plasma, at a concentration of 10 ng/ml, 48 h after ingestion of the onions.

1.3.3.3 Isoflavonoids

Current knowledge of isoflavonoid metabolism stems largely from sheep studies. Since a massive outbreak of permanent and temporary infertility in sheep grazing on certain cultivars of subterranean clover (*Trifolium subterraneum L.*) in Western Australia was reported in the 1940s (Bennetts *et al.*, 1946), a substantial amount of work on the metabolism and estrogenic effects of isoflavones on sheep has been conducted. For ruminants, the major metabolic transformation of isoflavones is performed by microorganisms in the rumen (Nilsson *et al.*, 1967). Biochanin A undergoes demethylation to genistein, which is converted to *p*-ethyl phenol and other phenolic acids by ring cleavage (Lundh, 1995). In contrast, formononetin, which is indirectly responsible for estrogenic disturbances in sheep (Millington *et al.*, 1964), is primarily demethylated to daidzein and subsequently to equol via hydrogenation (Lundh, 1995).

In spite of the purported significant role of isoflavonoids in human health, the pathways of metabolism of daidzein and genistein, the two principal isoflavones found in legumes and soy, remain unclear. These two isoflavones can be obtained from plant foods as the free, unconjugated forms, referred to as daidzein and genistein, or through the deconjugation of the glycosidic derivatives, daidzin and genistin. Additionally, they can be synthesized in the intestinal tract from their plant precursors, formononetin and biochanin A, respectively (Adlercreutz *et al.*, 1991, Hutchins *et al.*, 1995). *In vitro* anaerobic incubation of isoflavones with human feces suggests that intestinal half-life of genistein and daidzein may be as little as 3.3 h and 7.5 h respectively, indicating rapid degradation to other compounds by the gut microflora (Xu *et al.*, 1995).

Following ingestion of isoflavone-rich foods such as soy, the following compounds have been described in human urine: the isoflavones daidzein, genistein and formononetin; and their metabolites equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman), *O*-desmethylangolensin (*O*-Dma), dihydrodaidzein, dihydrogenistein, tetrahydrodaidzein, 6'-hydroxy-*O*-Dma, and dehydro-*O*-Dma (Adlercreutz *et al.*, 1986; Adlercreutz *et al.*, 1991; Axelson *et al.*, 1982; Axelson *et al.*, 1984; Bannwart *et al.*, 1984; Joannou *et al.*, 1995; Kelly *et al.*, 1993; Kelly *et al.*, 1995).

Biochanin A and formononetin undergo demethylation by intestinal bacteria, giving rise to genistein and daidzein respectively. Genistein is metabolized within the gut by ring cleavage to yield the non-estrogenic, hormonally inert compound *p*-ethylphenol (Kelly *et al.*, 1993). Recently, dihydrogenistein and 6'-OH-*O*-Dma were also identified as catabolic products of genistein metabolism in humans (Joannou *et al.*, 1995) (Figure 1.3). Daidzein undergoes intestinal microbial transformation in humans to yield the isoflavan equol (about 70%) and *O*-desmethylangolensin (5-20%) (Setchell and Adlercreutz, 1988). Equol does not appear to be degraded further and most of the absorbed equol is conjugated in the liver with glucuronic acid before being excreted in the urine (Axelson and Setchell, 1980; Axelson *et al.*, 1984). Other daidzein intermediates formed during this conversion are dihydrodaidzein, 4-hydroxy equol, 2-dehydro-*O*-Dma, and possibly dehydroequol (Joannou *et al.*, 1995) (Figure 1.4).

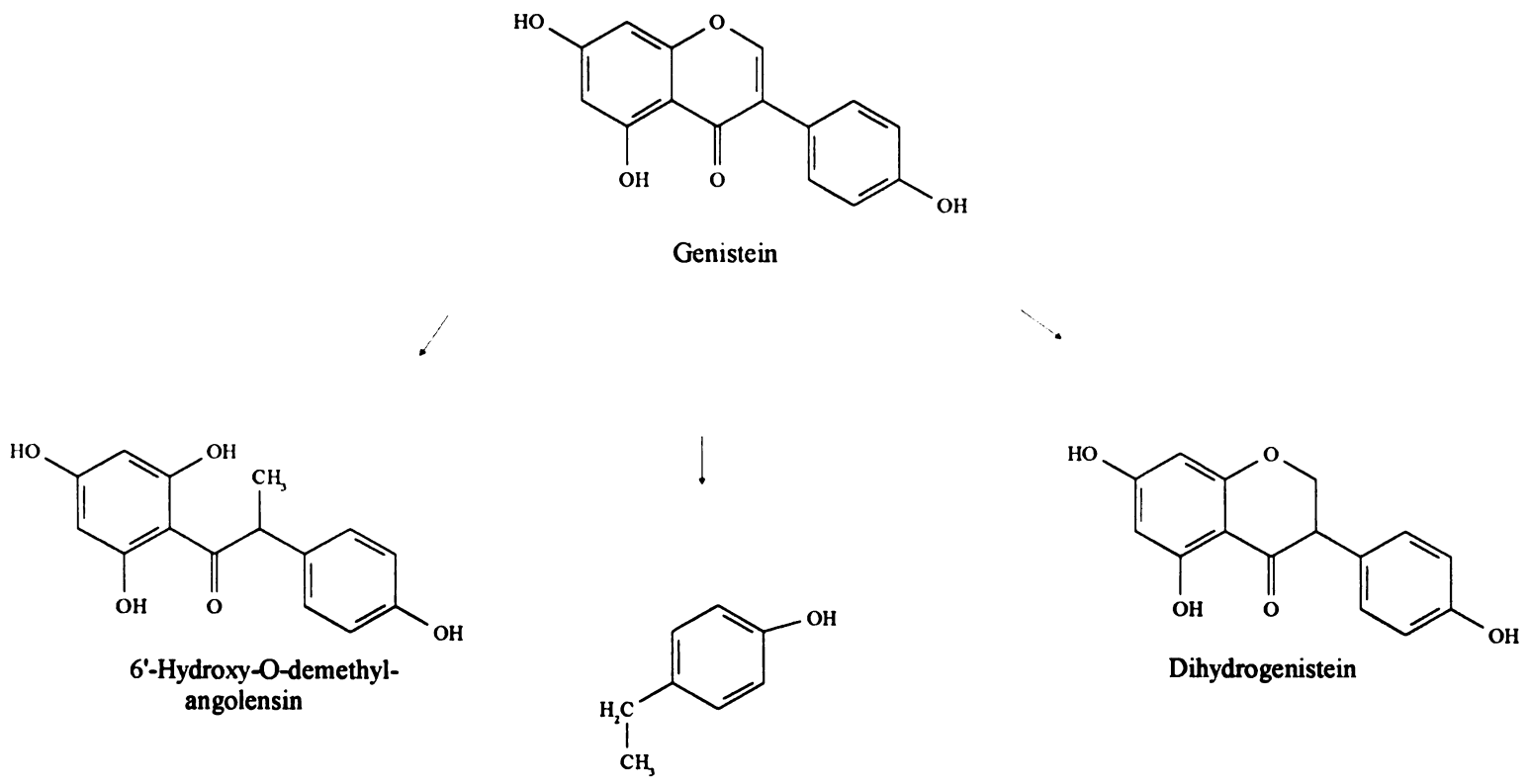


Figure 1.3 Metabolites of genistein detected in human urine.

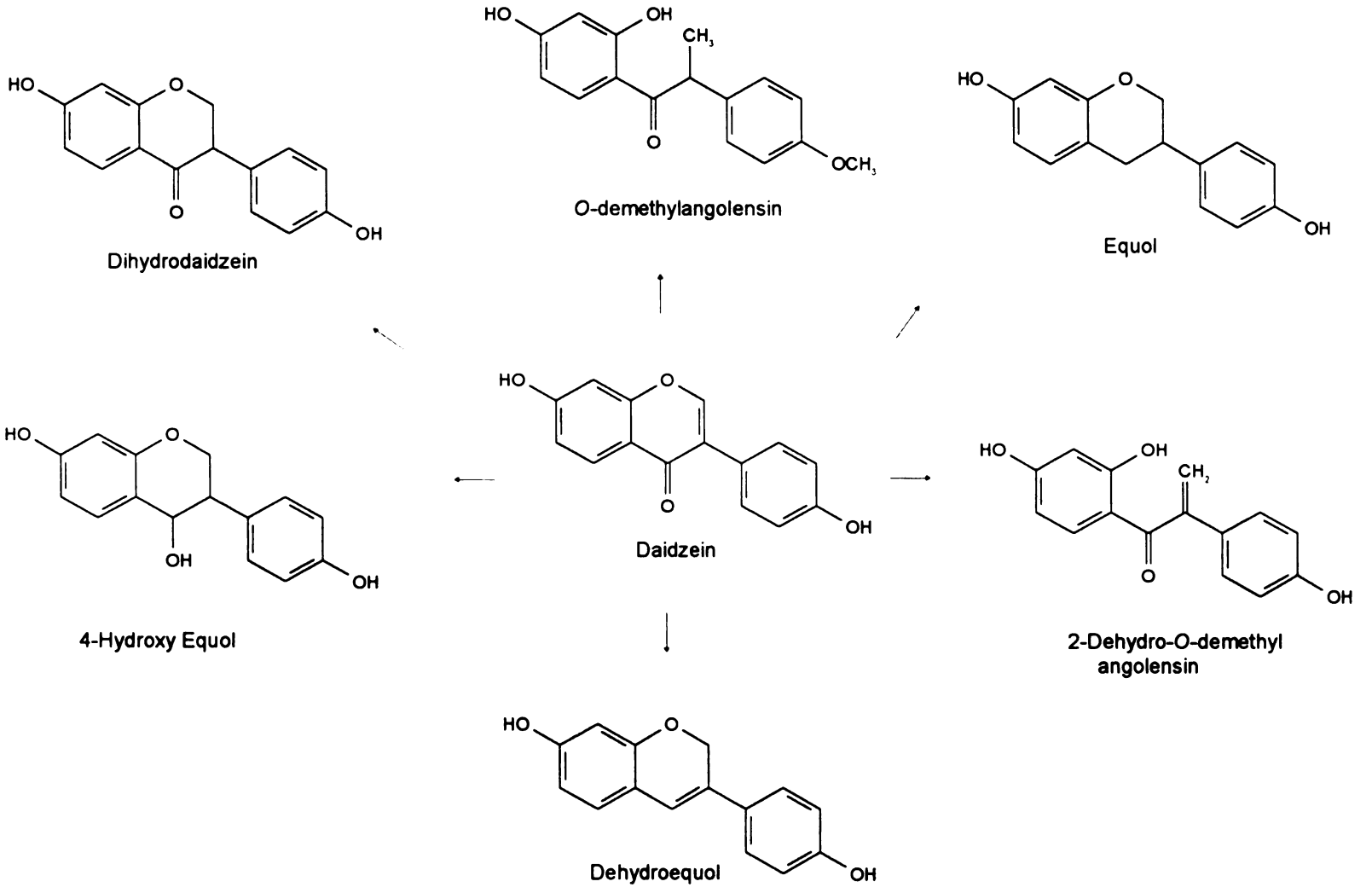


Figure 1.4 Metabolites of daidzein in human urine.

1.3.4 Biological Activities of Flavonoids and Isoflavonoids

Flavonoids and isoflavonoids are known to display a bewildering array of pharmacological and biochemical actions. Much of the recent interest in flavonoids and other polyphenolic compounds was created by the anomaly of the "French Paradox", the apparent compatibility of a high fat diet with a low incidence of coronary atherosclerosis (Renaud and De Lorgeril, 1992). It was suggested that the polyphenolic substances in red wine (flavonoids, catechins, anthocyanins and soluble tannins) may provide protection against coronary heart disease. *In vitro* (Frankel *et al.*, 1993) and *in vivo* (Fuhrman *et al.*, 1995) studies confirmed the inhibition of oxidation of low density lipoproteins by the phenolic substances in red wine.

In support of flavonoids exerting a protective effect *in vivo* were the results of a Dutch epidemiological study (the Zutphen Elderly Study) demonstrating that heart disease in elderly males was inversely correlated with their intake of flavonoids (Hertog *et al.*, 1993). The major sources of flavonols and flavones were tea (61%), onions (13%) and apples (10%). Mortality from coronary heart disease was strongly and inversely correlated with flavonoid intake in the Zutphen Elderly Study; a reduction in the mortality risk of more than 50% was found. A recent cohort study involving a 26-year follow-up of Finnish men and women found a similar inverse association between intake of flavonoids and coronary mortality (Knekt *et al.*, 1996).

In addition to their protective effect against heart disease, flavonoids are known to exhibit a broad spectrum of positive pharmacological properties, including antiinflammatory (Brasseur, 1989; Ferrándiz and Alcaraz, 1991), antiviral (Selway, 1986), antimetastatic

(Menon *et al.*, 1995), anticarcinogenic (Hirose *et al.*, 1994) and antitumor (Smith and Banks, 1986; So *et al.*, 1996) activities. While some of these effects (i.e., antiproliferative activity) may be attributed to the topoisomerase-II-dependent DNA cleavage induced by flavonoids (Yamashida *et al.*, 1990); others (such as vasoprotective and antiinflammatory) are believed to be related to their antioxidant properties (Rapta *et al.*, 1995).

Flavonoids have the ability to modify the activities of a host of enzyme systems including protein kinase C and various other kinases, tyrosine kinase, aldose reductase, myeloperoxidase, NADPH oxidase, xanthine oxidase, phospholipase A_2, phospholipase C, reverse transcriptases, ornithine decarboxylase, salidase, several ATPases, nucleotide phosphodiesterases, lipoxygenases, cyclooxygenase, cytochrome P450-dependent mixed function oxidases, epoxide hydrolase, glutathione-S-transferase, and aromatase, amongst others. Many of these enzyme systems are critically involved in immune function, carcinogenesis, cellular transformation, and tumor growth and metastasis (Kandaswami and Middleton, 1994).

Studies with mammalian cell systems demonstrate that flavonoids can modify the function of mast cells, basophils, neutrophils, eosinoiphils, macrophages/monocytes, B and T lymphocytes, platelets, nerve, smooth muscle and various cancer cells. This enables these compounds to affect diverse and numerous physiological and pathological processes, including secretion, platelet aggregation and adhesion to endothelial surfaces, mitogenesis, cell motility and malignant cell proliferation, cancer metastasis and, the function or expression of adhesion molecules in various mammalian cell types (Kandaswami and Middleton, 1993).

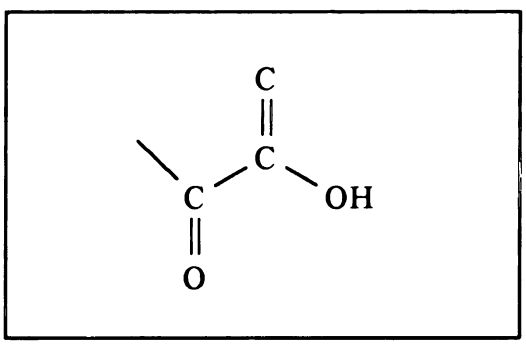
Isoflavonoids, being closely related in structure to estrogenic steroids, have also been shown to bind to estrogen receptors and exert weak estrogenic activity in various *in vivo* and *in vitro* assays (Martin *et al.*, 1978; Shutt and Cox, 1972); in addition, significant estrogenic effects in animals and in man have been observed (Setchell *et al.*, 1987; Van Thiel *et al.*, 1991). Definite antiestrogenic effects have also been reported *in vivo* since high levels of synthetic estrogens seem to be counteracted by administered isoflavonoids or their presence in the diet (Folman and Pope, 1966; Folman and Pope, 1969).

1.3.5 Antioxidant Activity of Flavonoids and Isoflavonoids

1.3.5.1 Flavonoids

Flavonoids are second only to the tocopherols as the most common and the most active antioxidant compounds naturally occurring in foods, possessing activity in both the hydrophilic and lipophilic systems (Kühnau, 1976). The antioxidant activity of these compounds is conferred by their polyphenolic structures. Polyphenols, depending on their precise structure, can act as antioxidants either by virtue of their free radical scavenging ability or their metal chelating activity (Salah *et al.*, 1995). The relative contributions of these two mechanisms are in dispute (van Acker *et al.*, 1996a). It is widely believed that the antioxidant ability of the flavonoids and isoflavonoids resides mainly in their ability to donate hydrogen atoms, thereby scavenging the free radicals generated during lipid peroxidation. Metal chelation has generally been regarded to play a minor role in the antioxidant activity of these compounds, and so, has not been studied much by researchers in the area (Morel *et*

al., 1994). This is despite the early realization by researchers that the structures of these compounds allowed them to form heavy metal complexes (Kühnau, 1976). Two points of attachment to the flavonoid were established: first, the *o*-diphenol (3',4'-dihydroxy-) grouping in ring B, and secondly, the ketol structure in ring C of the flavonols:



Lack of at least one of these groups was found to reduce or delete the chelating activity. However, the oxo group at C-4 could be reduced to a hydroxy group without loss of metal-complexing ability. The ability of the flavonoid aglycone quercetin and of its corresponding glycoside rutin to form stable, inert complexes with iron have also been demonstrated earlier (Afanas'ev *et al.*, 1989). In another study, the cytoprotective effects of the flavonoids catechin, quercetin and diosmetin were investigated on iron-loaded hepatocyte cultures, considering two parameters: the prevention of iron-increased lipid peroxidation and the inhibition of intracellular enzyme release (Morel *et al.*, 1993). The flavonoids tested were found to be capable of removing of iron from the hepatocytes; and their iron-chelating ability was correlated with their cytoprotective effect.

The redox chemistry of flavonoids is a predictor of their free radical scavenging activity. The redox properties of flavonoids enable them to act as reducing agents, hydrogen-donating antioxidants and singlet oxygen quenchers (Rice-Evans and Miller, 1996). The reduction potentials of flavonoid radicals are lower than those of the alkyl, peroxyl and superoxide radicals. This enables the flavonoid radicals to inactivate the damaging oxyl species generated during lipid peroxidation and prevent the deleterious consequences of their reactions (Jovanovic *et al.*, 1992; Wardman, 1989). While the general capability of flavonoids to scavenge superoxide radicals has been demonstrated in numerous studies (Cotelle *et al.*, 1992; Hanasaki *et al.*, 1994; Yuting *et al.*, 1990; Zhou and Zheng, 1991), the specific scavenging abilities proposed for hydroxyl (Husain *et al.* 1987), superoxide (Robak and Gryglewski, 1988) and peroxyl radicals (Torel *et al.* 1986) are still subject to debate (Bors *et al.* 1990). This is because of the non-specific techniques used to generate free radicals in these studies coupled with the nonspecific assay methods employed for their detection.

The structural requirements that are considered essential for effective radical scavenging by the flavonoids are indicated below:

(i). The *o*-dihydroxy (catechol) structure in the B ring, a radical target site for all flavonoids with a saturated 2,3-bond (Bors *et al.* 1990; Heilmann *et al.* 1995).

(ii). The presence of a hydroxyl group at position 3 on the C ring (Afanas'ev *et al.* 1989; Hu *et al.* 1995; Mora *et al.* 1990; Ratty and Das, 1988). Flavonoid aglycones with a 3-OH group such as fisetin, (+)-catechin, quercetin, myricetin, and morin are potent inhibitors of lipid peroxidation when compared with those lacking a 3-OH substitution such as the flavones

diosmetin and apigenin and the flavanones hesperetin and naringenin (Cook and Samman, 1996).

(iii). The 2,3-double bond in conjugation with a 4-oxo function, which participate in electron delocalization from the B ring (Bors *et al.* 1990).

(iv). The number of hydroxyl groups, a higher number providing maximal radical-scavenging potential and the strongest radical absorption (Bors *et al.* 1990; Chen *et al.* 1996, Cotelle *et al.* 1996; Roginsky *et al.* 1996).

(v). The patten of hydroxylation (Cholbi *et al.* 1991). Hydroxyl groups on positions C-5 and C-7 of the A-ring (De Whalley *et al.* 1990; Salvayre *et al.* 1988); C-3' and C-4' of the B-ring (Salvayre *et al.* 1988; Yuting *et al.* 1990); and position C-3 of the C ring appear to contribute to inhibition of lipid peroxidation (Cholbi *et al.* 1991). Flavonols appear to require a C-2' hydroxyl and the pyrogallol group (C-3', C-4', C-5') for antiperoxidative activity (Cook and Samman, 1996).

Despite the wealth of data on the importance of flavonoids in conferring protection against lipid peroxidation, the correlation between antioxidant activity and chemical structure is far from clear. This can be attributed, in part, to the different methods of assessment, varying substrate systems, and differential concentrations of active antioxidants used (Rice-Evans *et al.* 1996).

1.3.5.2 Isoflavonoids

In contrast to the numerous studies examining the abilities of flavonoids to inhibit lipid peroxidation, the subgroup of isoflavonoids have remained relatively unexplored in terms of

their antioxidant activities. This is despite the numerous potent biological activities associated with these compounds.

In a comparative study, the inhibition of *in vitro* microsomal lipid peroxidation induced by a Fe(II)-ADP complex and NADPH by naturally occurring isoflavones and their reduced derivatives (isoflavanones and isoflavans) was examined (Jha *et al.* 1985). While all the isoflavonoids examined had an inhibitory activity, the isoflavanones were found to be more effective than the parent isoflavones and the isoflavans were shown to be the most potent inhibitors.

The other studies examining the antioxidant activities of isoflavonoids have mainly focused on genistein. Genistein has been shown to strongly suppress tumor promoter-induced H_2O_2 formation in both *in vitro* and *in vivo* conditions (Wei *et al.* 1993). A later study by the same researchers (Wei *et al.* 1995) confirmed the antioxidant and antipromotional effects of genistein. Dietary administration of genistein enhanced activities of antioxidant enzymes, prolonged tumor latency and decreased tumor multiplicity in mice. The researchers concluded that the potent inhibition of oxidant formation and proto-oncogene expression by genistein suggested that the antioxidant and antiproliferative effects of genistein may, at least in part, be responsible for its anticarcinogenic mechanism.

In another *in vitro* study, genistein was found to be an effective scavenger of H_2O_2 but was less effective against other peroxidative systems (Record *et al.* 1995). The researchers could not find any evidence of iron chelation by genistein and concluded that *in vivo*, genistein would be unlikely to be sufficiently potent a chelator to compete with endogenous iron-binding ligands.

Another group of researchers found that dietary genistein enhanced the activities of antioxidant enzymes in various organs of mice, and this may be the mechanism of its chemopreventive actions (Cai and Wei, 1996).

None of the studies that were conducted attempted to elucidate the mechanism of action by which these compounds act as antioxidants or to establish the structural criteria necessary for a high activity.

CHAPTER 2

DEVELOPMENT AND VALIDATION OF FLUORESCENCE SPECTROSCOPIC ASSAYS TO EVALUATE ANTIOXIDANT EFFICACY. APPLICATION TO METAL CHELATORS.[1]

2.1 ABSTRACT

Two fluorescence-based assays were developed for rapid evaluation of compounds for antioxidant activity. These assays were based on the quenching of intensity of the fluorescent probe and an increase in its fluorescence anisotropy due to the free radicals generated during lipid peroxidation. A large unilamellar vesicle (LUV) system containing the fluorescence probe diphenylhexatriene-propionic acid (DPH-PA) was used to study the effects of chelators on metal-ion-induced lipid peroxidation. In this paper, the actions of the chelating agents ethylenediaminetetraacetic acid disodium salt (EDTA), nitrilotriacetic acid trisodium salt (NTA), adenosine-5'-diphosphate disodium salt (ADP) and sodium citrate on Fe(II) and Fe(III)-induced peroxidation were compared. The effects of chelators on metal-ion-induced peroxidation were found to be dependent on the type of metal used to initiate peroxidation and, in the case of citrate, also on the concentration of chelator used. EDTA

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strongly suppressed both Fe(II) and Fe(III)- induced peroxidation in this system. NTA and ADP inhibited Fe(III)-induced peroxidation but enhanced Fe(II)-induced peroxidation at all the concentrations tested. Citrate promoted both Fe(II) and Fe(III)-induced peroxidations at lower chelator to metal ratios; however, at higher ratios, it inhibited both peroxidations. The results of the two fluorescence-based assays agreed well with the quantitation of conjugated dienes and hydroperoxides by HPLC. The combination of sensitivity, speed and general utility associated with these methods suggest that they will be useful in rapid screening of extracts and purified compounds for antioxidant activity.

2.2 INTRODUCTION

The peroxidation of lipids via uncontrolled free radical chain reactions is a critically important reaction in physiological and toxicological processes in human health and disease as well as in the stability of food products during storage (Schaich, 1992). In biomembranes, it leads to a disruption in structure and a loss of protective function (Barclay, 1993).

Membrane lipid peroxidation is greatly stimulated by the presence of transition metals, partially through enhancement of initiation reactions as well as through metal ion catalysis of lipid hydroperoxide decomposition reactions (Braughler *et al.*, 1987). Chelators can alter the rate of metal-catalyzed peroxidation by steric effects, variations in redox potentials and alterations in solubility properties of the metal (Aust *et al.*, 1985). Several studies have been conducted to determine the effects of metal chelators on lipid peroxidation (Dikalov *et al.*, 1993; Fujii *et al.*, 1991; Miller *et al.*, 1992; Spear and Aust, 1994; Tampo *et al.*, 1994;

Yoshida *et al.*, 1993); however, the effects are complicated and not fully understood (Yoshida *et al.*, 1993).

The choice of model system and the methods used in the study of lipid peroxidation often introduce artifacts which complicate interpretations of the results. Results from many commonly used model systems such as methyl linoleate micelles may have little relationship to food products since they are not structurally representative of membrane phospholipids or triglycerides (Frankel, 1993). Sarcoplasmic reticulum microsomes (Dinis *et al.*, 1993; Monahan *et al.*, 1994; Tien *et al.*, 1982) and mammalian erythrocyte membranes (McKenna *et al.*, 1991; Thomas *et al.*, 1990) are useful models in membrane peroxidation studies. However, use of biological membranes as lipid substrates to evaluate antioxidant activity of compounds is complicated by the presence of numerous endogenous prooxidative and antioxidative factors, including transition metals, heme proteins, catalases, glutathione reductase, and superoxide dismutase (Girotti and Thomas, 1984; Kanner *et al.*, 1988; Kellogg and Fridovich, 1975; Svingen *et al.*, 1979). Although biological membranes may provide useful information on oxidative damage or antioxidant status in an individual, the inherent variability from preparation to preparation makes their use less attractive as systems for evaluation of antioxidant activity of compounds or plant extracts.

As an alternative model to study lipid peroxidation, artificial membranes such as liposomes offer clear advantages over biological membranes. In particular, large unilamellar vesicles (LUVs) of a defined lipid composition constitute a simple and convenient system for studying lipid peroxidation and its inhibition by antioxidants without the ambiguities

introduced by enzymes or scavengers that may be present in more complex biological systems (Vigo-Pelfrey and Nguyen, 1991).

Use of a defined lipid substrate and structure coupled with fluorescence spectroscopy offers the inherent advantages of speed, simplicity and sensitivity for probing membrane structure. This methodology can be readily adapted to peroxidation studies on the mechanism of action and efficacy of food antioxidants (Strasburg and Ludescher, 1995). The objective of this study was to develop a sensitive, quantitative and rapid fluorescence-based assay for measuring lipid peroxidation and to apply the assay to evaluate antioxidant efficacy. LUVs containing the fluorescent probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) were used to study the modulation of Fe(II), Fe(III) and Cu(II)-induced membrane peroxidation by the chelators disodium EDTA, nitrilotriacetic acid trisodium salt (NTA), adenosine-5'-diphosphate disodium salt (ADP) and sodium citrate. Results from these experiments were correlated with data obtained by measurement of conjugated dienes and the direct quantitation of hydroperoxides by HPLC-chemiluminescence to validate the fluorescence-based assays.

2.3 EXPERIMENTAL PROCEDURES

2.3.1 Materials

Synthetic 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC) of greater than 99% purity in chloroform solution was purchased from Avanti Polar Lipids (Alabaster, AL). The purity of the lipids was confirmed by thin layer chromatography using two different solvent systems (chloroform: methanol: water :: 65:25:4; chloroform: methanol: ammonium hydroxide :: 65:25:4). The lipids were stored in amber glass vials, layered with nitrogen, sealed with teflon tape and stored at -20^{0}C. The fluorescent probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) was purchased from Molecular Probes (Eugene, OR). Adenosine-5'-diphosphate disodium salt (ADP), nitrilotriacetic acid trisodium salt (NTA), 3-[N-morpholino] propanesulfonic acid (MOPS), potassium tetraborate, cytochrome c from horse heart (prepared without using TCA), 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol), Chelex 100, methylene blue and xylenol orange were from Sigma Chemicals (St. Louis, MO). 4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid (HEPES) was purchased from Boehringer Mannheim (Indianapolis, IN). Ethylenediaminetetraacetic acid disodium salt (EDTA), $FeCl_3 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$ and sodium phosphate were from Mallinckrodt (Paris, KY), citric acid monohydrate was from Fisher Scientific (Fair Lawn, NJ) and $CuCl_2 \cdot 2H_2O$ was from J.T. Baker (Phillipsburg, NJ). All the glassware used in the study was acid-washed. Contaminating transition metals were removed from the sodium citrate, sodium chloride and buffer stock solutions by maintaining the solutions in Chelex 100 (5g/100 mL, w/v). The solutions were sparged with nitrogen prior to use. The metal solutions were made up fresh

in nitrogen-sparged water immediately before use and stored on ice. The sodium citrate and ADP stock solutions were adjusted to a pH of 7.0.

2.3.2 Preparation of Large Unilamellar Vesicles

LUVs were prepared immediately before use according to the procedure outlined by MacDonald *et al.* (1991) with a few modifications. Briefly, the lipid and fluorescent probe stocks dissolved in chloroform and N,N-dimethylformamide respectively, were dried under vacuum, onto the wall of a round-bottomed flask, using a rotary evaporator. The mole ratio of fluorescent probe to lipid was maintained at 1:350. The lipid film obtained by evaporation was maintained under vacuum for at least an additional 0.5 h to remove any residual solvent, hydrated at a 10 mM lipid and 28.6 μM probe concentration in 500 μL of a solution containing NaCl (0.15 M), MOPS (pH 7.0) (0.01 M) and EDTA (0.1 mM) for 30 min at a temperature at least 10^0C higher than the transition temperature of the lipid (-16.2^{0}C), and freeze-thawed ten-times in a solid carbon-dioxide/ethanol bath. The resulting multilamellar vesicles were passed 29 times through 100 nm polycarbonate filters using a Liposofast extruder apparatus (Avestin, Ottawa, Canada). An odd number of passages was used to avoid any contamination of the sample by vesicles which may not have passed through the filters. The LUVs were characterized by freeze-fracture studies using a scanning electron microscope as described by MacDonald *et al.* (1991).

2.3.3 Fluorescence Experiments

For kinetic measurements, a 20 μL aliquot of the liposome suspension was diluted to 2 mL in buffer containing 100 mM NaCl and 50 mM Tris-HEPES (pH 7.0) to achieve final concentrations of 100 μM lipid and 0.286 μM DPH-PA. The suspension was pre-incubated at room temperature for 5 min with continuous stirring, using a magnetic stirrer to incorporate oxygen into the experimental system. Another 5 min incubation was done in the cuvette for temperature equilibration. An aliquot of the stock solution of the chelator to be tested was added to achieve the desired final concentration in the cuvette. Peroxidation was initiated by the addition of 20 μL of a 100 μM stock metal ion solution to achieve a final metal concentration of 1 μM. The control sample did not contain either added metal ions or chelator. The reaction was monitored over 21 min with a reading being taken at 0 min, 1 min and every 3 min thereafter.

Cuvette temperature was maintained at 22°C with a circulating water bath. The cuvette holder was also fitted with a magnetic stirring mechanism to maintain a well dispersed suspension of vesicles. Fluorescence experiments were conducted using a SLM Instruments, Model 4800, spectrofluorometer (Urbana, IL) interfaced to a computer with data acquisition hardware from On-Line Instrument Systems (Bogart, GA). The samples were excited with polarized light at 384 nm (slit width 2 nm), and vertical and horizontal components of the sample fluorescence, $I_{\parallel}$ and $I_{\perp}$, emitted through optical filters (KV 418, Schott, Duryea, PA), were detected in the T-format. The steady-state anisotropy, r_s, was calculated as:

$$r_s = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescent intensities of the vertically ($\parallel$) and horizontally ($\perp$) polarized emission when the sample is excited with vertically polarized light (Lakowicz, 1983). Light scattering by the vesicle suspension was determined by measuring intensity in the absence of fluorescent probe using vertically polarized excitation light with the emission polarizer set to 55^0. The ratio of this signal to that determined for membranes containing the probe gave the fraction of signal resulting from light scattering. The light scattering fraction was negligible relative to the fluorescence signal and no corrections were made to the anisotropy values.

2.3.4 Preparation of SLPC Hydroperoxides

SLPC hydroperoxides were prepared according to the procedure described by Miyazawa *et al.* (1987) and modified by Zhang *et al.* (1995). Twenty five milligrams of lipid dissolved in 25 mL of methanol containing 0.1 mM methylene blue were placed in a 250-mL beaker cooled with ice water and photoirradiated with two 150 watt-lamps for 8 h. After photooxidation, the methylene blue was removed from the solution by solid phase sequential extraction using two Supelclean LC-Si 6-mL, 1 g columns (Supelco, Bellefonte, PA). The resulting hydroperoxide-containing solutions were placed in amber glass vials (Avanti Polar Lipids), layered with nitrogen, sealed with teflon tape and maintained at -80°C until use. The concentration of hydroperoxides was determined using the xylenol orange assay developed by Jiang *et al.* (1991).

2.3.5 HPLC-Chemiluminescence Assay and Conjugated Diene Determination

LUVs (2 mM) were suspended in 100 mM NaCl and 50 mM Tris-HEPES (pH 7.0). Oxidation was initiated with or without addition of chelator to a final concentration of 400 μM, followed by addition of Fe(II) ions to a final concentration of 200 μM. The reaction mixture of a total volume of 2.5 mL was maintained at 22°C throughout the experiment. Aliquots of 500 μL were removed at 0 min, 1 min and at 30 min intervals thereafter, during the 90 min experiment. Lipid was extracted from the LUV suspension by adding 3 volumes of ice-cold chloroform:methanol (2:1, v/v). The suspension was vortexed for 1 min, centrifuged at 750 x g for 10 min and the lower chloroform layer was collected. The extraction procedure was repeated twice. The chloroform fractions were combined, dried under nitrogen, immediately resuspended in 30 μL of methanol and analyzed by HPLC-chemiluminescence using a normal phase Supelcosil LC-NH_2 (5 μm, 250 x 4.6 mm) column with a Supelguard LC-NH_2 guard column (Supelco, Bellefonte, PA). The mobile phase, consisting of a 90:10 (v/v) solution of methanol and 10 mM sodium phosphate monobasic buffer, was adjusted to a final pH of 6.5 and was used at a flow rate of 1 mL/min with continuous helium sparging. A Waters 991 photodiode array (PDA) detector (Milford, MA) set at a range of 200-240 nm was used to detect the shift from unconjugated diene absorption at 205 nm to conjugated diene absorption at 234 nm.

The eluate passing through the PDA detector was mixed with a chemiluminescence reagent at a post-column mixing tee. The luminescence reagent was prepared as described by Miyazawa *et al.* (1987). It consisted of cytochrome c (10 μg/mL) and luminol (1 μg/mL) dissolved in 50 mM nitrogen-sparged borate buffer (pH 10.0) containing 1% methanol to

improve mixing with the mobile phase. The reagent was prepared daily, placed in amber glass containers, degassed and continuously sparged with helium. The flow rate of the reagent was 1 mL/min. The chemiluminescence generated by the presence of hydroperoxides was detected by a Waters 474 fluorescence detector. The peak areas of the prepared standards chromatographed under the same conditions as the samples were used to construct the calibration curves. Separate calibration curves were generated for the quantitation of conjugated dienes and the hydroperoxides.

2.4 RESULTS

2.4.1 Characterization of the LUVs

Figures 2.1A and 2.1B consist of electron micrographs of replicas of LUVs that were slam-frozen, fractured and shadowed. The micrograph in Figure 2.1A confirms that virtually all the vesicles formed by extrusion contained single bilayers, a critical feature of our experimental system. Figure 2.1B illustrates the relatively homogenous size distribution of the vesicles. The average diameter of the LUVs was 80 nm.

2.4.2 DPH-PA Fluorescence Properties in the LUVs

Initial experiments were conducted to determine the optimal concentrations of DPH-PA and the ratio of probe to phospholipid to be used in the LUVs. DPH-PA had negligible fluorescence in the absence of phospholipids (Figure 2.2A). Fluorescence intensity increased and reached saturation at 100 μM phospholipid as increasing amounts of LUVs were added

Figure 2.1. Freeze-fracture electron micrographs of the LUVs at a 10 mM concentration in a solution containing 0.15 M NaCl, 0.01 M MOPS (pH 7.0) and 0.1 mM EDTA. The lengths of the bars represent 100 nm. These micrographs confirm the unilamellar nature of the vesicles (A) and their relatively homogenous size distribution (B).

Figure 2.1 A

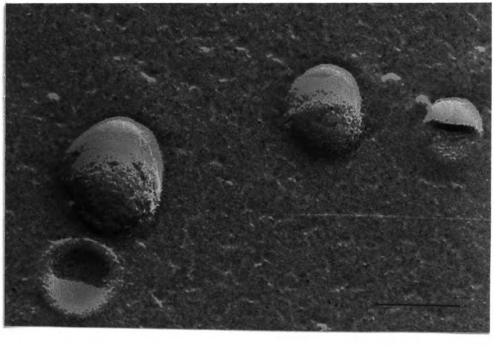


Figure 2.2 B

Figure 2.2. Fluorescence intensity (●) and anisotropy (▪) of DPH-PA in LUVs as a function of concentration. (A) 2 mL of buffer (100 mM NaCl/50 mM Tris-HEPES, pH 7.0) containing 0.286 μM DPH-PA and various concentrations of phospholipid and (B) 2 mL of buffer containing 100 μM phospholipid and various concentrations of DPH-PA.

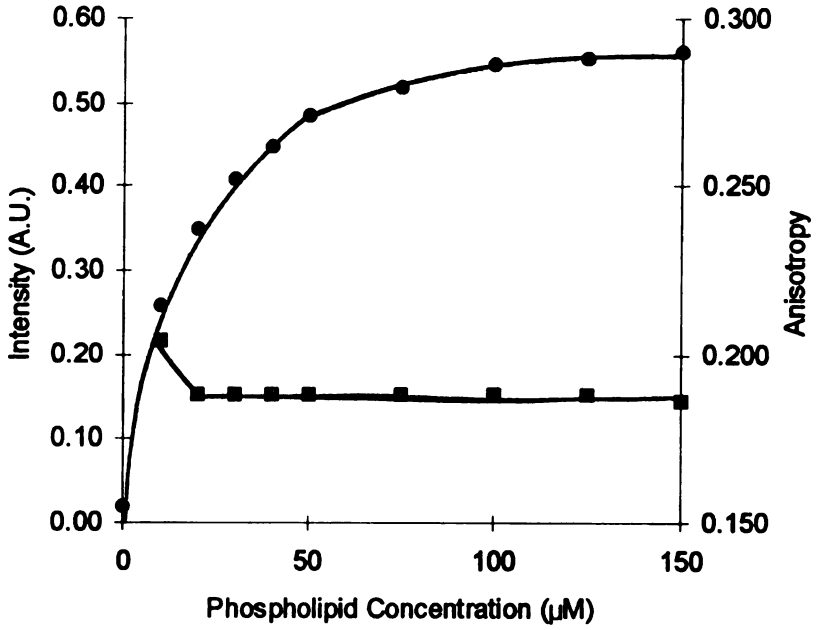


Figure 2.2A

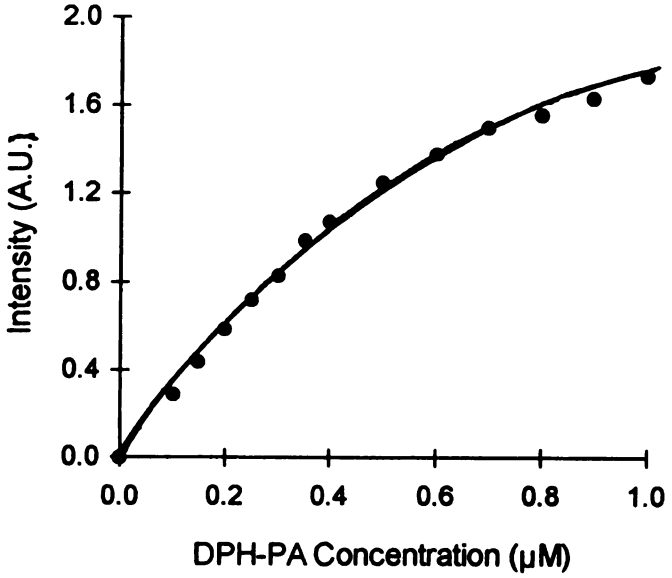


Figure 2.2B

to a fixed concentration of DPH-PA. The anisotropy values were stable over the phospholipid concentration range of 20-125 μM. When the phospholipid concentration was held constant at 100 μM (Figure 2.2B), the fluorescence intensity increased linearly with the concentration of DPH-PA at lower concentrations. Above a concentration of 0.4 μM DPH-PA, the increase in fluorescence became non-linear though saturation was not observed at the probe concentrations tested. In all subsequent experiments, concentrations of 0.286 μM DPH-PA and 100 μM phospholipid were used. Under these conditions, the fluorescence intensity of DPH-PA was a linear function of its concentration in the membrane and the lowest possible probe to lipid ratio was used, while maintaining a strong fluorescence signal.

2.4.3 Fe(II)-Induced Peroxidation Monitored by Fluorescence Intensity Decay

Figure 2.3 illustrates the effects of chelators on the rates of Fe(II)-induced peroxidation in the LUVs, studied at a 2:1 molar ratio of chelator to metal. The rate of peroxidation was monitored by quenching of the fluorescence intensity of the probe by the free radicals generated during lipid peroxidation. The control LUVs containing DPH-PA showed very stable intensity values over the twenty-one minute time period, indicative of the stability of the probe in the absence of initiators of peroxidation. When Fe(II) ions were added to initiate peroxidation in the vesicles, the fluorescence intensity decreased to approximately 30% of the original value by the end of the twenty-one minute assay. Addition of EDTA followed by Fe(II) ions resulted in only a small decrease in fluorescence intensity values of the probe, indicating that EDTA behaved as an antioxidant under these conditions. However, the other three chelators studied - ADP, NTA and citrate - all acted as

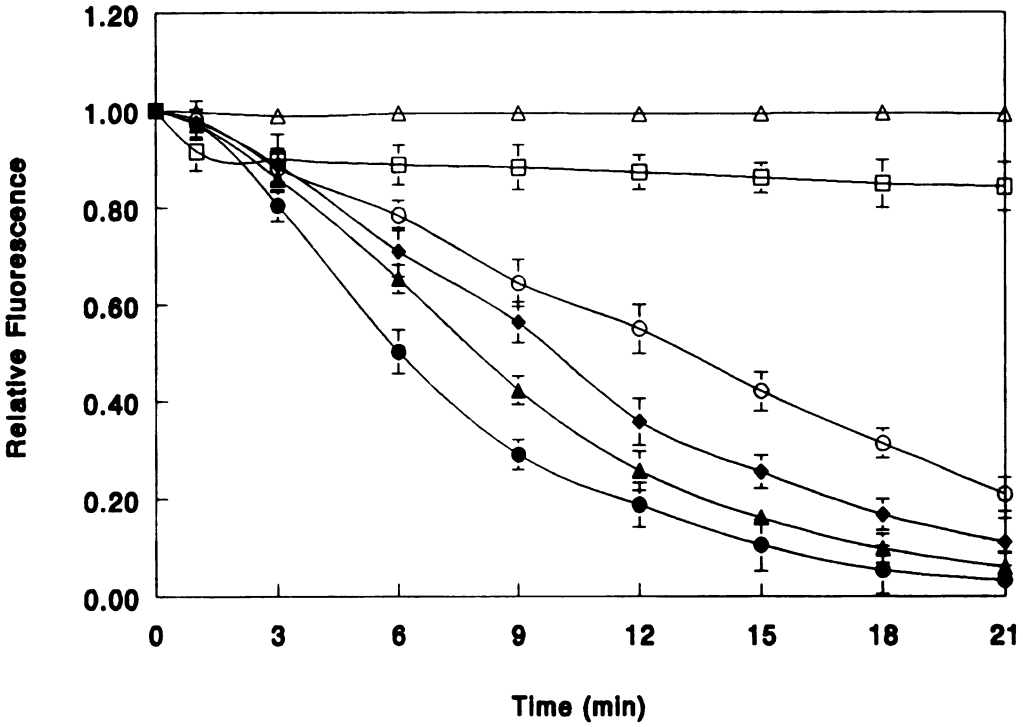


Figure 2.3. Peroxidation of 100 μM SLPC LUVs induced by 1 μM Fe(II) and 2 μM chelator. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time, as described under Experimental Procedures. Relative fluorescence on the y-axis represents the ratio of the fluorescence intensity after a given time of oxidation versus the initial fluorescence intensity at time = 0 min. Values represent the mean ± standard deviation of triplicate measurements. △, Control; ○, Fe(II); □, Fe(II)-EDTA; ◆, Fe(II)-ADP; ▲, Fe(II)-NTA; ●, Fe(II)-Citrate.

prooxidants, as evidenced by a more rapid drop in fluorescence intensity of the probe than that observed with the Fe(II) ions alone. When the chelators were added to the LUVs in the absence of metal ions, there was no change in fluorescence intensity values (data not shown).

2.4.4 Fe(II)-Induced Peroxidation Monitored by Increase in Fluorescence Anisotropy

Fluorescence anisotropy was used to monitor the change in membrane fluidity that accompanied peroxidation (Figure 2.4). Membrane peroxidation in the LUVs was accompanied by an increase in the steady-state anisotropy parameter for DPH-PA. This increase in anisotropy, indicating a reduction of DPH-PA mobility in the lipid bilayer and a decrease in membrane fluidity, may be attributed to an increase in the molecular order of the fatty acyl chains in the bilayer (Monahan *et al.*, 1994).

The fluorescent anisotropy values for the control LUVs were stable over the course of the assay, indicative of a membrane of unchanging fluidity. Similarly, there was no significant change in anisotropy in the LUVs in the presence of the EDTA-Fe(II) complex at a 2:1 molar ratio. However, the chelators ADP, NTA and citrate, when complexed to Fe(II), all caused a greater increase in anisotropy values than did the Fe(II) ions alone, indicating a substantial loss of membrane fluidity upon peroxidation. These results were consistent with the time course of the loss of fluorescence intensity upon peroxidation observed in Figure 2.3.

2.4.5 Fe(III)-Induced Peroxidation of LUVs

Using either the decrease in fluorescence intensity (Figure 2.5) or the increase in anisotropy (Figure 2.6) to monitor the progress of membrane peroxidation, Fe(III) ions were

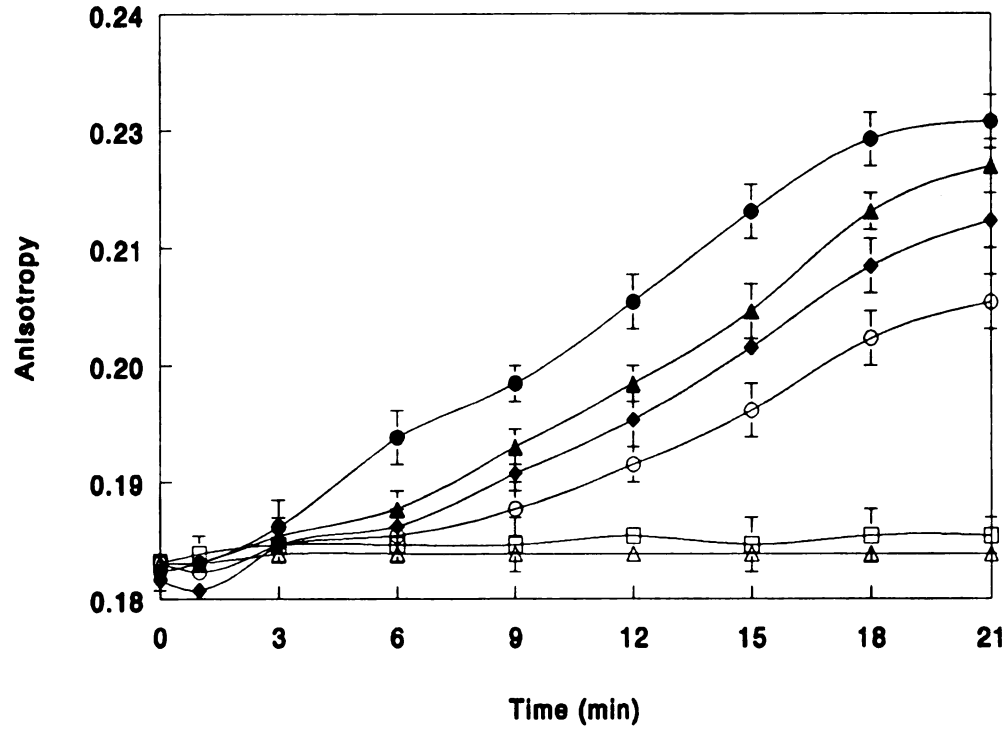


Figure 2.4. Peroxidation of 100 μM SLPC LUVs induced by 1 μM Fe(II) and 2 μM chelator. The rate of peroxidation was followed by an increase in fluorescence anisotropy as a function of time, as described under Experimental Procedures. Values represent the mean ± standard deviation of triplicate measurements. △, Control; ○, Fe(II); □, Fe(II)-EDTA; ◆, Fe(II)-ADP; ▲, Fe(II)-NTA; ●, Fe(II)-Citrate.

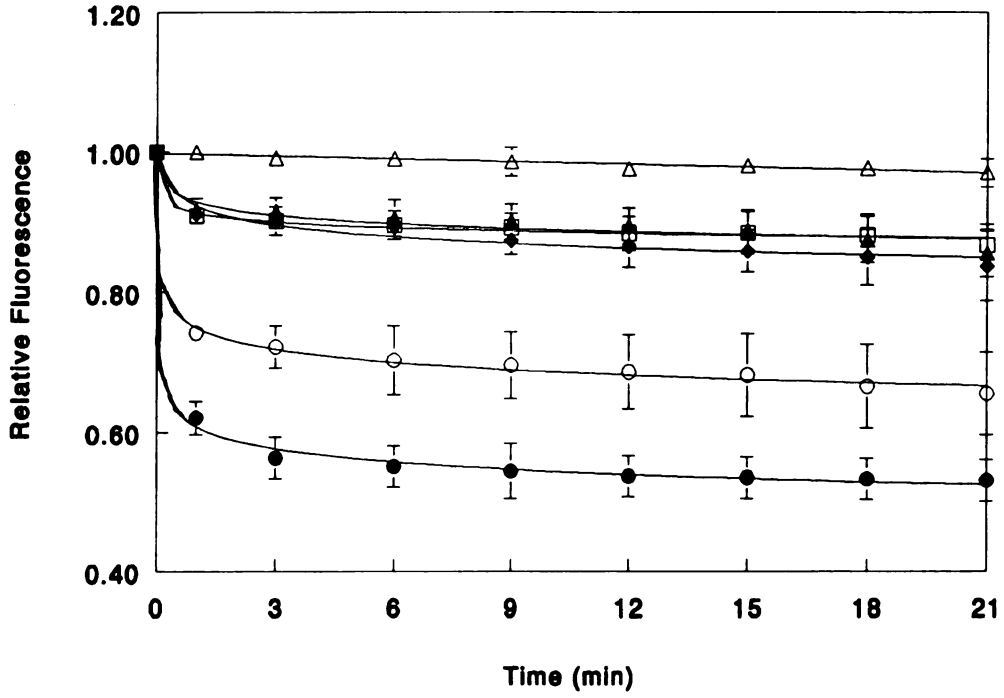


Figure 2.5. Peroxidation of 100 μM SLPC LUVs induced by 1 μM Fe(III) and 2 μM chelator. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time, as described under Experimental Procedures. Relative fluorescence on the y-axis represents the ratio of the fluorescence intensity after a given time of oxidation versus the initial intensity at time = 0 min. Values represent the mean ± standard deviation of triplicate measurements. △, Control; ○, Fe(III); □, Fe(III)-EDTA; ◆, Fe(III)-ADP; ▲, Fe(III)-NTA; ●, Fe(III)-Citrate.

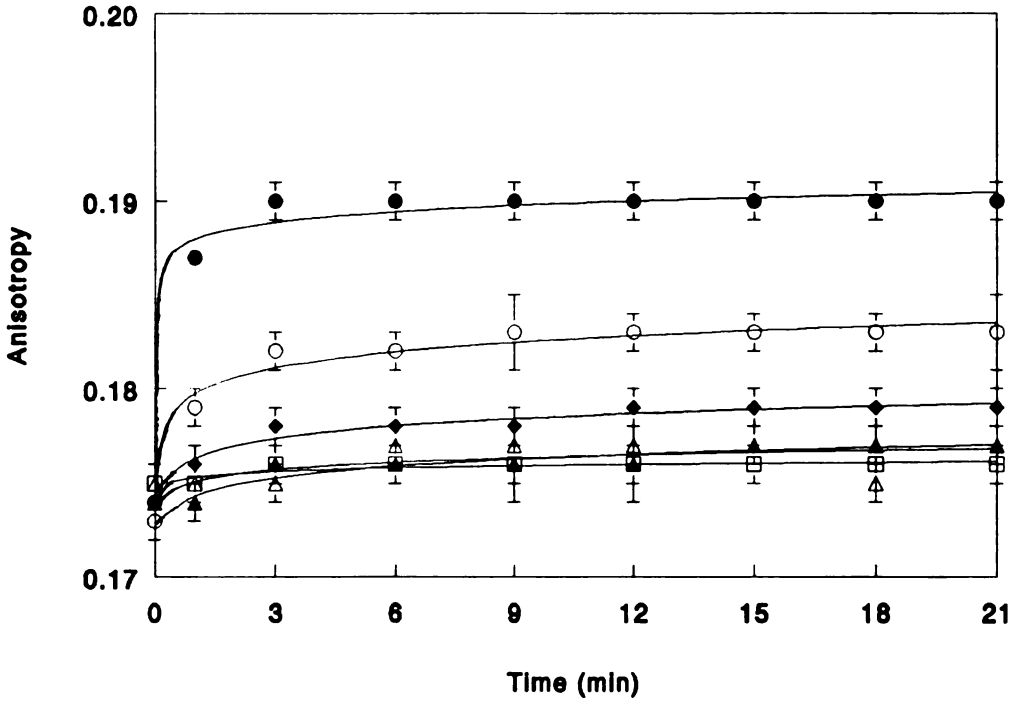


Figure 2.6. Peroxidation of 100 μM SLPC LUVs induced by 1 μM Fe(III) and 2 μM chelator. The rate of peroxidation was followed by an increase in fluorescence anisotropy as a function of time, as described under Experimental Procedures. Values represent the mean ± standard deviation of triplicate measurements. △, Control; ○, Fe(III); □, Fe(III)-EDTA; ◆, Fe(III)-ADP; ▲, Fe(III)-NTA; ●, Fe(III)-Citrate.

less effective than Fe(II) ions in inducing peroxidation in the LUVs. Furthermore, the effects of chelators on the rate of Fe(III)-induced lipid peroxidation were very different from the Fe(II)-induced peroxidation in the LUVs. Of all the chelators examined, it was found that only the citrate-Fe(III) complex acted as a prooxidant. EDTA, ADP and NTA all acted as antioxidants in the presence of Fe(III) ions, as indicated by the reduction in fluorescence-intensity decline (Figure 2.5) or by the smaller increase in anisotropy values (Figure 2.6) compared to the results obtained with Fe(III) alone.

2.4.6 Cu(II)-Induced Peroxidation of LUVs

Under the assay conditions employed in this study, neither the Cu(II) ions alone (present in the upper, non-reductive state) nor the Cu(II) ions complexed with chelators initiated peroxidation in the vesicles (data not shown).

2.4.7 Effect of Chelator Concentrations

For the chelators that exhibited a prooxidant effect in the presence of Fe (II) or Fe(III) ions at the initial molar ratios of 2:1 tested, the effects of varying the concentration of chelators relative to the metal ions was also examined. Figure 2.7 shows the effects of a 1:1, 5:1, 10:1 and 20:1 molar ratio of NTA relative to Fe(II) on rates of peroxidation. NTA maintained its prooxidant effect at all the ratios tested. Similar results were obtained when varying ratios of ADP to Fe(II) (Figure 2.8) were evaluated, with ADP enhancing the rates of LUV peroxidation at all the concentrations tested. Citrate, on the other hand, reversed its prooxidant effect at higher chelator concentrations in the presence of both Fe(II) (Figure 2.9)

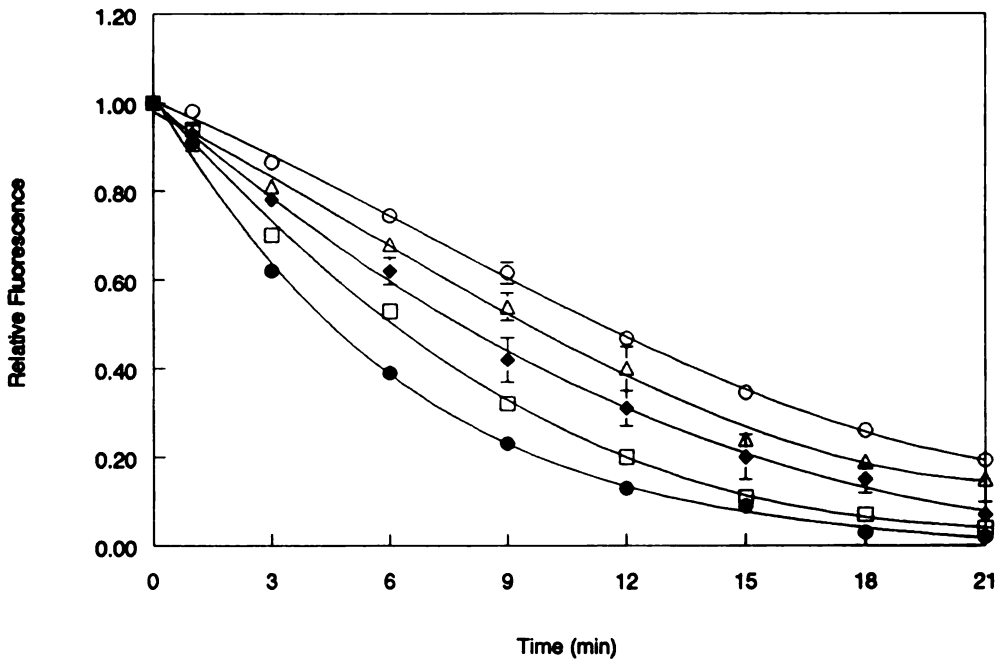


Figure 2.7. Peroxidation of the LUVs induced by varying molar ratios of NTA to Fe(II). The concentration of metal was kept constant at 1 μM (○) and different concentrations of NTA were used for final NTA to metal molar ratios of 1:1 (△), 5:1 (◆), 10:1 (□) and 20:1 (●). Values represent the mean ± standard deviation of triplicate measurements.

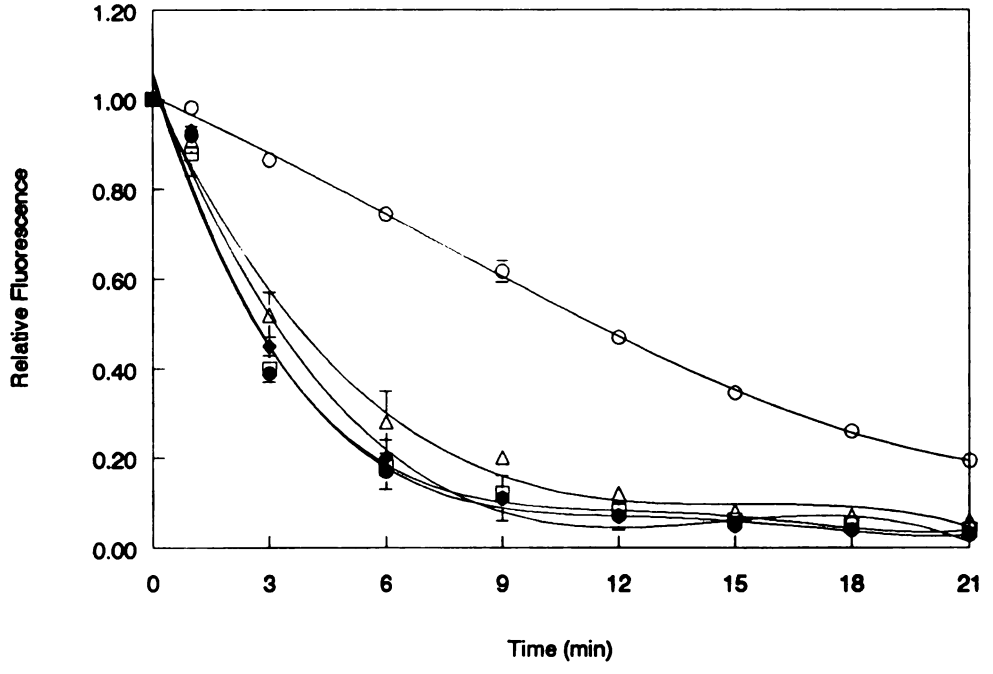


Figure 2.8. Peroxidation of the LUVs induced by varying molar ratios of ADP to Fe(II). The concentration of metal was kept constant at 1 μM (○) and different concentrations of ADP were used for final ADP to metal molar ratios of 1:1 (△), 5:1 (◆), 10:1 (□) and 20:1 (●). Values represent the mean ± standard deviation of triplicate measurements.

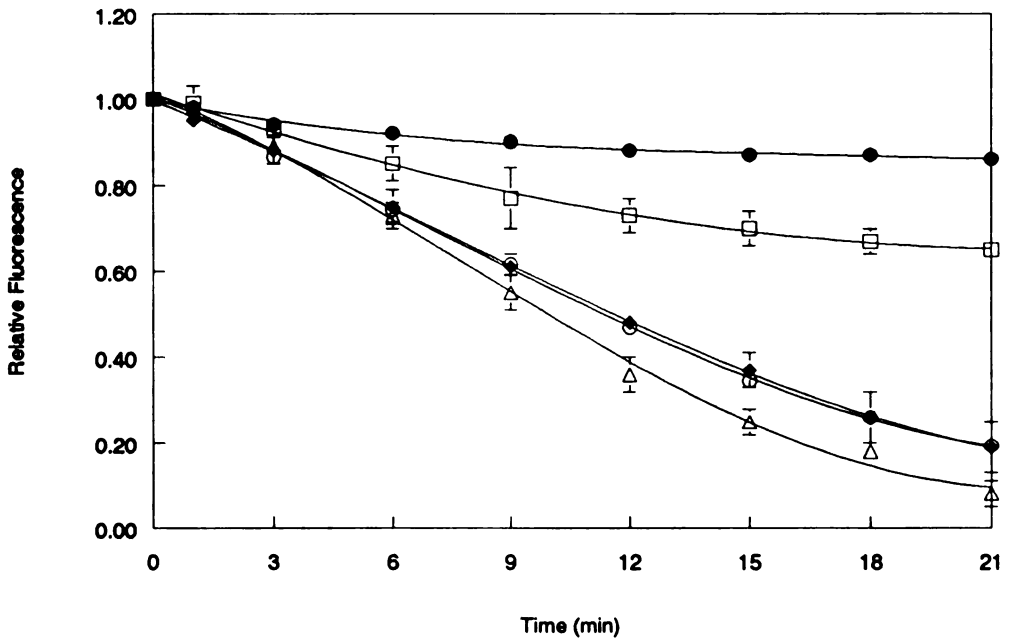


Figure 2.9. Peroxidation of the LUVs induced by varying molar ratios of citrate to Fe(II). The concentration of metal was kept constant at 1 μM (○) and different concentrations of citrate were used for final citrate to metal molar ratios of 1:1 (△), 5:1 (◆), 10:1 (□) and 20:1 (●). Values represent the mean ± standard deviation of triplicate measurements.

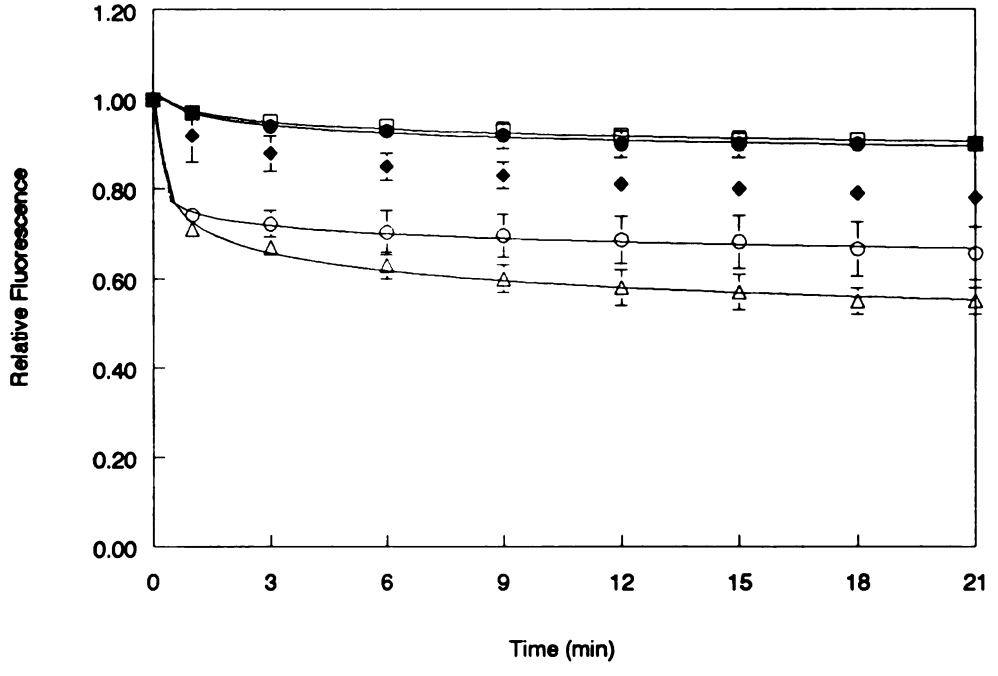


Figure 2.10. Peroxidation of the LUVs induced by varying molar ratios of citrate to Fe(III). The concentration of metal was kept constant at 1 μM (○) and different concentrations of citrate were used for final citrate to metal molar ratios of 1:1 (△), 5:1 (◆), 10:1 (□) and 20:1 (●). Values represent the mean ± standard deviation of triplicate measurements.

and Fe(III) ions (Figure 2.10). With Fe(II) ions, citrate did not exhibit any antioxidant activity up to a chelator to metal molar ratio of 5:1. At chelator to Fe(III) molar ratios of 10:1 and 20:1 though, citrate strongly suppressed peroxidation in the LUVs, as evidenced by the small drop in intensity over the assay period. In the presence of Fe(III) ions, citrate reversed its prooxidant effect at chelator to metal ratios of 5:1 and higher.

2.4.8 Validation of the Fluorescence-Based Assays

Quantitation of conjugated diene and hydroperoxide formation as a function of time was done by HPLC to establish the validity of the fluorescence intensity and anisotropy measurements in the model system as assays for lipid peroxidation. The data in Figures 2.11 and 2.12 show the amounts of conjugated dienes and phosphatidylcholine hydroperoxides (PCOOHs), respectively, that were formed during the Fe(II)-induced peroxidation of SLPC vesicles over a 90 minute time period. The PCOOHs were specifically detected by chemiluminescence as a single peak eluting at 6.5 min. By UV detection at 234 nm, an unoxidized PC peak was seen at 3.7 min and an oxidized PC peak at 6.4 min. Formation of conjugated dienes and PCOOHs were consistent with the peroxidation measurements by fluorescence. At a chelator to metal molar ratio of 2:1, citrate caused the greatest acceleration of Fe(II)-induced formation of conjugated dienes and PCOOHs in the LUVs, followed by NTA and ADP. EDTA strongly suppressed the Fe(II)-induced formation of conjugated dienes and PCOOHs.

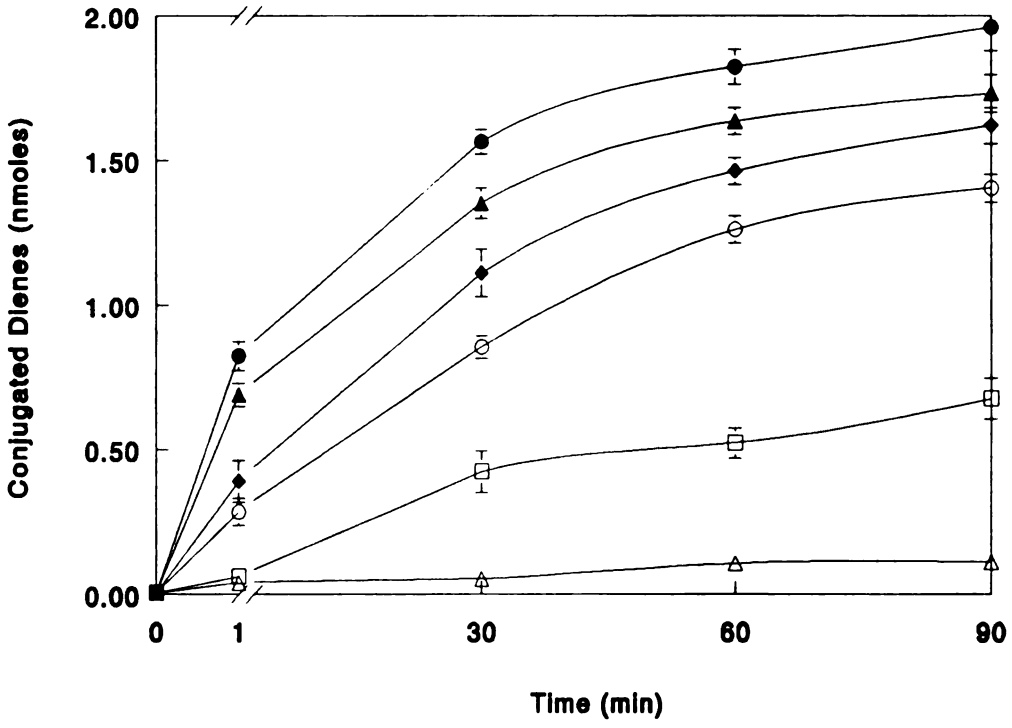


Figure 2.11. Peroxidation of 2 mM SLPC LUVs induced by 200 μM Fe(II) and 400 μM chelator. The rate of peroxidation was monitored by the measurement of conjugated diene absorption at 234 nm, as described under Experimental Procedures. Values represent the mean ± standard deviation of triplicate measurements. △, Control; ○, Fe(II); □, Fe(II)-EDTA; ◆, Fe(II)-ADP; ▲, Fe(II)-NTA; ●, Fe(II)-Citrate.

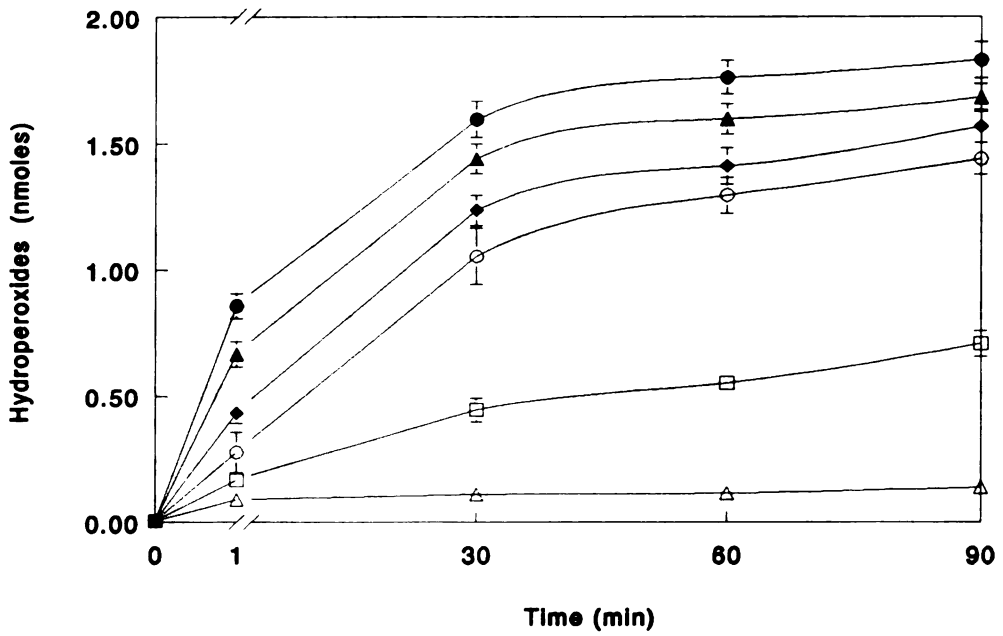


Figure 2.12. Peroxidation of 2 mM SLPC LUVs induced by 200 μM Fe(II) and 400 μM chelator. The rate of peroxidation was monitored by quantitation of hydroperoxides (PCOOHs) using HPLC-chemiluminescence, as described under Experimental Procedures. Values represent the mean ± standard deviation of triplicate measurements. △, Control; ○,

2.5 DISCUSSION

Fluorescence spectroscopy has previously been used successfully to monitor lipid peroxidation in submitochondrial particles (de Hingh *et al.*, 1995), low-density lipoproteins (Laranjinha *et al.*, 1992), microsomes (Dinis *et al.*, 1994), human erythrocyte membranes (McKenna *et al.*, 1991; Van den Berg *et al.*, 1988) and phospholipid vesicles (Kuypers *et al.*, 1987). However, the probe used in these studies, *cis*-parinaric acid, requires special handling, has a relatively low quantum yield in membranes and is subject to complicating photochemical and oxidative reactions, including dimerizations (Lentz, 1993). DPH-PA overcomes these disadvantages, and therefore, offers great potential as a probe for membrane studies.

DPH-PA (Figure 2.13) is an anionic derivative of the parent DPH molecule. In contrast to DPH which is non-specifically partitioned in various domains of the membrane (Ivessa *et al.*, 1988), the polar substituent of DPH-PA provides a surface anchor that ensures that the polar head of the probe is localized at the lipid-water interface of the membrane, with the hydrophobic tail portion lying parallel to the acyl chains of the membrane. In addition, the probe is similar to long-chain free fatty acids both in its anionic carboxylic group and in its approximate chain length, making it a useful probe for lipid peroxidation studies. The probe also lacks the extrinsic bulk of some other membrane probes like the *n*-(9-anthroyloxy) free fatty acid and pyrene derivatives, and can be expected to cause minimal disturbance to the lipid bilayer (Trotter and Storch, 1989). In contrast to *cis*-parinaric acid, DPH-PA is relatively photostable and requires little special handling, it exhibits strong fluorescence enhancement in a lipid environment and offers sensitive fluorescence anisotropy responses to lipid ordering (Mateo *et al.*, 1991).

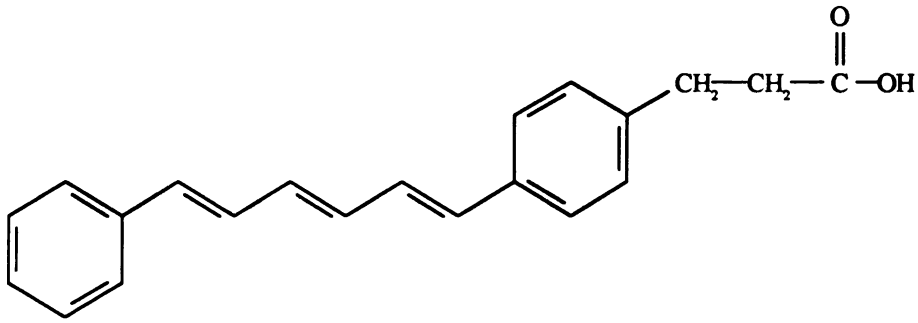


Figure 2.13. Structure of 3-(*p*-(6-phenyl)-1, 3, 5-hexatrienyl)phenylpropionic acid.

These favorable properties of the probe DPH-PA were utilized in developing fluorescence intensity and anisotropy assays for monitoring lipid peroxidation in membranes. The basis for the fluorescence intensity assay is the structure of the DPH-PA molecule, its conjugated double bond system giving rise to its fluorescent properties, as well as to its susceptibility to peroxidation. The probe reacts readily with a variety of free radicals to yield non-fluorescent products. Degradation of the probe that accompanies membrane peroxidation is indicated by a decrease in fluorescence, which can then be measured directly. In contrast, the fluorescence anisotropy assay developed here follows the changes in membrane structure that accompany peroxidation. Free radical reactions such as lipid peroxidation have been implicated in decreased membrane fluidity (Yu *et al.*, 1992; Choe *et al.*, 1995). A decrease in membrane fluidity is indicated by an increase in the steady-state fluorescence anisotropy parameter of the probe.

The two fluorescence-based assays developed in this study were used to explore the effects of chelators on metal-ion-induced peroxidation. Of the three metal ions tested, Fe(II) ions caused the greatest acceleration of peroxidation in the liposomes followed by Fe(III) ions. By contrast, Cu(II) ions were unable to induce peroxidation under the conditions used in this assay.

These differences in the abilities of the three metal ions to initiate peroxidation in the LUVs are probably a result of the differences in accessibility of these oxidizing species to the site of peroxidation in the membranes. Cu(II) ions have previously been shown to be less effective at initiating lipid peroxidation in liposomes as compared to micelles (Maiorino *et al.*, 1995). These researchers found that liposomal peroxidation by Cu(II) occurred after an initial

lag phase during which peroxidation did not take place, suggesting that a physical constraint had to be overcome before massive lipid peroxidation could occur. When the lipid organization was shifted from bilayer to micellar dispersion, this lag phase was abolished.

The different coordination geometry of Cu(II) and Cu(I) may also affect their ability to participate in redox reactions of lipid peroxidation. Cu (II) complexes have a square planar geometry whereas Cu(I) complexes are mostly tetrahedral. On the other hand, both Fe(II) and Fe(III) complexes are almost always octahedral or distorted octahedral. It is thought that the fact that the two valence states of copper usually have different coordination environments may impose a kinetic hindrance on redox reactions involving copper (Miller *et al.*, 1990).

Another factor that may explain the ability of iron and the inability of copper to initiate peroxidation in our system may be the different mechanisms through which these compounds catalyze lipid peroxidation. The presence of hydroperoxides is essential for a peroxidative response to copper, suggesting that copper catalyzes the degradation of lipid hydroperoxides and thus promotes the propagation phase of the peroxidation mechanism (Ding and Chan, 1984). Iron functions in both the initiation and propagation phases of lipid peroxidation (Minotti, 1993). Since the lipid used in this study was of a very high purity and contained no detectable amounts of hyroperoxides, it may explain why Cu(II) was unable to initiate peroxidation in this liposomal system.

The two valence states of iron gave different rates of peroxidation and also showed different effects in the presence of chelators. Fe(II) ions caused greater acceleration of lipid peroxidation than did the Fe(III) ions. A possible explanation for this difference in activity for the two metal ions is that only Fe(II) ions are active in peroxide decomposition and

although the system is seemingly pure, it is probable that trace peroxides and trace Fe(II) are responsible for much of the small peroxidation seen with Fe(III) ions.

All the chelators examined in this study had a marked effect on the rates of metal-ion-induced peroxidations. Depending on the concentration of the chelator and on the metal ion used for peroxidation, these chelators either suppressed or promoted the rates of peroxidation. EDTA was an effective antioxidant in this system, irrespective of the valence state of iron used as the initiator of peroxidation. NTA and ADP, on the other hand, behaved as antioxidants in the presence of Fe(III) ions, but were prooxidants in the presence of Fe(II) ions. They maintained their prooxidant effect even at the higher concentrations of chelators tested. In contrast, citrate displayed a prooxidant effect in the presence of Fe(II) and Fe(III) at the lower concentrations of chelators tested. However, when the concentration of chelators used was in high molar excess over that of the metal ions, the prooxidant effect of citrate was reversed and it became an effective antioxidant.

This biphasic, paradoxical behavior of citrate may be attributed to the fact that at the lower concentrations, citrate may be preferentially complexing the oxidized form of iron, leaving traces of the lower valence form in solution to participate in the Haber-Weiss reaction, reductively activating peroxides through formation of the hydroxide ion and the extremely reactive alkoxyl or hydroxyl free radicals (Porter, 1993). This would also explain the promotion of citrate to antioxidant status, at the higher concentrations, when all of the iron would be chelated. Since one would expect citrate to be in much greater preponderance to the metal in a real food or biological system, the antioxidant activity of citrate would be the predominant one observed.

Another factor that may explain the different effects of the chelators studied on the rates of metal-catalyzed lipid peroxidation may be their effect on the redox potential of the metal ions. Transition metals have a range of accessible oxidation states that enables them to transfer electrons. The redox potential for such a transfer is altered by chelation of the metals (Kanner *et al.*, 1987). The reduction potential of Fe(III)/Fe(II) (aqueous) is +110 mV at pH 7.0. The reduction potential of the chelated Fe(III)EDTA/Fe(II)EDTA increases to +120 mV, whereas those of the chelated Fe(III)citrate/Fe(II)citrate and Fe(III)ADP/Fe(II)ADP complexes decrease to +100 mV at neutral pH (Buettner, 1993).

In general, chelators in which oxygen atoms ligate the metal tend to preferentially bind to the oxidized forms of iron or copper, thereby decreasing the redox potential of these metals. In contrast, chelators in which nitrogen atoms primarily bind the metal favor the reduced forms of iron and copper and tend to increase the redox potential of the metal (Miller *et al.*, 1990). This alteration of reduction potential influences the reactions in which iron can participate, which in turn changes the yields of the different reactive oxygen species obtained during iron autooxidation.

Alteration in the accessibility of the metal ions to the site of peroxidation after complexation with the different chelators can also influence the ability of the metal to participate in lipid peroxidation. Since metal ions are present in the aqueous phase and catalysis of membrane peroxidation must occur at the phase interface or membrane surface, any chelator which increases the binding of the metal to the membrane will increase the catalytic effectiveness of the metal (Willson, 1982). Chelators like ADP are known to increase fatty acid solubilization of the metals, thereby placing the metal at the site of

peroxidation and enhancing the rate of lipid peroxidation (Schaich and Borg, 1988). Other factors like the charge on the chelator and the steric effects of the complex formed can also influence the rate of peroxidation. The net effect on lipid peroxidation often results from a complex competition between the individual effects (Schaich, 1992).

Numerous other studies have demonstrated the complexity imparted by chelators on the reactivity of metals in lipid peroxidation. Chelators have variously been shown to have a prooxidant, antioxidant or no effect on lipid peroxidation depending on the source and preparation of tissues, cells, membranes or purified lipids, the solvents used, the concentrations of metal complexes, the presence or absence of oxygen sources, and also, how these effects are measured (Schaich, 1992).

This illustrates the need of working with a clean and simple model system that is free from other complicating factors, in order to better elucidate the molecular bases of these reactions. The SLPC lipid substrate used in this model system has a composition that is representative of the phospholipids found in biological membranes, with a saturated fatty acid at the *sn*-1 position, an unsaturated fatty acid at the *sn*-2, and a phosphate-containing polar group at the *sn*-3 position (Stanley, 1991). The defined composition and high purity of this lipid also offered other obvious advantages. Since the model system was free of contaminating endogenous pro- or antioxidants associated with biologically derived materials and extreme care was taken to keep the system free from oxygen and metal contaminants until the time of initiation of oxidation, interpretations of the effects of chelators on membranal metal-induced peroxidation were subject to fewer ambiguities.

The results of the fluorescence studies characterizing the effects of chelators on Fe(II)-induced peroxidation were compared with the rates of PCOOH and conjugated diene formation, for validation of the fluorescence assays. The HPLC data showed that both the hydroperoxides and conjugated dienes increased in concert with the rates of decrease in fluorescence intensity and membrane fluidity. This was strong evidence to support our hypothesis that the fluorescence changes observed upon the initiation of peroxidation stem from the metal-ion-induced generation of free radicals.

The fluorescence assays are especially useful in monitoring the initial stages of lipid peroxidation. The advantages of the fluorescence assays include extremely high sensitivity (< 1 μmol of pure lipid is required to run an assay), very small concentration of probe required relative to lipid (ratio of probe molecules to lipid molecules is 1:350) so that the overall composition of the membrane is not significantly altered, and the low quantum yield of DPH-PA in water, assuring that the measured fluorescence is emanating from within the membrane and not from the surrounding aqueous environment. In addition, these assays offer the speed of other accelerated tests, while avoiding the artifacts introduced by the high temperature-abuse conditions of other accelerated tests.

The complexity and variability of food systems and biological materials precludes the use of any single method to define antioxidant activity. However, the experimental system developed here offers a promising alternative as a screening tool to evaluate antioxidant efficacy of purified compounds or plant extracts on membrane lipids prior to long-term studies in foods or in biological systems.

CHAPTER 3

STRUCTURE-ACTIVITY RELATIONSHIPS FOR ANTIOXIDANT ACTIVITIES OF A SERIES OF FLAVONOIDS IN A LIPOSOMAL SYSTEM

3.1 ABSTRACT

Structurally diverse plant phenolics were examined for their abilities to inhibit lipid peroxidation induced either by Fe(II) and Fe(III) metal ions or by azo-derived peroxyl radicals in a liposomal membrane system. The antioxidant abilities of the flavonoids were compared against coumarin and the widely used synthetic antioxidant *tert*-butylhydroquinone (TBHQ). The antioxidant efficacies of these compounds were evaluated using a previously developed fluorescence spectroscopic assay based on their abilities to inhibit the fluorescence intensity decay of an extrinsic probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) caused by the free radicals generated during lipid peroxidation. All the flavonoids tested exhibited higher antioxidant efficacies against metal-ion-induced peroxidations as opposed to peroxyl-radical-induced peroxidation, indicating that metal chelation plays a larger role in determining the antioxidant activities of these compounds than has previously been believed. Distinct structure-activity relationships were also revealed for the antioxidant abilities of the flavonoids. Presence of hydroxyl substituents on the flavonoid nucleus enhanced activity,

whereas substitution by methoxy groups diminished antioxidant activity. Substitution patterns on the B-ring were especially important towards antioxidant potencies of the flavonoids. In cases where the B-ring could not contribute to the antioxidant activities of flavonoids, hydroxyl substituents in an catechol structure on the A-ring were able to compensate and become a larger determinant of flavonoid antioxidant activity.

3.2 INTRODUCTION

Apart from the fat-soluble tocopherols, the most common and active antioxidant compounds naturally occurring in foods are the flavonoids, possessing activity in both the hydrophilic and lipophilic systems (Kühnau, 1976). In recent years, there has been a renewed interest in investigating the many positive pharmacological properties of flavonoids. Much of this interest in the bioactivity of flavonoids has been spurred by the dietary anomaly referred to as the "French Paradox", the apparent compatibility of a high fat diet with a low incidence of coronary atherosclerosis (Renaud and De Lorgeril, 1992). It was suggested that the polyphenolic substances such as flavonoids in red wine may provide protection against coronary heart disease. *In vitro* (Frankel *et al.*, 1993; Pace-Asciak *et al.*, 1995) and *in vivo* (Fuhrman *et al.*, 1995) studies confirmed the inhibition of oxidation of low density lipoproteins by the phenolic substances in red wine.

The mechanism of this protective action of the flavonoids is a subject of considerable debate. As polyphenolic compounds, flavonoids have the ability to act as antioxidants by a free radical scavenging mechanism (Cotelle *et al.*, 1992; Hanasaki *et al.*, 1994; Heilman *et al.*, 1995; Montinsinos *et al.*, 1995) with the formation of less reactive flavonoid phenoxyl

radicals (Equations 1 and 2); on the other hand, through their known ability to chelate transition metals (Afanas'ev *et al.*, 1989; Morel *et al.*, 1993; Laughton *et al.*, 1991; Thompson and Williams, 1976; van Acker *et al.*, 1996a), these compounds may inactivate iron ions through complexation, thereby suppressing the superoxide-driven Fenton reaction (Equations 3 and 4), which is currently believed to be the most important route to active oxygen species (Afanas'ev *et al.*, 1989).

$$ROO^{\cdot} + \text{Fl-OH} \rightarrow ROOH + \text{Fl-O}^{\cdot} \quad (1)$$

$$HO^{\cdot} + \text{Fl-OH} \rightarrow H_2O + \text{Fl-O}^{\cdot} \quad (2)$$

$$O_2^{\cdot -} + Fe(III) \rightarrow O_2 + Fe(II) \quad (3)$$

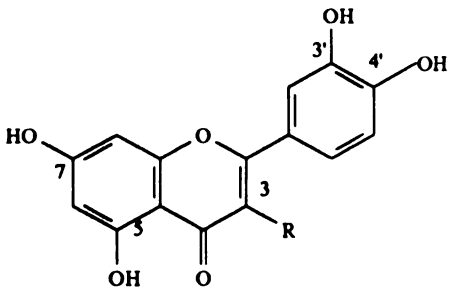
$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^{\cdot} + HO^{-} \quad (4)$$

There is much discussion regarding the relative contributions of these two mechanisms (van Acker *et al.*, 1996a). It is widely believed that the antioxidant ability of flavonoids resides mainly in their ability to donate hydrogen atoms, thereby scavenging the free radicals generated during lipid peroxidation. Despite the early realization by researchers that the structures of these compounds allow them to form heavy metal complexes (Kühnau, 1976), metal chelation has generally been regarded to play a minor role in the antioxidant activity of these compounds and so, has not been studied much by researchers in the area (Morel *et al.*, 1994).

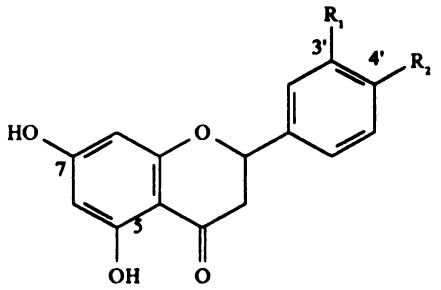
In the present study, we assessed the abilities of a range of structurally diverse flavonoids to inhibit lipid peroxidation induced by Fe(II) and Fe(III) ions. In addition, we

examined the abilities of these compounds to scavenge peroxyl radicals generated by the aqueous-phase azo compound 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH). Quercetin, the most abundant flavonol in fruits and vegetables (Hollman *et al.*, 1995), was one of the flavonoids examined in the study (Figure 3.1). Since flavonoids principally enter the diet as glycosides (Hollman *et al.*, 1996), rutin, a glycoside of quercetin, was also included in the study as it represents the naturally occurring form of quercetin. The other commonly-occurring, structurally-related flavonoids investigated were luteolin, chrysin, naringenin and hesperetin. In addition, a series of seven flavones with different hydroxyl and methoxy substitution patterns were evaluated to better elucidate the structural criteria necessary for a high antioxidant activity of these compounds (Figure 3.2). The antioxidant activities of the flavonoids were compared against two other compounds: the structurally-related coumarin which is sometimes referred to as a neoflavonoid and *tert*-butylhydroquinone (TBHQ), a widely used food antioxidant .

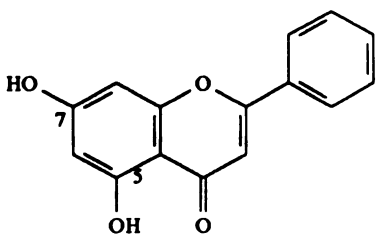
In a preceding chapter (Chapter 2), we described the development of a fluorescence-based assay utilizing the flourescent probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) for monitoring lipid peroxidation. The basis of the assay is provided by the structure of DPH-PA, its conjugated double bond system giving rise to its fluorescent properties as well as a susceptibility to peroxidation. Peroxidation of the probe molecule results in a decrease in fluorescence, which can then be measured directly and sensitively. Another aim of this study was to test the effectiveness of this new method for its ability to discriminate between antioxidant activities of structurally related compounds.



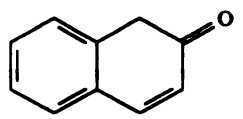
R = OH Quercetin
R = O-rutinose Rutin
R = H Luteolin



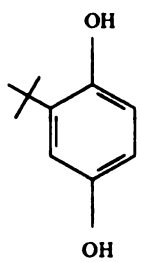
R_1 = H, R_2 = OH Naringenin
R_1 = OH, R_2 = OCH_3 Hesperetin



Chrysin

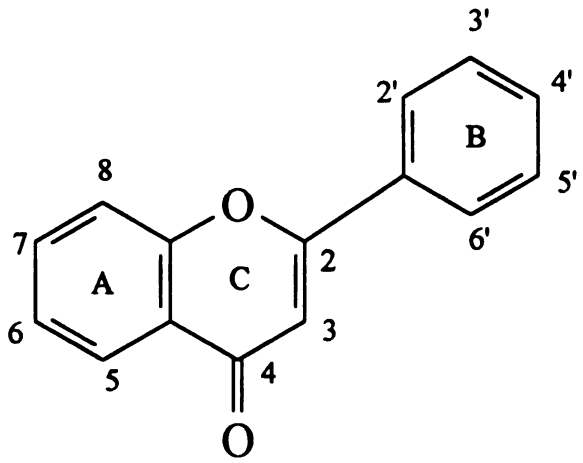


Coumarin



TBHQ

Figure 3.1. Structures of the commonly-occurring flavonoids and the other antioxidants, coumarin and TBHQ, included in this study.



Substituents				
Number	**C-5**	**C-7**	**C-8**	**C-4'**
1	H	H	H	OCH_3
2	H	OH	OH	H
3	OH	OH	H	OCH_3
4	OCH_3	H	H	OCH_3
5	H	OH	H	OCH_3
6	OCH_3	OCH_3	H	OCH_3
7	H	OCH_3	H	OCH_3

Figure 3.2. Substitution patterns for the series of flavones examined for their antioxidant activity.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 Materials

Synthetic 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC) (Avanti Polar Lipids, Alabaster, AL) was the lipid substrate used in the study. Its purity was confirmed by silica gel TLC using two different solvent systems (chloroform : methanol : water :: 65 : 25 :4; chloroform : methanol : ammonium hydroxide :: 65 : 25 : 4). The lipid stock solutions, dissolved in chloroform, were maintained at -20^{0}C in amber glass vials that were layered with nitrogen. The fluorescent probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) was obtained from Molecular Probes (Eugene, OR). Quercetin and rutin were obtained from Sigma Chemical Co. (St. Louis, MO). The other flavonoids were purchased from Indofine Chemical Company, Inc. (Somerville, NJ). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was from Wako Chemical Company (Richmond, VA). $FeCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$ of greater than 99% purity were obtained from Aldrich (Milwaukee, WI) and stored at -20^{0}C under anaerobic conditions. All other reagents were of the highest grade available.

The DPH-PA and flavonoid stock solution were prepared in N,N-dimethylformamide and dimethyl sulfoxide (DMSO) respectively. The iron and AAPH stock solutions were prepared immediately before use in nitrogen-sparged, double-distilled, deionized water and maintained on ice under anaerobic conditions. To remove metal contaminants, all the glassware was acid-washed prior to use and the salt and buffer solutions maintained in a chelating resin Chelex 100 (sodium form, mesh 50-100, Bio-Rad, Richmond, CA).

3.3.2 Preparation of Large Unilamellar Vesicles

LUVs containing the probe DPH-PA were prepared fresh for each day of experimentation using the extrusion procedure of MacDonald *et al.* (1991). Briefly, a mixture containing 5 μmol of SLPC and 15 nmol of DPH-PA was dried under vacuum onto the wall of a 15 mL round-bottomed flask. The resulting lipid film was hydrated in 500 μL of a solution containing 0.15 M NaCl, 0.01 M MOPS (pH 7.0) and 0.1 mM EDTA. After 10 freeze-thaw cycles using a dry ice/ethanol bath, the suspension was passed 29 times through two stacked polycarbonate filters (pore size 100 nm) using a Liposofast extruder apparatus (Avestin, Ottawa, Canada).

3.3.3 Antioxidant Evaluation of Flavonoids

The fluorescent intensity assay described in Arora and Strasburg (1997) was used to evaluate the antioxidant efficacy of the flavonoids. In the assay, the peroxidative degradation of the probe, indicated by a decrease in its fluorescence, is used to monitor the sensitivity of the membrane towards oxidative stress. A 20 μL aliquot of the LUV suspension was diluted to 2 mL in a buffer containing 100 mM NaCl and 50 mM Tris-HEPES (pH 7.0) to achieve final concentrations of 100 μM lipid and 300 nM probe in the cuvette. After a 5 min pre-incubation at room temperature with continuous stirring, the quartz cuvette containing the suspension was transferred to a thermostatted cuvette holder (37^{0}C) in the spectrofluorometer and incubated for an additional 10 min for temperature equilibration. An aliquot of the flavonoid stock solution, dissolved in DMSO, was added to yield a final concentration of 10

μM. The volume of added stock solution never exceeded 0.3 % of the total volume; under these conditions, DMSO had no effect on the rate of peroxidation.

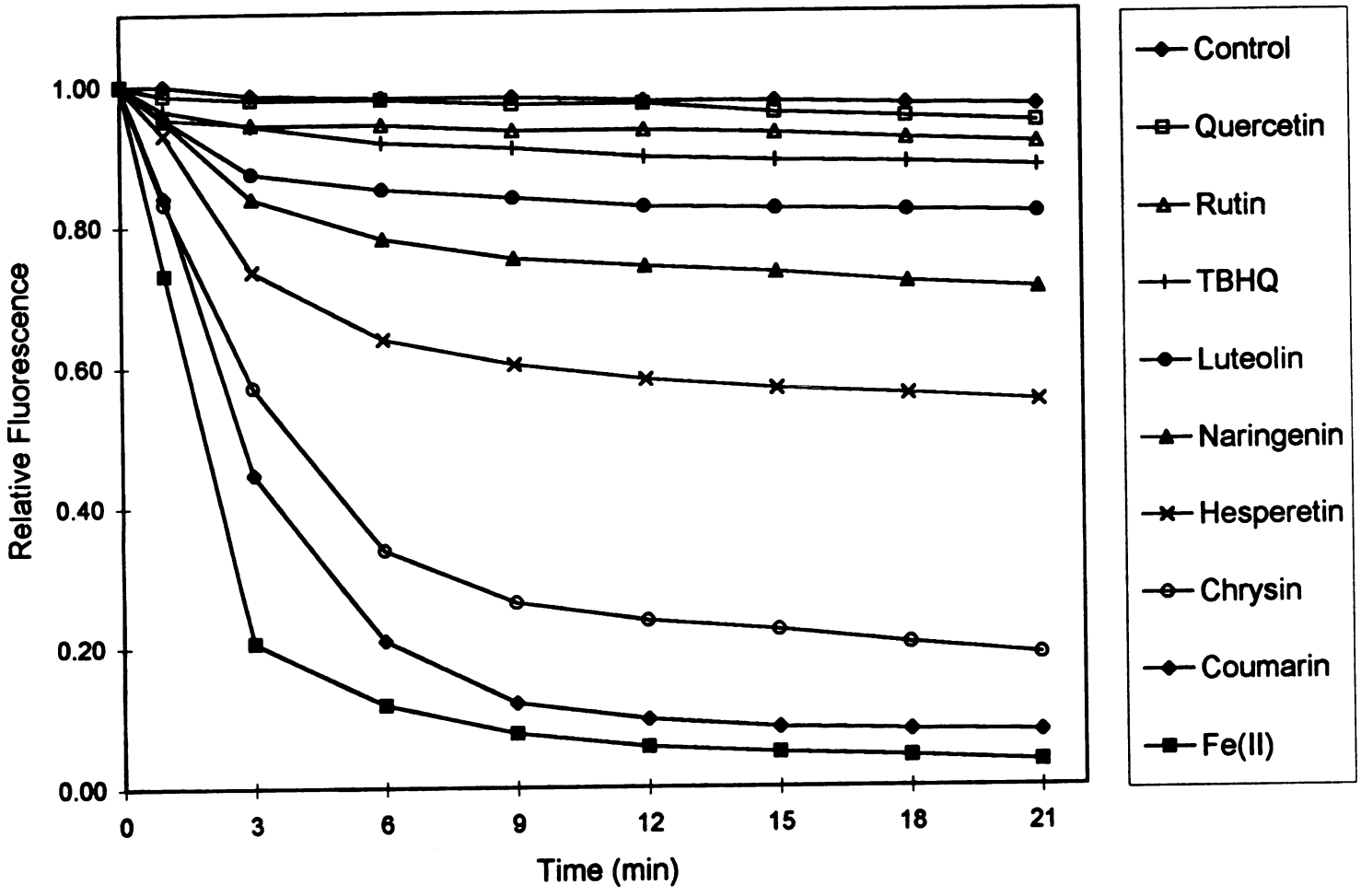
Following a 5 min incubation to allow partitioning of the flavonoid into the membrane, peroxidation was initiated by addition of 20 μL of 1 mM stock $FeCl_2$ or $FeCl_3$ solutions or 40 μL of stock AAPH solution for final concentrations of 10 μM for $FeCl_2$ and $FeCl_3$, and 10 mM for the AAPH. The control samples did not contain any peroxidation initiator or flavonoid to be tested. The decay in fluorescence intensity was monitored over 21 min, with readings being taken at 0 min, 1 min and every 3 min thereafter. The fluorescent experiments were conducted using a SLM Instruments, Model 4800, spectrofluorometer (Urbana, IL) with data acquisition hardware and software from On-Line Instrument Systems (Bogart, GA). The samples were excited with light of 384 nm, and the emitted light was passed through optical filters (KV 418, Schott, Duryea, PA), prior to detection.

3.4 RESULTS

3.4.1 Effects of Flavonoids on Rates of Fe(II)-Catalyzed Peroxidation in LUVs

Figure 3.3 illustrates the inhibitory effects of the commonly-occurring flavonoids, the neoflavonoid coumarin and the commercial food antioxidant TBHQ on rates of Fe(II)-induced lipid peroxidation. The antioxidant efficacies of these compounds were evaluated by their degrees of inhibition of the decay in fluorescence intensity of the extrinsic probe DPH-PA. The abilities of the flavonoids to inhibit rates of Fe(II)-induced peroxidation varied widely among the compounds tested, with the degree of inhibition depending primarily on the

Figure 3.3. Effects of the commonly-occurring flavonoids, coumarin and TBHQ on rates of Fe(II)-induced peroxidation in the LUVs at 37^{0}C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM test compound suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of Fe(II) for a final concentration of 10 μM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements.


Relative Fluorescence
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0.80
0.60
0.40
0.20
0.00
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3
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9
12
15
18
21
Time (min)
Control
Quercetin
Rutin
TBHQ
Luteolin
Naringenin
Hesperetin
Chrysin
Coumarin
Fe(II)

Figure 3.3

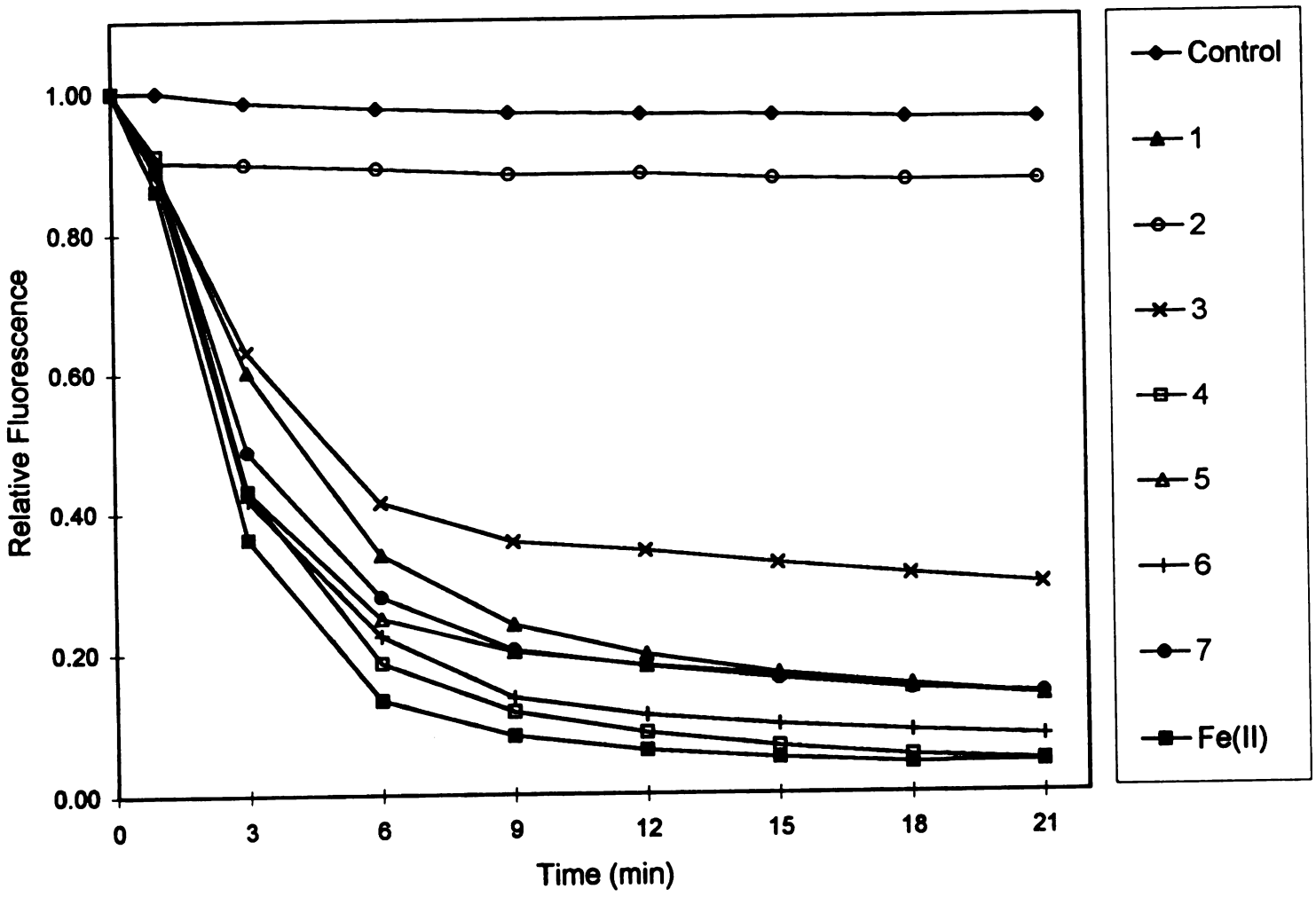
structure of these compounds. The flavon-3-ol quercetin, with five hydroxyl groups, was the superior antioxidant under these conditions. Its corresponding glycoside, the flavone rutin, was also extremely effective in this system. Both quercetin and rutin possessed antioxidant activities that were comparable to the potent food antioxidant TBHQ. Luteolin, a flavone with 4 hydroxyl groups, also demonstrated a high antioxidant activity under these conditions. Next, in decreasing order of effectiveness, were the two flavanones naringenin and hesperetin. Of the two, naringenin with three hydroxyl groups was a superior antioxidant to hesperetin which contains a methoxy group in addition to the three hydroxyl groups. The flavone chrysin, which lacked any hydroxyl substitution on the B ring, possessed minimal antioxidant activity against Fe(II)-induced peroxidation. Coumarin, which lacks a B ring completely, also exhibited negligible antioxidant activity.

A series of flavones with different substitution patterns were also tested to precisely define the chemical features required for a high antioxidant activity of these compounds. In the Fe(II)-catalyzed system, only **2** was effective as an antioxidant (Figure 3.4). **3** also inhibited the Fe(II)-induced LUV peroxidation, although to a much lesser degree as compared to **2**. The other five flavones examined in this study were quite ineffective under these conditions. All these flavones lacked hydroxyl groups on the B-ring. In addition, with the exception of **2**, they all contained at least one methoxy group.

3.4.2 Effects of Flavonoids on Rates of Fe(III)-Catalyzed Peroxidation in the LUVs

Similar trends were observed for antioxidant activity of these compounds when Fe(III) ions were used to catalyze lipid peroxidation. Again, among the commonly-occurring

Figure 3.4. Effects of the series of seven flavones on rates of Fe(II)-induced peroxidation in the LUVs at 37°C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM flavone suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of Fe(II) for a final concentration of 10 μM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements. For a description of the chemical structures of the flavones, refer to Figure 3.2.


Relative Fluorescence
1.00
0.80
0.60
0.40
0.20
0.00
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3
6
9
12
15
18
21
Time (min)
Control
1
2
3
4
5
6
7
Fe(II)

Figure 3.4

flavonoids, the flavonol aglycone quercetin was the most effective antioxidant in the liposomal system used in the study (Figure 3.5). Substitution of the 3-OH group of quercetin with the disaccharide rutinose in rutin only led to a minimal drop in activity. Luteolin was also an excellent inhibitor of Fe(III)-catalyzed LUV peroxidation. These three compounds had superior antioxidant activities in comparison to TBHQ. Similarly, as was observed with the Fe(II) ions, naringenin appeared to be slightly more effective as an antioxidant than hesperetin, whereas chrysin and coumarin were very ineffective in this system.

The series of seven flavones tested were less effective at inhibiting Fe(III)-catalyzed LUV peroxidation than the flavonoids shown above (Figure 3.6). **2** was the only flavone in this series that possessed an appreciable amount of antioxidant activity. **3** possessed a minimal amount of antioxidant activity. The other five flavones studied did not inhibit the Fe(III)-induced lipid peroxidation to any appreciable extent.

3.4.3 Effects of Flavonoids on Rates of AAPH-Induced Peroxidation in the LUVs

Next, as opposed to metal-catalyzed peroxidation, the aqueous-phase, free-radical-generator AAPH was used to initiate peroxidation and the inhibitory effects of the flavonoids was studied. All the flavonoids were less effective at inhibiting AAPH-induced peroxidation than the metal-ion-induced peroxidations (Figure 3.7). TBHQ was superior to all the flavonoids studied at scavenging AAPH-induced peroxyl radicals. Among the set of commonly-occurring flavonoids, quercetin was more effective than the other compounds tested. Rutin, luteolin, naringenin and hesperetin possessed only marginally higher antioxidant activities than chrysin and coumarin.

Figure 3.5. Effects of the commonly-occurring flavonoids, coumarin and TBHQ on rates of Fe(III)-induced peroxidation in the LUVs at 37°C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM test compound suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of Fe(II) for a final concentration of 10 μM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements.

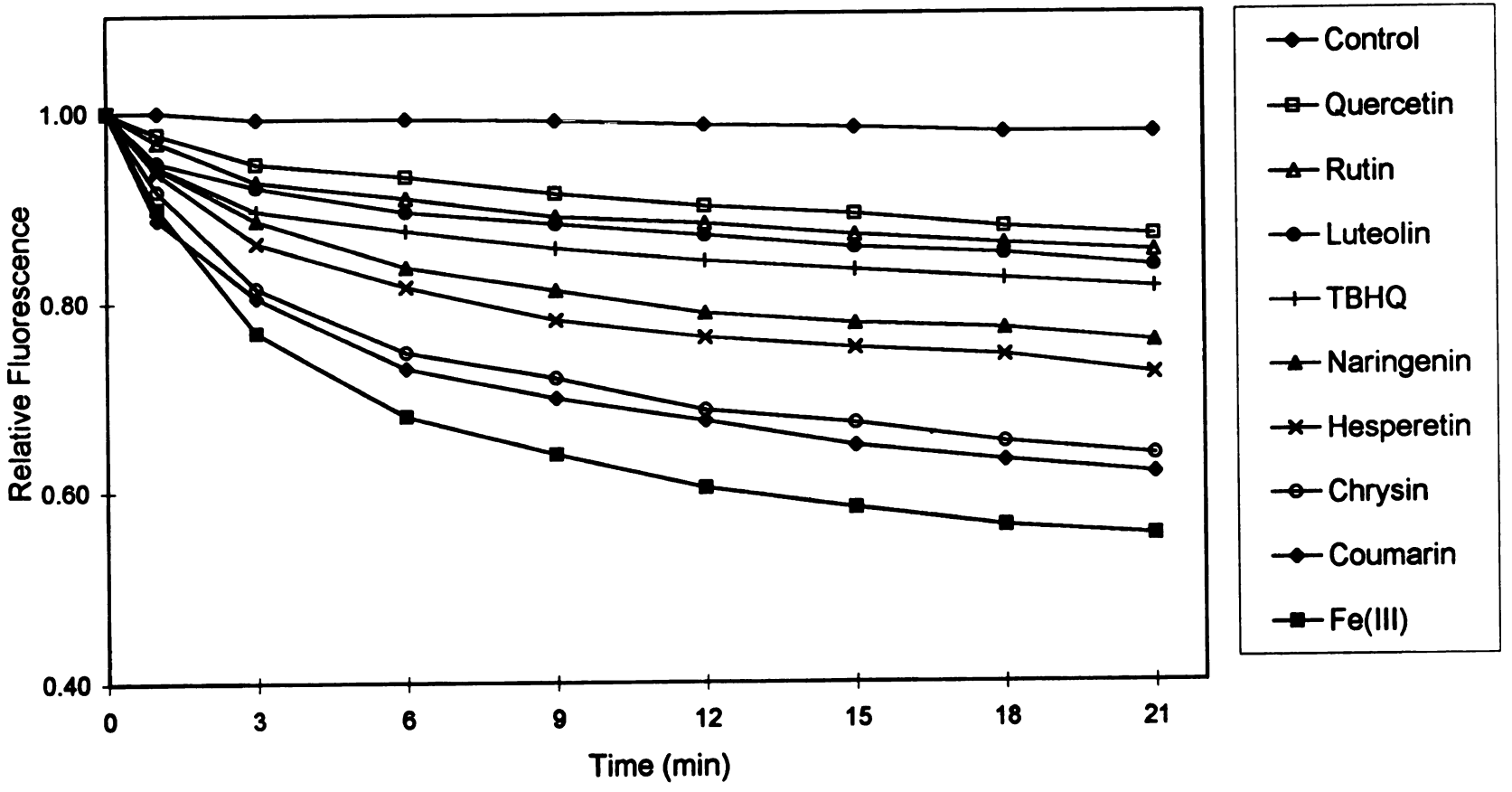
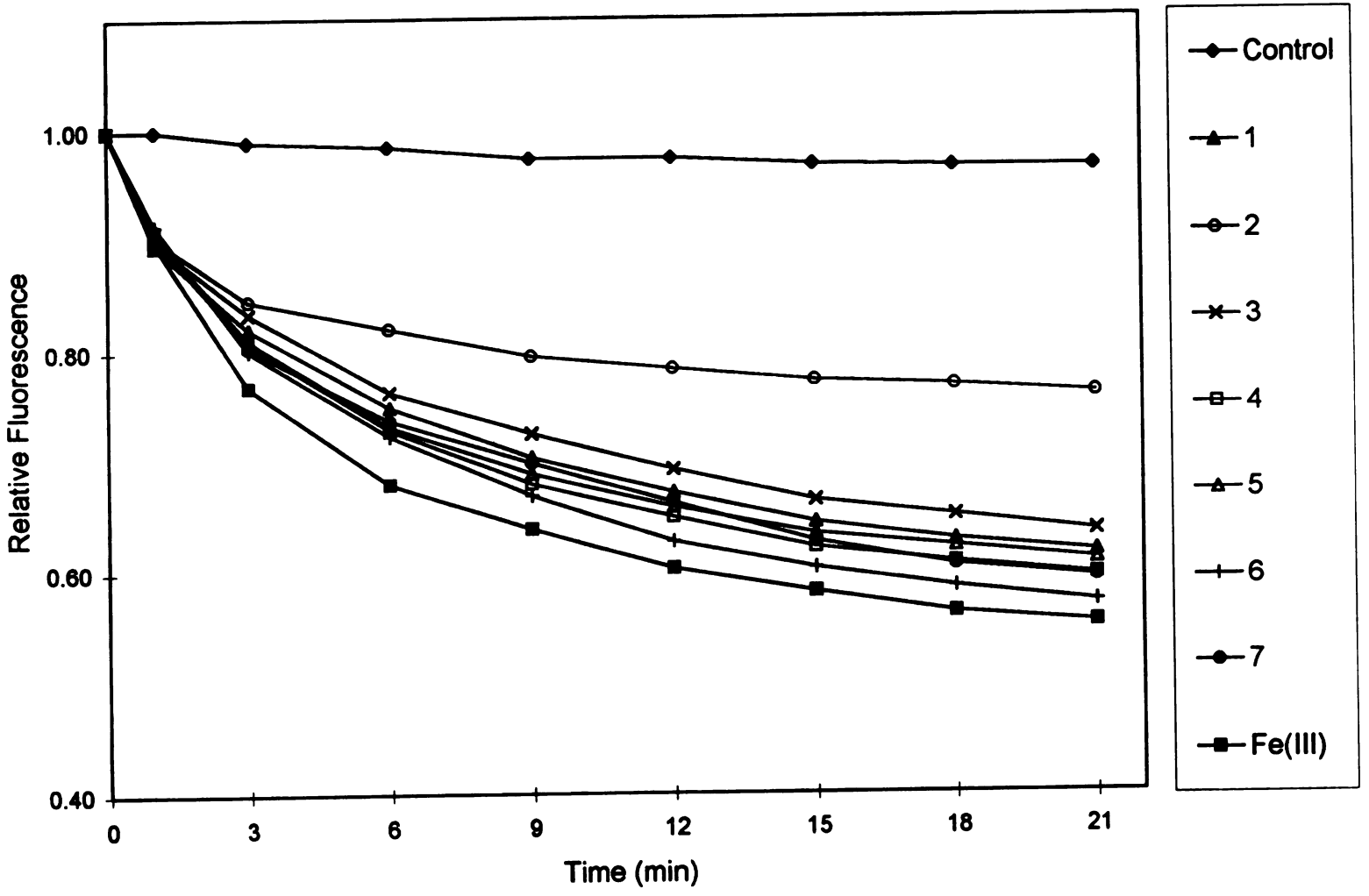

Relative Fluorescence
1.00
0.80
0.60
0.40
0
3
6
9
12
15
18
21
Time (min)
Control
Quercetin
Rutin
Luteolin
TBHQ
Naringenin
Hesperetin
Chrysin
Coumarin
Fe(III)

Figure 3.5

Figure 3.6. Effects of the series of seven flavones on rates of Fe(III)-induced peroxidation in the LUVs at 37°C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM flavone suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of Fe(II) for a final concentration of 10 μM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements. For a description of the chemical structures of the flavones, refer to Figure 3.2.


Relative Fluorescence
1.00
0.80
0.60
0.40
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3
6
9
12
15
18
21
Time (min)
Control
1
2
3
4
5
6
7
Fe(III)

Figure 3.6

Figure 3.7. Effects of the commonly-occurring flavonoids, coumarin and TBHQ on rates of AAPH-induced peroxidation in the LUVs at 37^{0}C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM test compound suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of AAPH for a final concentration of 10 mM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements.

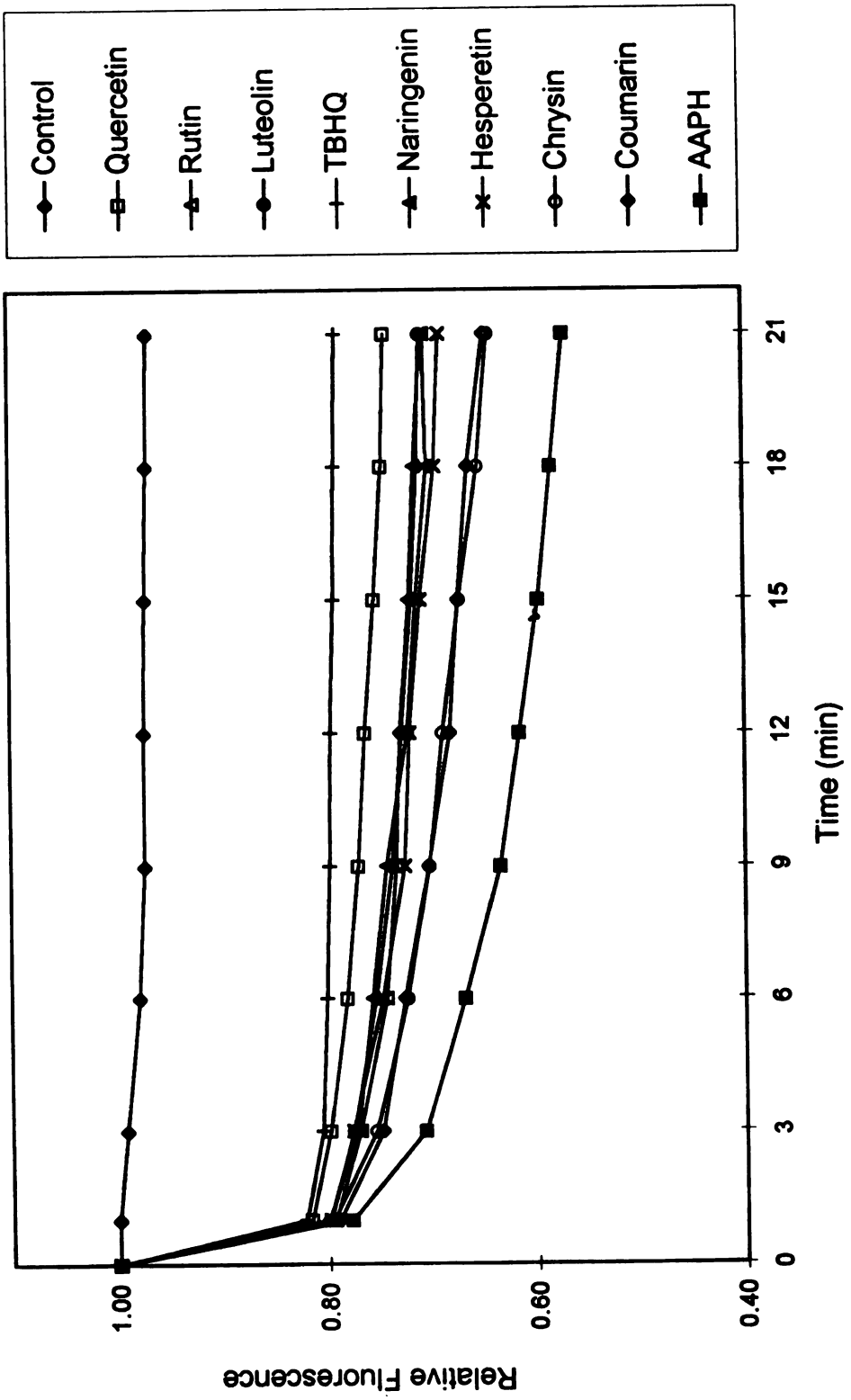

Control
Quercetin
Rutin
Luteolin
TBHQ
Naringenin
Hesperetin
Chrysin
Coumarin
AAPH
Relative Fluorescence
1.00
0.80
0.60
0.40
0
3
6
9
12
15
18
21
Time (min)

Figure 3.7

The series of seven flavones studied also demonstrated lower effectiveness against AAPH-induced peroxidation (Figure 3.8). In this series, **2, 3** and **5** were moderately effective at scavenging AAPH-induced peroxyl radicals. **1, 4, 6** and **7** possessed minimal antioxidant activities against AAPH-induced peroxidation.

3.5 DISCUSSION

In the present study, the protective effects of a range of structurally diverse flavonoids against aqueous-phase generators of LUV peroxidation were assessed. The aim was to compare the effectiveness of these compounds at inhibiting metal-ion-induced peroxidations versus peroxidations induced by the water-soluble free radical generator AAPH. We also sought to ascertain the relationship between chemical structure of the flavonoids and their antioxidant activity. Another goal was to test the abilities of the previously developed fluorescence assay to distinguish between antioxidant activities of structurally similar compounds. The flavonoids tested in this study demonstrated vastly different inhibitory effects on rates of lipid peroxidation in liposomes. In general, all the compounds examined were more effective at inhibiting metal-ion-induced peroxidation than AAPH-induced peroxidation.

Quercetin, a flavon-3-ol, was the most effective antioxidant in this system, indicating the importance of the hydroxyl group at the C-3 position for a high antioxidant activity. Replacement of the hydroxyl group at the C-3 position of quercetin by the disaccharide rutinose in rutin led to some drop in activity; however, the drop in activity was very small. Interestingly, rutin was still more effective than the flavone luteolin which has the same

Figure 3.8. Effects of the series of seven flavones on rates of AAPH-induced peroxidation in the LUVs at 37°C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM flavone suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of AAPH for a final concentration of 10 mM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements. For a description of the chemical structures of the flavones, refer to Figure 3.2.

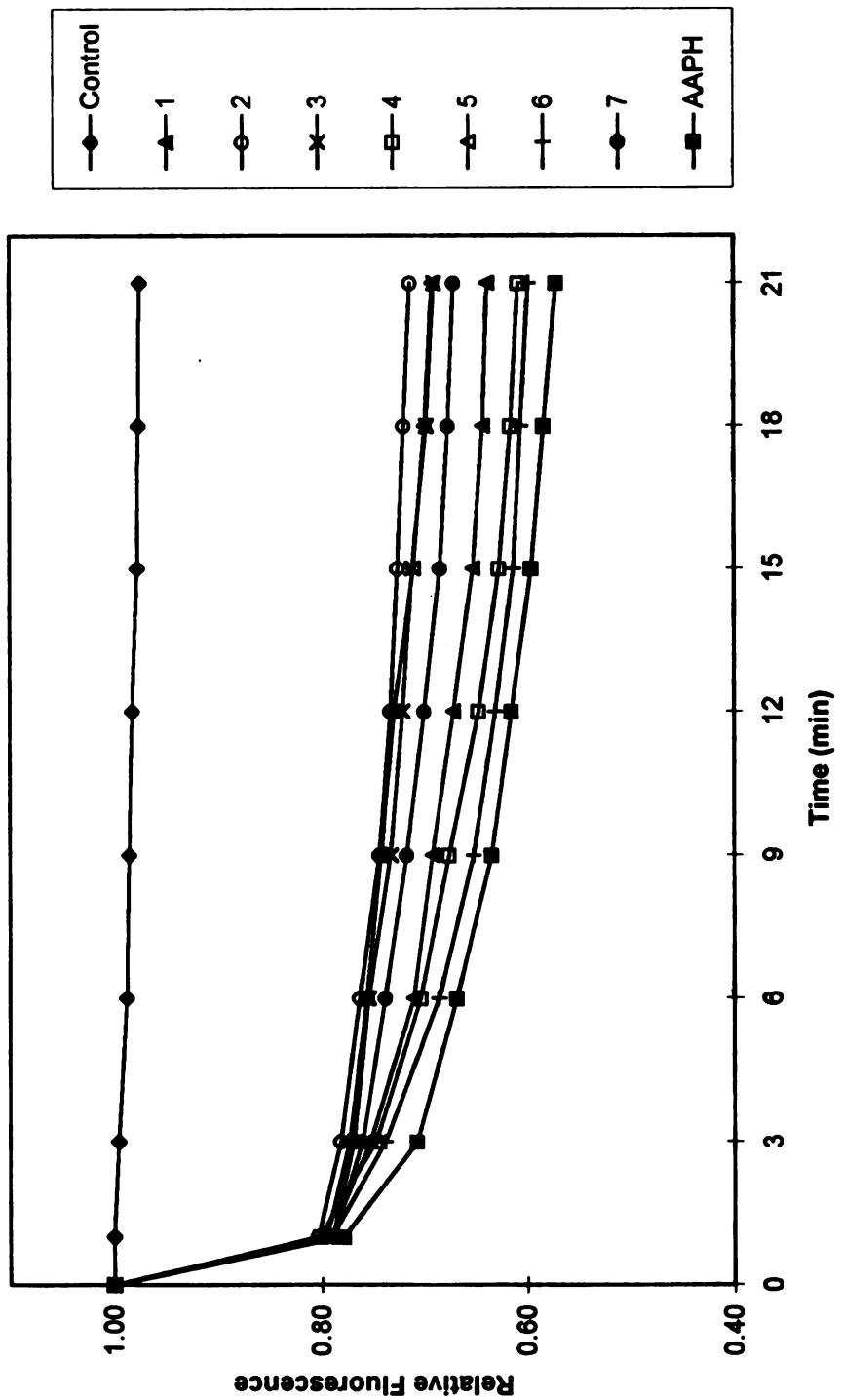

Control
1
2
3
4
5
6
7
AAPH
Relative Fluorescence
1.00
0.80
0.60
0.40
0
3
6
9
12
15
18
21
Time (min)

Figure 3.8

substitution pattern as quercetin and rutin, with the only difference being the presence of a hydrogen at the C-3 position.

These small differences in antioxidant activity based on differences in substitution at the C-3 position are probably a result of the planarity of the molecule. van Acker *et al.* (1996b) determined that the torsion angle of ring B with the rest of the flavonoid molecule was correlated with scavenging activity of the flavonoids. In quercetin, the 3-OH moiety anchored the position of the ring B in the same plane as rings A and C via hydrogen-bonds, resulting in a torsion angle of ring B with the rest of the molecule of close to 0^0 and thereby yielding a completely planar molecule. This extra hydrogen bond formed as a result of the presence of the 3-OH group had a large stabilizing effect on quercetin. The researchers further demonstrated that removal of the 3-OH moiety from the flavonoids induced a torsion angle of approximately 20^0 of ring B with the rest of the molecule and decreased the conjugation. In rutin, the sugar moiety caused some loss of coplanarity of ring B with the rest of the flavonoid. Similarly, in luteolin, replacement of the 3-OH with a hydrogen atom and the subsequent loss of a hydrogen bond caused a slight twist of the ring.

Hendrickson *et al.* (1994) investigated the electrochemical properties of quercetin, rutin and luteolin and determined that their initial oxidation at a glassy carbon electrode was a $2e^-$-$2H^+$ process yielding an *o*-quinone species. The *o*-quinone further underwent homogenous rearrangement by at least two processes: one zero-order and the other pseudo first-order. The zero-order process was likely an intra-molecular rearrangement involving the substituent at C-3. The catechol group on these compounds was oxidized to quinone which further underwent intra-molecular rearrangement followed by keto-enol tautomerization.

This zero-order process was strongly dependent on the leaving group at C-3; with the poorer the leaving group at this position, the slower the rearrangement kinetics of the electrochemically generated intermediate. Quercetin with a proton attached to the oxygen at C-3 exhibited the fastest zero-order kinetics. Rutin with a disaccharide attached to this oxygen exhibited slower zero-order kinetics. Luteolin, being unsubstituted at C-3, could not react by this mechanism and exhibited the highest degree of reversibility.

As a group, quercetin, rutin and luteolin exhibited higher antioxidant activities than the rest of the flavonoids studied. This provided evidence for the highly significant contribution of the 3',4'-dihydroxy (catechol) substitution pattern on the B-ring towards the antioxidant activity of these compounds. The presence of a *o*-dihydroxy (catechol) structure in the B-ring confers a higher degree of stability to the flavonoid phenoxyl radicals by participating in electron delocalization and is, therefore, an important determinant for antioxidative potential (Bors *et al.*, 1990).

The flavanones naringenin and hesperetin had moderate antioxidant activities and were not as effective as the flavones quercetin, rutin and luteolin. Presence of the 2,3-double bond in conjugation with a 4-oxo group has previously been shown to be important for antioxidant activities of these compounds (Bors *et al.*, 1990; Rice-Evans *et al.*, 1995; Rice-Evans and Miller, 1996) and saturation of the 2,3-double bond is believed to cause a loss of antioxidative potential. However, our study could not provide conclusive evidence for the requirement of a 2,3-double bond. The lower antioxidant activities observed for the flavanones naringenin and hesperetin as compared to the flavones quercetin, rutin and luteolin appeared to be related more to the substitution pattern on the B-ring rather than to the presence of a 2,3-double

bond. Naringenin, with a hydroxyl group at C-4', had a higher antioxidant activity as compared to hesperetin, which carries a hydroxyl group at the C-3' position and a methoxy group at the C-4' position. This reiterated the importance of substitution patterns on the B-ring where electron-withdrawing substituents like hydroxyl groups enhanced antioxidant activity for the flavonoids whereas electron-donating groups like methoxy groups decreased antioxidant activity. Presence of electron-donating or withdrawing groups at the aromatic system has previously been shown to strongly influence the redox potential of phenols (Steenken and Neta, 1982). This altered reduction potential of the flavonoids has an effect on the lipid peroxidation reactions in which they can participate which in turn, alters their ability to scavenge deleterious oxy radicals.

The flavone chrysin, which lacks hydroxyl substitution at the B-ring and coumarin, which lacks a B-ring, demonstrated negligible activity in this system. This is further proof that the antioxidant activity of these compounds is governed by the position and number of hydroxyl groups on the B-ring. Hydroxyl groups on the A-ring did not appear to be significant contributors to antioxidant activity in these compounds. These results are in line with other studies that suggested that hydroxyl groups associated with the A-ring or the pyran ring of flavonoids had poor reactivity towards peroxyl radicals and were not significant contributors towards their antioxidant activity (Roginsky *et al.*, 1996). van Acker *et al.* (1996b) examined spin distributions on flavonoids and determined that, upon oxidation of the flavonoid molecule, an event occurring mainly in the B-ring, almost all the spin remained in the B-ring, even in the case of a completely conjugated molecule. This agreed with the

hypothesis that ring B mainly determined the antioxidative potential of these compounds and that the rest of the flavonoid molecule only had a small influence.

The experiments conducted with the series of seven flavones extended these findings. Interestingly, regardless of the initiator of peroxidation employed, **2** was the most effective of this set of compounds. Though **2** lacked any hydroxyl substitution on the B-ring, it carried two hydroxyl groups on the A-ring. Unlike chrysin, where the two hydroxyl groups on the A-ring did not form a catechol structure and the compound did not display any significant antioxidant activity, the hydroxyl groups on **2** were at the C-7 and C-8 positions, thereby forming a catechol structure. This surprisingly high activity for **2** was quite unexpected and suggested that, in cases where the B-ring could not contribute to the antioxidant activity of the flavonoids, certain substituents on the A-ring may be able to compensate and become a larger determinant of the antioxidant efficacy of these compounds.

Of the other flavones, **3** showed some antioxidant activity, whereas, none of the other compounds studied inhibited the liposomal peroxidation to any considerable extent. All these flavones shared one common feature - absence of any hydroxyl groups on the B-ring and presence of at least one methoxy group on the B-ring. This, too, confirmed our earlier observations that electron-withdrawing groups at the B-ring enhanced activity of these compounds while electron-donating groups suppressed it. This is probably a consequence of the altered spin-distribution on the molecule. Extensive spin delocalization would enhance activity through an increased stabilization of the flavonoid phenoxyl radical (Mayouf and Lemmetyinen, 1993).

Another striking observation of the study was that all the flavonoids investigated were more effective at inhibiting the metal-ion-induced peroxidation than the peroxidation catalyzed by AAPH-induced peroxyl radicals. This is despite the fact that flavonoids possess lower reduction potentials than peroxyl radicals and can therefore inactivate these damaging oxyl species and prevent the deleterious consequences of their reactions (Jovanovic *et al.*, 1994). This suggests that metal chelation may play a larger role in determining the antioxidant activity of these compounds than has previously been believed. The higher antioxidative potentials of flavonoids against metal-ion-induced peroxidations are probably a consequence of their combined metal chelating and free radical scavenging abilities. In the AAPH-induced peroxidations, however, only the free radical scavenging mechanism contributes towards the inhibitory action of these compounds, thereby accounting for the lower antioxidant potencies.

In an earlier study, the inhibitory effects of quercetin and rutin on Fe(II)-ion-dependent and independent lipid peroxidation were examined to elucidate the chelating and free radical scavenging activities of these compounds (Afanas'ev *et al.*, 1989). Both flavonoids were significantly more effective at inhibiting Fe(II)-dependent lipid peroxidation due to their ability to chelate iron ions with the formation of inert complexes unable to initiate peroxidation. Additionally, the iron complexes of flavonoids retained their free radical scavenging activities. Morel *et al.* (1993) studied the antioxidant and iron-chelating actions of flavonoids on iron-loaded rat hepatocyte cultures. They directly demonstrated that the flavonoids possessed good chelating activities and were capable of removing iron already

inside the hepatocytes, thereby suggesting that the metal-chelating mechanism was a significant contributor to the antioxidant mechanism of these compounds.

In our study, another possible explanation for the higher antioxidant activities observed against metal-ion-dependent peroxidation versus AAPH-induced peroxidation may be that the aqueous-phase, AAPH-derived-peroxyl radicals, being significantly bulkier than the radicals generated by the metal ions, may not be able to localize into the same region of the membrane as the flavonoids and hence may not be accessible to the flavonoids for scavenging. Determination of the precise localization of flavonoids in the membrane bilayer is the subject of a later chapter in the dissertation. The results from that study indicate that the flavonoids preferentially partition into the hydrophobic core of the bilayer, a region that may be different than that in which the AAPH-derived peroxyl radicals localize.

Figure 3.9 summarizes the structural criteria that enhanced the antioxidant activity of flavonoids. Substitution patterns on the B-ring were found to be the most important contributor to the antioxidant activity of flavonoids. Hydroxyl groups boosted the antioxidant activity; whereas methoxy groups suppressed antioxidant activity. Presence of *o*-dihydroxy substitution on the flavonoids substantially enhanced the antioxidant activity of these compounds. A hydroxyl group at the C-3 position was also beneficial to the ability of flavonoids to inhibit lipid peroxidation.

This study also confirms the sensitivity of the fluorescence-based assay previously developed in our laboratory in evaluating plant-derived compounds for antioxidant activity. The simple and rapid assay can be used to characterize structure-activity relationships within a class of structurally related compounds.

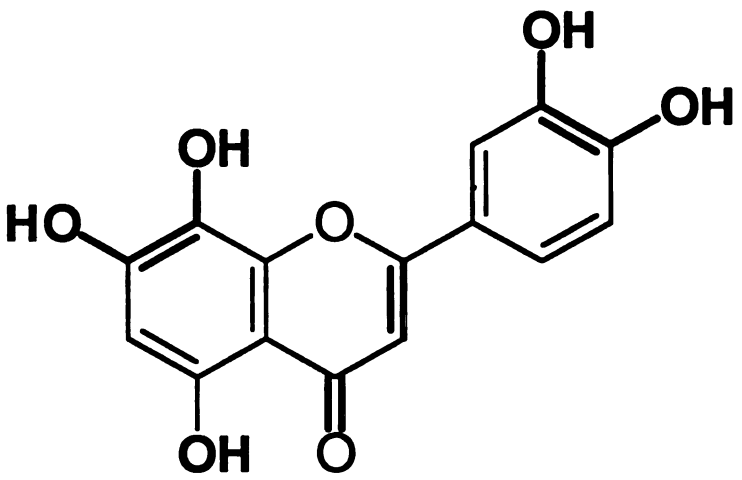


Figure 3.9. Structural criteria that enhance the antioxidant activity of flavonoids.

CHAPTER 4

ANTIOXIDANT ACTIVITIES OF ISOFLAVONES AND THEIR BIOLOGICAL METABOLITES IN A LIPOSOMAL SYSTEM

4.1 ABSTRACT

Genistein and daidzein, the two major soy isoflavones, principally occur in nature as their glycosylated or methoxylated derivatives, which are cleaved in the large intestine to yield the free aglycones and further metabolites. The objective of this study was to compare the antioxidant activities of genistein and daidzein with their glycosylated and methoxylated derivatives, and their human metabolites. The abilities of these compounds to inhibit lipid peroxidation in a liposomal system were evaluated using fluorescence spectroscopy, and structural criteria that enhance antioxidant activity were established. The peroxidation initiators employed in the study were Fe(II) and Fe(III) metal ions, and aqueous-phase, azo-derived peroxyl radicals. Both the parent isoflavonoids and their metabolites were more effective at suppressing metal-ion-induced peroxidations than the peroxyl-radical-induced peroxidation. Antioxidant activities for the isoflavone metabolites were comparable to or superior to those for the parent compounds themselves. Equol and its 4-hydroxy and 5-hydroxy derivatives were the most potent antioxidants in the study, suggesting that absence

of the 2,3-double bond coupled with a loss of the 4-oxo group enhanced antioxidant activity. Additionally, the number and position of hydroxyl groups were determining factors for isoflavonoid antioxidant activity, with hydroxyl substitution being of utmost importance at the C-4' position, of moderate importance at the C-5 position and of little significance at the C-7 position.

4.2 INTRODUCTION

Certain phytochemicals in fruits, vegetables and grains are now being recognized as possessing possible cancer-preventive properties through inhibition of tumor initiation, prevention of oxidative damage, or by affecting steroid hormones or prostaglandin metabolism to suppress tumor promotion (Caragay, 1992). In recent years, there has been a surge of interest in studying the beneficial physiological effects of one such class of compounds, the isoflavonoids. Isoflavonoids have a very restricted distribution in the plant kingdom and occur almost exclusively in legumes (*Leguminosae* family); with soybeans, chickpeas, lentils and beans representing the major dietary sources (Ingham, 1983).

The principal isoflavonoids occurring in legumes are genistein and daidzein which are present primarily as their glycoside conjugates (genistin and daidzin) or their respective 4'-methoxy derivatives (biochanin A and formononetin) (Price and Fenwick, 1985). Upon ingestion, the isoflavonoids are subjected to acidic and enzymatic hydrolysis and demethylation by the gut microflora to yield the free aglycones, demethylated products and further metabolites, with both the metabolites and the unfermented parent aglycones being liable for further absorption (Kelly *et al.*, 1995).

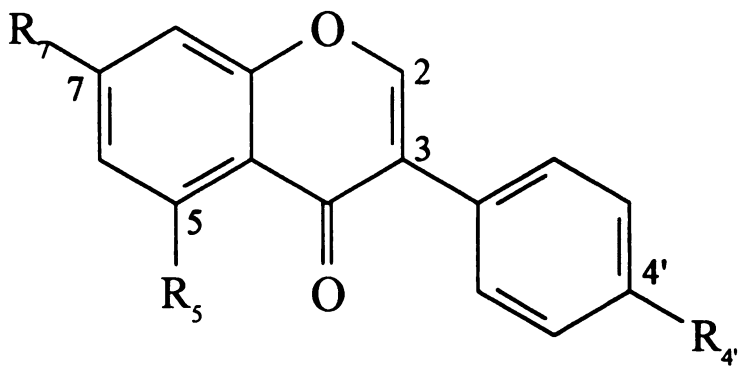
The pathways for human metabolism of isoflavones have not been clearly established. Biochanin A and formononetin are known to undergo demethylation by intestinal bacteria, giving rise to genistein and daidzein respectively. Daidzein undergoes intestinal microbial transformation in humans to yield the isoflavan equol (7-hydroxy-3-(4'-hydroxyphenyl)-chroman) (about 70%) and a ring-cleavage compound *O*-desmethylangolensin (*O*-Dma) (5-20%) (Setchell and Adlercreutz, 1988). Other daidzein intermediates formed during this conversion are dihydrodaidzein, 4-hydroxy equol, 2-dehydro-*O*-Dma, and possibly dehydroequol (Joannou *et al.*, 1995). Genistein is metabolized within the gut by ring cleavage to yield the non-estrogenic, hormonally inert compound *p*-ethyl phenol (Kelly *et al.*, 1993). Other compounds that have recently been identified as catabolic products of genistein metabolism in humans include dihydrogenistein and 6'-OH-*O*-Dma (Joannou *et al.*, 1995; Kelly *et al.*, 1993).

The reported presence of isoflavones of plant origin in human urine (Adlercreutz *et al.*, 1991; Adlercreutz *et al.*, 1986; Axelson *et al.*, 1984; Axelson *et al.*, 1982; Bannwart *et al.*, 1984) has renewed intense scientific interest in the study of these compounds because of their association with a broad range of biological activities which include antiestrogenic (Cassidy *et al.*, 1995), anticancer (Peterson and Barnes, 1996), antiinflammatory (Yamamoto *et al.*, 1996), cardioprotective (Anthony *et al.*, 1996) and enzyme-inhibitory effects (Yamashita *et al.*, 1990). Since it is believed that many of these protective effects of isoflavonoids may be related to their antioxidant activities, there has been a surge of interest in exploring the antioxidant activities of these compounds. However, most of the studies have focused solely on the antioxidant activity of genistein (Cai and Wei, 1996; Record *et al.*,

1995; Wei *et al.*, 1993). As genistein is principally obtained from the diet as its 7-β-glucoside, genistin, it would be appropriate to include both the free aglycone and the conjugated glycoside in studies on antioxidant evaluation.

In addition, *in vitro* anaerobic incubation of isoflavones with human feces has shown that intestinal half-life of genistein and daidzein may be as little as 3.3 h and 7.5 h respectively (Xu *et al.*, 1995), indicating rapid degradation to other compounds by gut microflora (Chang and Nair, 1995). It would, therefore, also be logical to include these metabolites in studies related to antioxidant activity of isoflavonoids. However, other than a recent report on the antioxidant activities of daidzein metabolites equol and *O*-Dma (Hodgson *et al.*, 1996), there have been no studies examining the antioxidant efficacies of isoflavone metabolites.

The aims of this study were to do a comprehensive study comparing the antioxidant activities of isoflavone aglycones, glycosides and their biological metabolites, and to establish structural criteria that enhance the antioxidant activity of these compounds. The parent isoflavones included in this study were the free aglycones genistein and daidzein, the methoxy derivatives biochanin A and formononetin and the glycosides genistin and daidzin (Figure 4.1). Daidzein metabolites equol, 4-hydroxy equol and dihydrodaidzein, and genistein metabolite dihydrogenistein were evaluated for their antioxidant abilities (Figure 4.2). 5-hydroxy equol, a possible metabolite of genistein, has to date not been detected in human urine. However, it represents the fully reduced form of genistein (just as equol represents the fully reduced form of daidzein) and hence, was examined in the study for better elucidation of structure-activity relationships. Other known metabolites like *p*-ethylphenol and *O*-Dma, where the isoflavone nucleus was no longer intact, were not included in the study.



Substituents			
Isoflavonoid	**R-5**	**R-7**	**R-4'**
Genistein	OH	OH	OH
Genistin	OH	O-glucose	OH
Daidzein	H	OH	OH
Daidzin	H	O-glucose	OH
Biochanin A	OH	OH	OCH_3
Formononetin	H	OH	OCH_3

Figure 4.1. The structures of the plant-derived isoflavonoids included in this study.

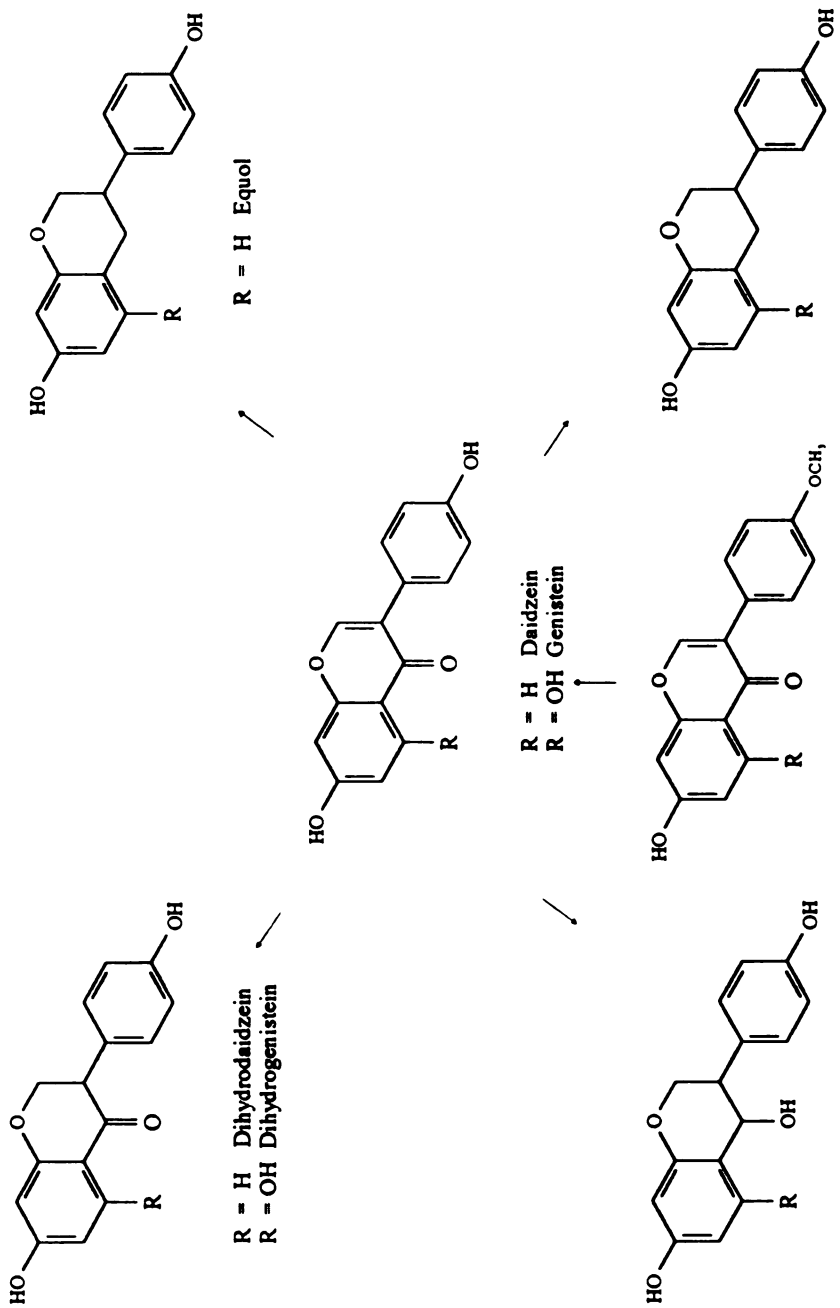


Figure 4.2. Structures of the isoflavone metabolites examined for their antioxidant activity.

Another aim of this investigation was to better understand the mechanism of action by which these compounds act as antioxidants. To accomplish that objective, the inhibitory effects of these compounds against lipid peroxidation induced by metal ions versus peroxidation induced by peroxyl radicals generated in the aqueous phase by thermal decomposition of an azo compound were studied. For the sake of comparison, the well-known antioxidant *tert*-butylhydroquinone (TBHQ) was also included in the study.

Many different methods are available for evaluating the oxidative stability of lipids (Gutteridge and Halliwell, 1990). Most of these approaches suffer from serious methodological drawbacks. In addition, they can be time-consuming, have limited sensitivity and require large amounts of material. A recently developed assay in our laboratory (Chapter 2) overcomes many of these disadvantages. The approach is based on the susceptibility of the extrinsic probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) to peroxidative damage with concomitant loss of its fluorescent character. The decay of DPH-PA fluorescence is directly related to the extent of lipid peroxidation and can be followed continually. This fluorescence assay was used in this study for evaluation of isoflavonoid antioxidant efficacy. This study was also aimed at testing the ability of this assay to discriminate between antioxidant activities of compounds that are closely related in structure.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 Materials

Synthetic 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC) was obtained from Avanti Polar Lipids (Alabaster, AL). Lipid purity was confirmed by silica gel TLC using two different solvent systems (chloroform : methanol : water :: 65 : 25 :4; chloroform : methanol : ammonium hydroxide :: 65 : 25 : 4). The lipid stock solutions in chloroform were maintained at -20°C in amber glass vials that were layered with nitrogen. The fluorescent probe 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) was obtained from Molecular Probes (Eugene, OR). The isoflavone glycosides were purified from soy molasses, and the aglycones and their metabolites were obtained by synthesis (Chang *et al.*, 1994; Chang *et al.*, 1995). 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) was obtained from Wako Chemical Company (Richmond, VA). $FeCl_2 \cdot 4H_2O$ and $FeCl_3 \cdot 6H_2O$ of greater than 99% purity were obtained from Aldrich (Milwaukee, WI) and stored at -20°C under anaerobic conditions. All other reagents were of the highest grade available.

The DPH-PA and isoflavonoid stock solution were prepared in N,N-dimethylformamide and dimethyl sulfoxide (DMSO) respectively. The iron and AAPH stock solutions were prepared immediately before use in nitrogen-sparged, double-distilled, deionized water and maintained on ice under anaerobic conditions. To remove metal contaminants, all the glassware was acid-washed prior to use and the salt and buffer solutions maintained in a chelating resin Chelex 100 (sodium form, mesh 50-100, Bio-Rad, Richmond, CA).

4.3.2 Preparation of Large Unilamellar Vesicles

LUVs containing the probe DPH-PA were prepared fresh for each day of experimentation using the extrusion procedure of MacDonald *et al.* (1991). Briefly, a mixture containing 5 µmol of SLPC and 15 nmol of DPH-PA was dried under vacuum onto the wall of a 15 mL round-bottomed flask. The resulting lipid film was hydrated in 500 µL of a solution containing 0.15 M NaCl, 0.01 M MOPS (pH 7.0) and 0.1 mM EDTA. After 10 freeze-thaw cycles using a dry ice/ethanol bath, the suspension was passed 29 times through two stacked polycarbonate filters (pore size 100 nm) using a Liposofast extruder apparatus (Avestin, Ottawa, Canada).

4.3.3 Antioxidant Evaluation of Flavonoids

The fluorescent intensity assay described in Arora and Strasburg (1997) was used to evaluate the antioxidant efficacy of the isoflavonoids. A 20 µL aliquot of the LUV suspension was diluted to 2 mL in a buffer containing 100 mM NaCl and 50 mM Tris-HEPES (pH 7.0) to achieve final concentrations of 100 µM lipid and 300 nM probe in the cuvette. After a 5 min pre-incubation at room temperature with continuous stirring, the suspension was transferred to a thermostatted cuvette holder (37^{0}C) in the spectrofluorometer and incubated for an additional 10 min for temperature equilibration. An aliquot of the isoflavonoid stock solution, dissolved in DMSO, was added to yield a final concentration of 10 µM. The volume of added stock solution never exceeded 0.3 % of the total volume; under these conditions, DMSO had no effect on the rate of peroxidation.

Following a 5 min incubation to allow partitioning of the isoflavonoid into the membrane, peroxidation was initiated by addition of 20 μL of 1 mM stock $FeCl_2$ or $FeCl_3$ solutions or 40 μL of stock AAPH solution for final concentrations of 10 μM for $FeCl_2$ and $FeCl_3$, and 10 mM for the AAPH. The control samples did not contain any peroxidation initiator or flavonoid to be tested. The decay in fluorescence intensity was monitored over 21 min, with readings being taken at 0 min, 1 min and every 3 min thereafter.

Fluorescence measurements were performed in a 2 mL thermostated quartz cuvette at 37°C, and the fluorescent signal was followed with a SLM Instruments, Model 4800, spectrofluorometer (Urbana, IL) with data acquisition hardware and software from On-Line Instrument Systems (Bogart, GA). The excitation wavelength used was 384 nm, slit width 2 nm, and the emitted light was passed through optical filters (KV 418, Schott, Duryea, PA), prior to detection.

4.4 RESULTS

4.4.1 Effect of Isoflavonoids on Rates of Fe(II)-Catalyzed Peroxidation in LUVs

The inhibitory effects of genistein, daidzein, and their methoxy and glycoside derivatives on rates of Fe(II)-induced peroxidation are illustrated in Figure 4.3. The efficacies of these compounds as antioxidants were evaluated as the degree of inhibition of the fluorescence intensity of the probe DPH-PA. All the isoflavonoids examined in the study inhibited this drop in intensity to some degree; however, the rate of inhibition varied widely depending on the structures of these compounds. The isoflavone genistein, with hydroxyl

Figure 4.3. Effects of the plant-derived isoflavones on rates of Fe(II)-induced peroxidation in the LUVs at 37^{0}C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM test compound suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of Fe(II) for a final concentration of 10 μM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements.

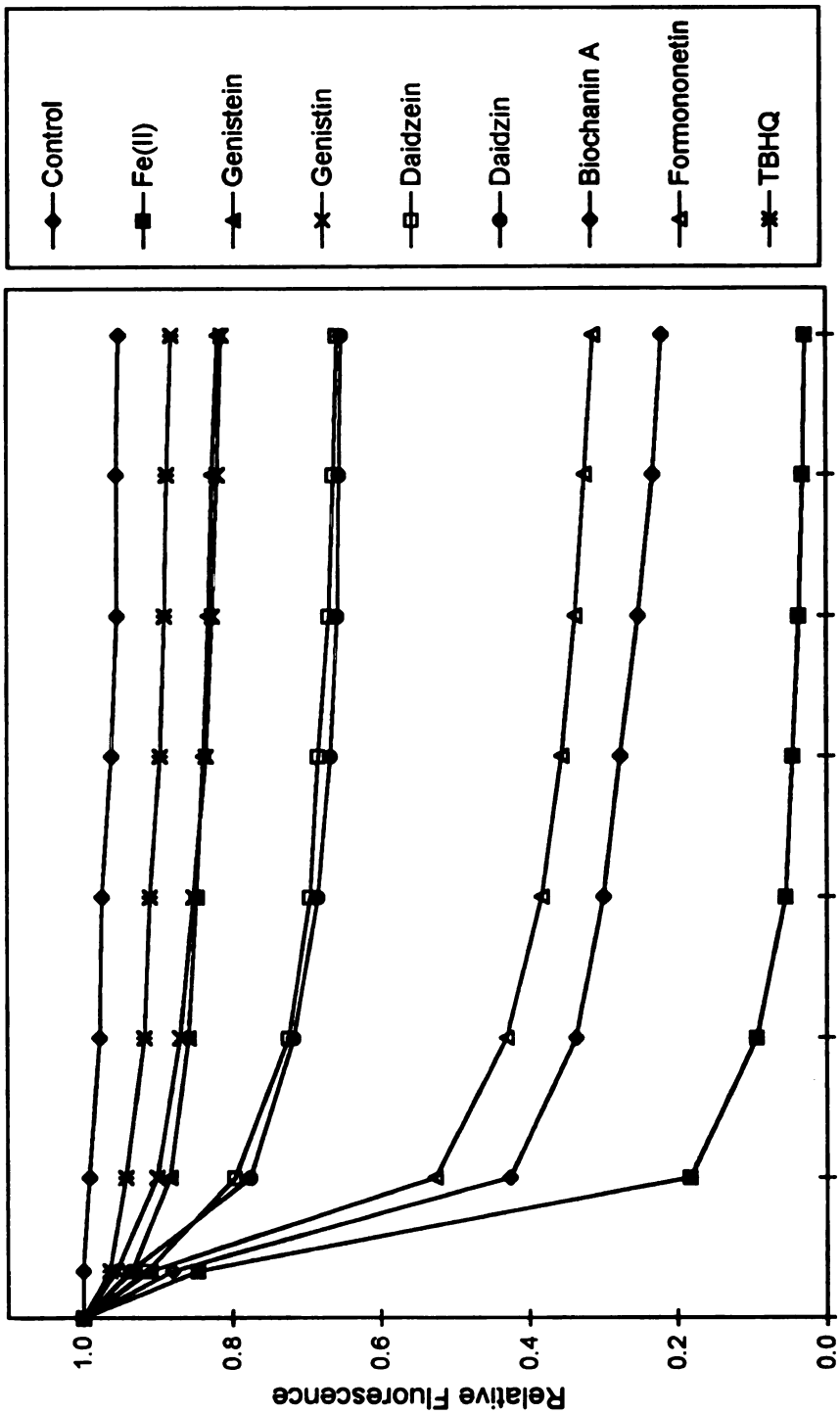

Control
Fe(II)
Genistein
Genistin
Daidzein
Daidzin
Biochanin A
Formononetin
TBHQ
Relative Fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
0
3
6
9
12
15
18
21
Time (min)

Figure 4.3

groups at 5, 7 and 4' positions exhibited the highest antioxidant activity among this group of isoflavonoids. Blocking the hydroxyl group at the C-7 position of genistein by a glucose in genistin did not have any effect on the antioxidant activity, as evidenced by the identical decay in fluorescence in the presence of either of the two compounds. Daidzein, which lacks the C-5 hydroxyl of genistein, was less effective as an antioxidant. Again, the antioxidant activity for daidzin, the 7-β-glucoside of daidzein, was identical to the aglycone itself. However, substitution of the C-4' hydroxyl of genistein and daidzein by a methoxy in biochanin A and formononetin, respectively, substantially reduced their antioxidant activities. The antioxidant activity of the commercial food antioxidant TBHQ against Fe(II)-induced peroxidation was comparable to or surpassed that of the isoflavones examined in the study.

The isoflavone metabolites inhibited the rates of Fe(II)-induced peroxidation in the LUVs at rates that were comparable to or superior than the parent compounds themselves (Figure 4.4). Of the metabolites, the isoflavans (with a saturated 2,3 bond and no keto group at the C-4 position) displayed the highest antioxidant activities. 5-hydroxy equol, the fully reduced derivative of genistein, was the most potent antioxidant under these conditions, showing intensity values that were almost indistinguishable from the control experiment. 4-hydroxy equol and equol were also very effective under these conditions. The antioxidant activities displayed by equol and its 4-and 5-hydroxy derivatives against Fe(II)-induced peroxidation were even superior to the highly effective antioxidant TBHQ. The isoflavanones (with a saturated 2,3 bond) dihydrogenistein and dihydrodaidzein were less effective as antioxidants than the isoflavans.

Figure 4.4. Effects of the isoflavone metabolites on rates of Fe(II)-induced peroxidation in the LUVs at 37°C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM test compound suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of Fe(II) for a final concentration of 10 μM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements.

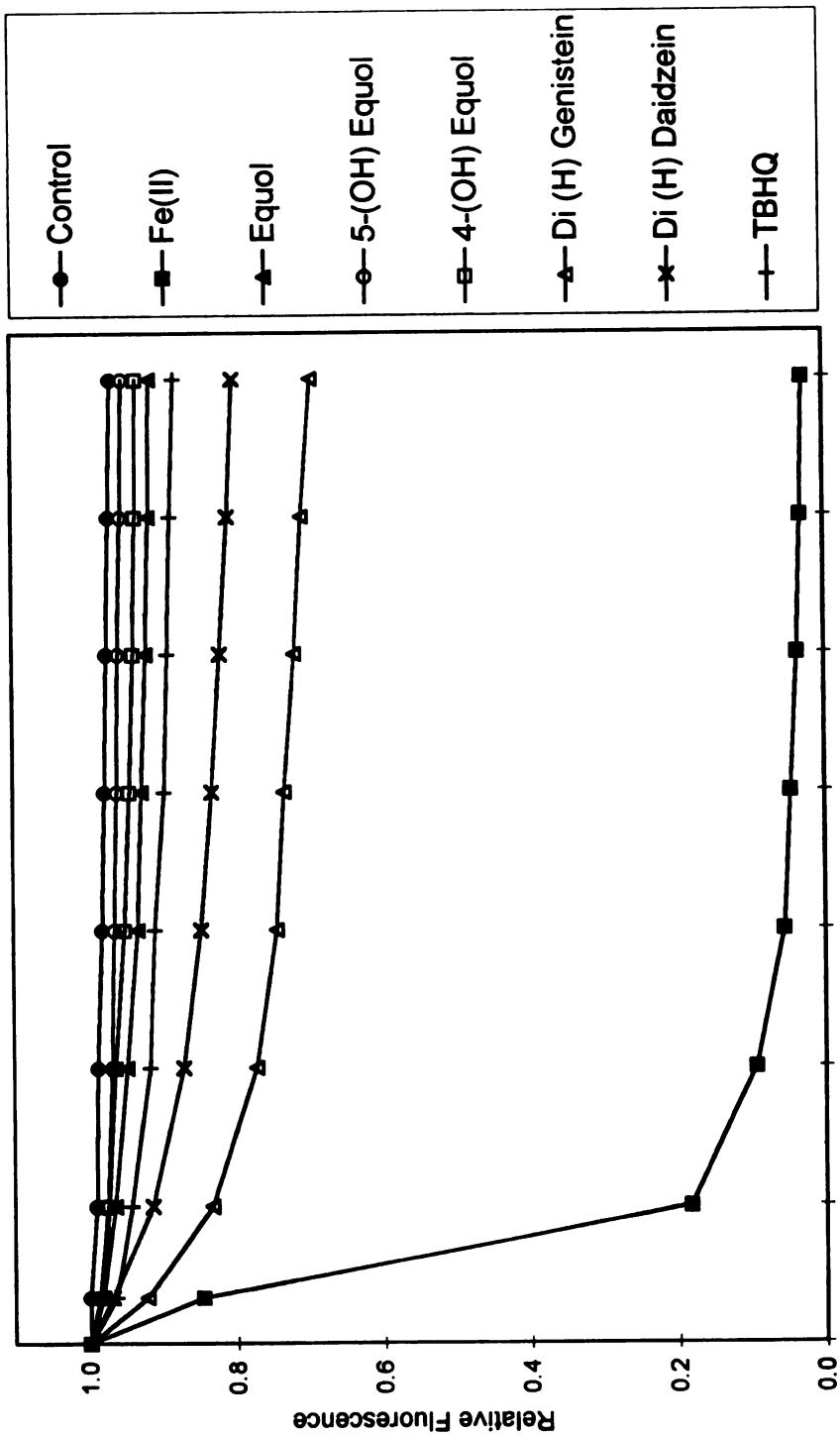

Control
Fe(II)
Equol
5-(OH) Equol
4-(OH) Equol
Di (H) Genistein
Di (H) Daidzein
TBHQ
Relative Fluorescence
1.0
0.8
0.6
0.4
0.2
0.0
0
3
6
9
12
15
18
21
Time (min)

Figure 4.4

4.4.2 Effects of Isoflavonoids on Rates of Fe(III)-Catalyzed Peroxidation in the LUVs

The trends for the antioxidant activities of the isoflavonoids remained essentially unchanged when Fe(III) ions were used to initiate peroxidation instead of Fe(II) ions (Figure 4.5). Here too, the aglycone genistein and its corresponding 7-β-glucoside, genistin, were the most effective of the set of dietary isoflavanones examined. In fact, genistein and genistin surpassed the inhibitory effects observed with TBHQ. Daidzein and its glycoside daidzin also displayed very similar rates of inhibition. These inhibitory effects of daidzein and daidzin were less than those seen for genistein and genistin. Methoxylation of the C-4' hydroxyl of genistein and daidzein led to a dramatic drop in their antioxidant activities.

The isoflavone metabolites were very effective at inhibiting Fe(III)-induced liposomal peroxidation (Figure 4.6). Again, 5-hydroxy equol, a possible genistein metabolite, and equol and 4-hydroxy equol, daidzein metabolites, exhibited the highest antioxidant potencies. These activities were greater than those observed with TBHQ. The dihydro derivatives of daidzein and genistein were slightly less effective than TBHQ; however, they still showed substantial rates of inhibition against Fe(III)-induced peroxidation.

4.4.3 Effects of Isoflavonoids on Rates of AAPH-Induced Peroxidation in the LUVs

Figure 4.7 illustrates the inhibitory effects of the parent isoflavones against peroxidation induced by peroxyl radicals generated at a constant rate in the aqueous phase by thermal decomposition of the azo compound AAPH. The isoflavones studied were less effective at inhibiting the AAPH-induced peroxidation. TBHQ exhibited greater antioxidant potencies against AAPH-induced peroxidation than the isoflavones. The antioxidant trends

Figure 4.5. Effects of the plant-derived isoflavones on rates of Fe(III)-induced peroxidation in the LUVs at 37^{0}C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM test compound suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of Fe(II) for a final concentration of 10 μM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements.

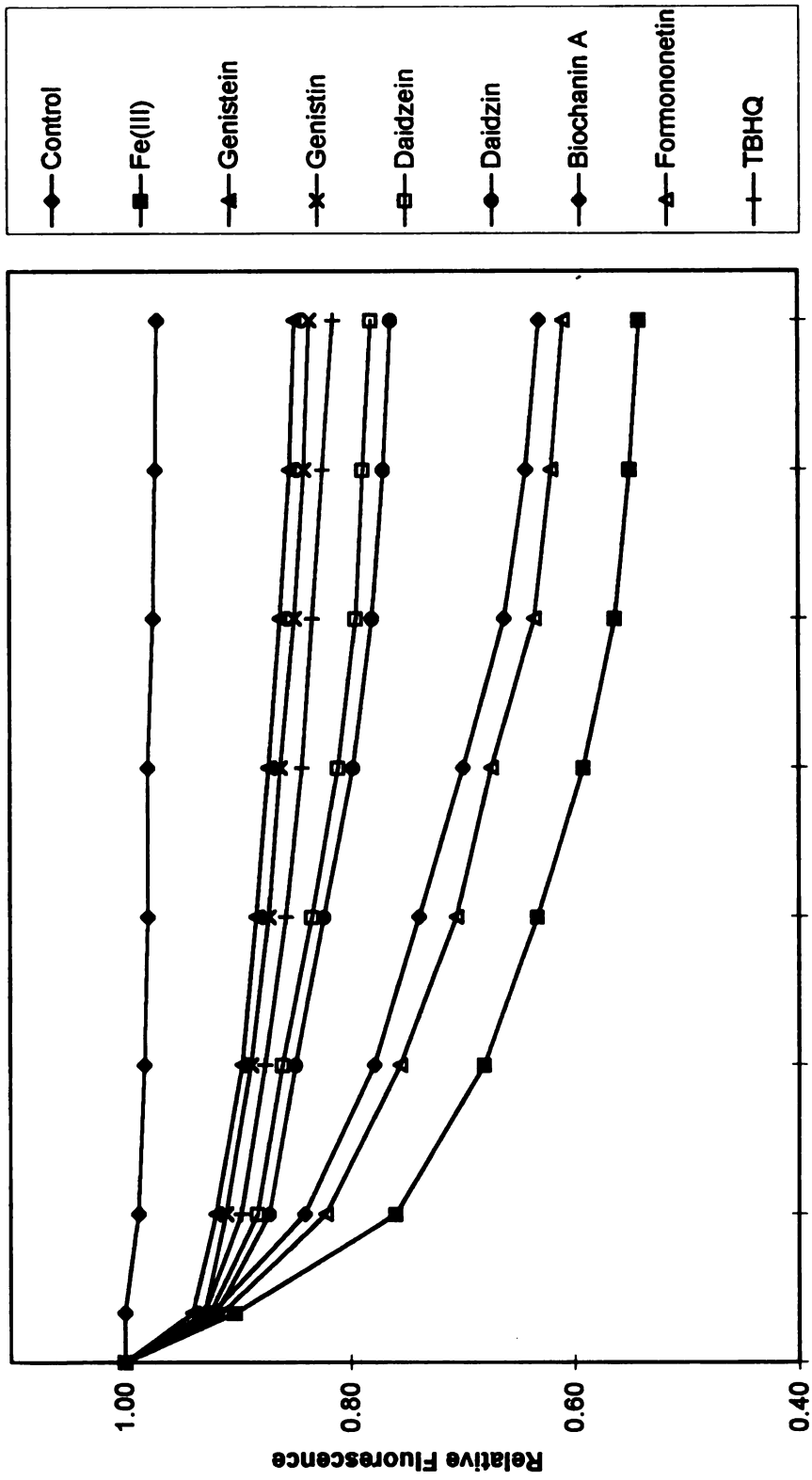

Control
Fe(III)
Genistein
Genistin
Daidzein
Daidzin
Biochanin A
Formononetin
TBHQ
Relative Fluorescence
1.00
0.80
0.60
0.40
0
3
6
9
12
15
18
21
Time (min)

Figure 4.5

Figure 4.6. Effects of the isoflavone metabolites on rates of Fe(III)-induced peroxidation in the LUVs at 37°C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM test compound suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of Fe(II) for a final concentration of 10 μM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements.

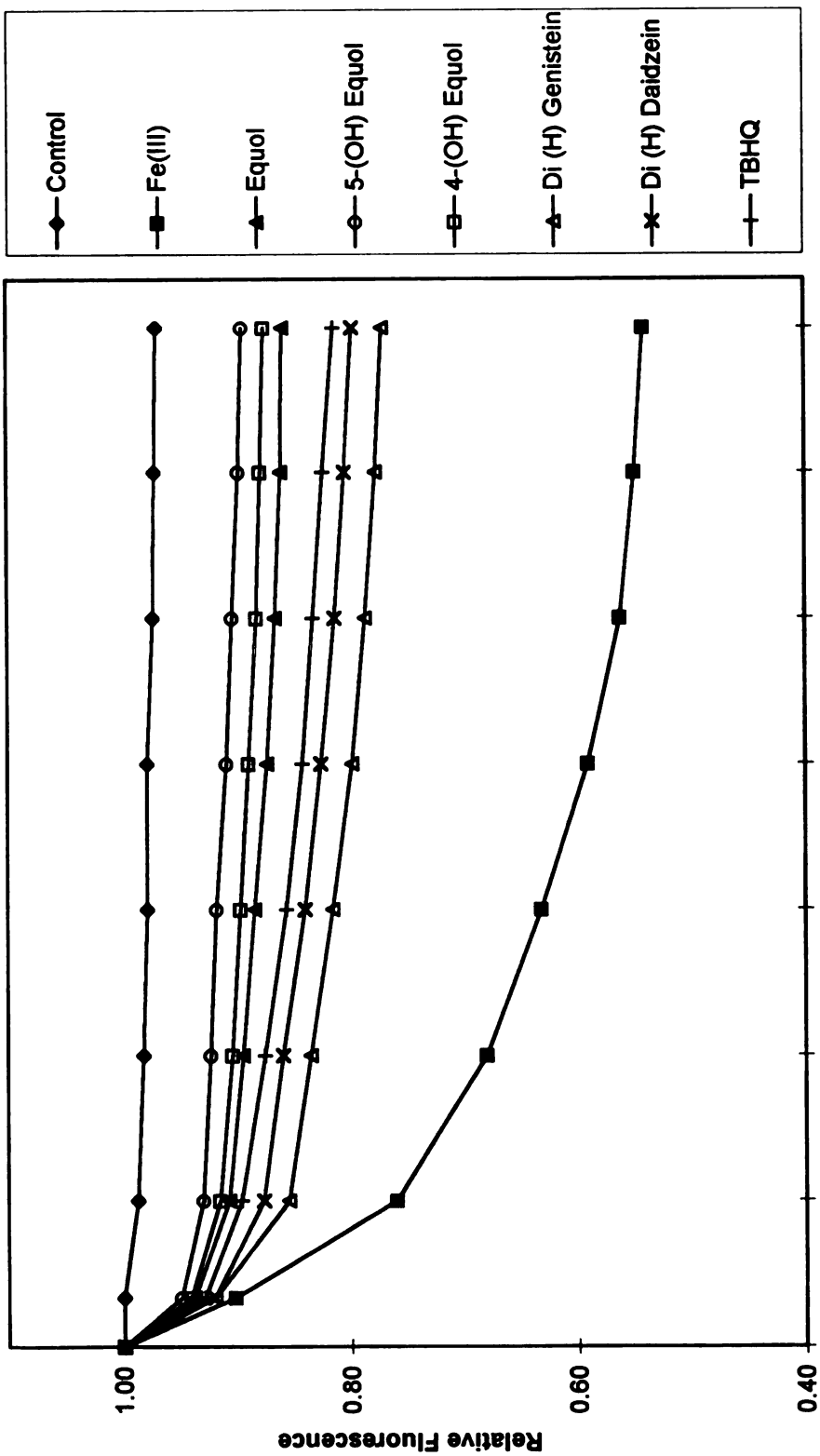

Control
Fe(III)
Equol
5-(OH) Equol
4-(OH) Equol
Di (H) Genistein
Di (H) Daidzein
TBHQ
Relative Fluorescence
1.00
0.80
0.60
0.40
0
3
6
9
12
15
18
21
Time (min)

Figure 4.6

Figure 4.7. Effects of the plant-derived isoflavones on rates of AAPH-induced peroxidation in the LUVs at 37°C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM test compound suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of AAPH for a final concentration of 10 mM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements.

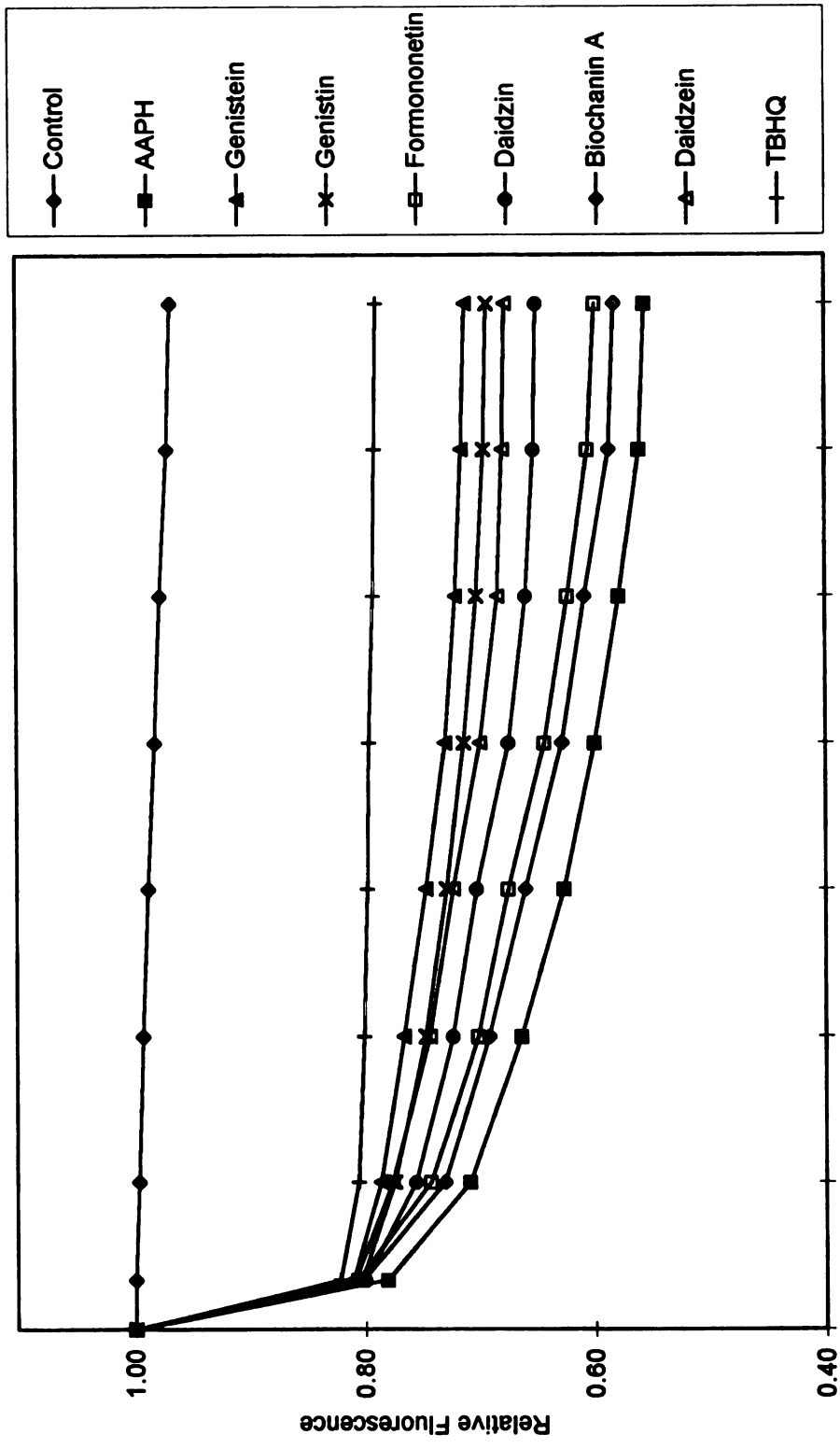

Control
AAPH
Genistein
Genistin
Formononetin
Daidzin
Biochanin A
Daidzein
TBHQ
Relative Fluorescence
1.00
0.80
0.60
0.40
0
3
6
9
12
15
18
21
Time (min)

Figure 4.7

observed earlier against Fe(II) and Fe(III)-induced peroxidations were maintained here; however, there were only marginal differences between the antioxidant activities of the different isoflavones.

The trends remained unchanged with the metabolites of the parent isoflavones (Figure 4.8). Again, these compounds were less effective at inhibiting the AAPH-induced peroxidations than the inhibitions observed earlier against Fe(II) and Fe(III) ions. Equol and its 4- and 5-hydroxy derivatives had a greater inhibitory effect than the dihydro derivatives of daidzein and genistein. All the metabolites displayed lower antioxidant activities than TBHQ.

4.5 DISCUSSION

In spite of the various reports linking many of the beneficial properties of isoflavonoids to their antioxidant activities, no comprehensive studies have been conducted examining the antioxidant efficacies of the dietary isoflavonoids versus their biological metabolites. The aim of this work was to compare the antioxidant potencies of the naturally occurring glycosidic and methoxylated forms of isoflavones, the free aglycones and their biological metabolites. The goal was to establish structural features that enhance the antioxidant activities of this class of compounds. The different initiators of peroxidation used to test the inhibitory actions of these compounds were the Fe(II) and Fe(III) metal ions and peroxyl radicals generated from thermal decomposition of the water-soluble azo compound AAPH. Another objective of the study was to test the sensitivity of the fluorescence

Figure 4.8. Effects of the isoflavone metabolites on rates of AAPH-induced peroxidation in the LUVs at 37°C. Peroxidation was initiated in the LUVs containing 100 μM lipid, 300 nM probe and 10 μM test compound suspended in 2 mL of buffer (100 mM NaCl, 50 mM Tris-HEPES, pH 7.0) by addition of AAPH for a final concentration of 10 mM. The rate of peroxidation was monitored by a decrease in fluorescence intensity as a function of time. Relative fluorescence represents the fluorescence intensity after a given time of oxidation over the fluorescence intensity at time = 0 min. Values represent the means of triplicate measurements.

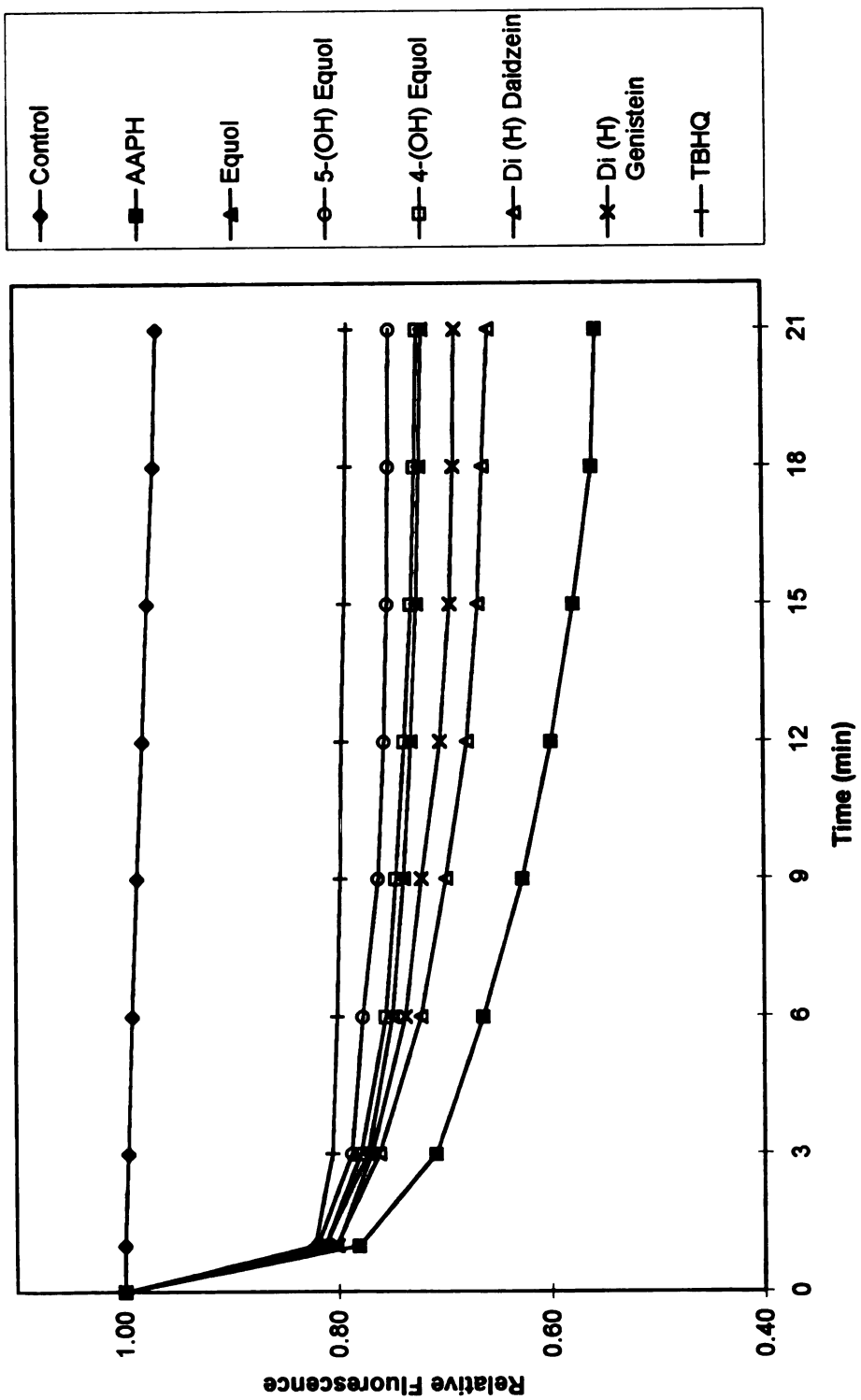

Control
AAPH
Equol
5-(OH) Equol
4-(OH) Equol
Di (H) Daidzein
Di (H) Genistein
TBHQ
Relative Fluorescence
1.00
0.80
0.60
0.40
Time (min)
0
3
6
9
12
15
18
21

Figure 4.8

spectroscopic assay previously developed in our laboratory in distinguishing between antioxidant activities of structurally-related compounds.

The inhibitory potencies of isoflavonoids were dependent on the system used to initiate peroxidation. All the compounds studied had a more pronounced inhibitory effect against the metal-ion-induced peroxidations. The inhibitory effects of the isoflavonoids decreased considerably when AAPH was used instead to initiate lipid peroxidation in the vesicles. This was in spite of the fact that the reduction potential of isoflavonoids is lower than that of peroxyl radicals, thereby allowing them to reduce these damaging oxy radicals (Jovanovic *et al.*, 1994).

These results suggest that the free radical scavenging ability of these compounds only partly accounts for their antioxidant capabilities. Because of their polyphenolic structures, these compounds can donate hydrogen atoms to deleterious oxy radicals and form the less reactive phenoxyl radicals in the process. The same structure also confers isoflavonoids with an ability to chelate metal ions. The greater antioxidative potential of the isoflavonoids against metal-ion-induced peroxidation observed in the study is probably a consequence of their combined metal chelating and free radical scavenging abilities.

Metal chelation, though, cannot solely account for the greater antioxidant activities of the isoflavonoids in the presence of metal ions. This is because the molar ratio of isoflavonoid to metal ions was maintained at one to one in this study and most chelators of similar structure complex iron with a 3:1 (chelator : iron) stoichiometry (Ryan and Petry, 1993).

In a study comparing antioxidant effectiveness of genistein against AAPH and Fe(II)ions, Record *et al.* (1995) also observed that genistein afforded significant protection against Fe(II)-induced peroxidation but was less effective against AAPH-induced peroxidation. Using the catechol-binding assay, these researchers were unable to demonstrate any evidence of a substantial chelating ability for genistein. However, the assay used in the study lacked sensitivity and was also limited by solubility properties of genistein.

Though no similar studies have been conducted with isoflavonoids, a study conducted with a series of estrogens, compounds with structures similar to isoflavonoids, found that inhibitory actions of these compounds were greater when Fe(II) ions were used as initiators and much less pronounced against AAPH (Lacort *et al.*, 1995). It was suggested that these compounds may either be exerting their protective effects through chelation of metals or by altering iron redox chemistry (Ruiz-Larrea *et al.*, 1995).

In addition to being dependent on the peroxidation initiator used in the assay, the antioxidant potencies for the isoflavonoids were also influenced by their chemical structures. Although all the isoflavonoids examined in the study inhibited lipid peroxidation to a certain extent, the degree of inhibition varied widely according to the structure of these compounds. These structure-activity relationships were valid in all of the three peroxidation systems used in the study.

Genistein, with hydroxyl groups at C-5, 7 and 4' positions, was an effective antioxidant in the liposomal system. Daidzein, lacking the C-5 hydroxyl group of genistein, was less effective as an antioxidant. This suggested that the hydroxyl at the C-5 position contributed towards the antioxidant activity of these compounds. In contrast, blocking the

C-7 hydroxyl of genistein or daidzein by a glucose in genistin or daidzin had no effect on the antioxidant activities of the aglycones, thereby indicating that the hydroxyl at the C-7 position had a negligible effect on the antioxidant activities of these compounds.

On the other hand, the contribution of the C-4' hydroxyl on the B-ring towards the antioxidative potentials of the isoflavones was highly significant, as demonstrated by the dramatic drop in the abilities of these compounds to inhibit lipid peroxidation when this 4'-hydroxyl group of genistein or daidzein was substituted by a methoxy group. Biochanin A and formononetin were very weak antioxidants in this *in vitro* system. In an *in vivo* system, however, since biochanin A and formononetin undergo fast demethylation to genistein and daidzein, their antioxidant potencies may be vastly increased.

Comparing antioxidant potencies of genistein, daidzein and biochanin A, Wei *et al.* (1995) also determined that the loss of the hydroxyl group at the C-4' position of isoflavones totally diminished their inhibitory activity towards lipid peroxidation whereas replacement at other positions had less of an influence. Genistein was found to be a more potent inhibitor of lipid peroxidation than daidzein, and biochanin A was very ineffective under their assay conditions.

An interesting observation of our study was that the metabolites of genistein and daidzein exhibited antioxidant potencies that were comparable to or superior than the parent compounds themselves. Reducing the isoflavone nucleus to yield the isoflavan structure substantially enhanced the antioxidant activities of these compounds. This was evidenced by the superior antioxidant actions of the derivatives equol, 4-hydroxy equol and 5-hydroxy equol as compared to the parent isoflavones. This indicated that the absence of the 2,3-

double bond in conjunction with a loss of the 4-oxo group enhanced antioxidant activities of these compounds.

These results were surprising since it is widely believed that presence of a 2,3-double bond coupled with a 4-oxo group increases the antioxidant activities for flavonoids due to the greater stability conferred to the flavonoid phenoxyl radical by this increase in conjugation (Bors *et al.*,1990). The only other study comparing the antioxidant activities of equol with daidzein and genistein also determined that equol was a more potent antioxidant, as compared to the parent isoflavones (Hodgson *et al.*, 1996). Since the inhibitory actions in the study were evaluated against Cu(II)-induced lipoprotein oxidation in serum, the authors suggested that equol may be inhibiting oxidation via additional mechanisms such as by acting as co-antioxidants. However, use of our well-defined system with no contaminating pro- or antioxidants present, ensures that the activity observed is attributable to the test compound alone. The higher antioxidant activity of equol may be a result of its non-planar structure that confers equol with a greater flexibility for conformational changes, thereby enabling it to penetrate into the interior of the membrane with greater ease than some of the other isoflavonoids that are more rigid in structure.

With the dihydro metabolites of genistein and daidzein, however, no conclusive differences could be seen with respect to the parent compounds. The antioxidant activities observed for dihydrogenistein and dihydrodaidzein were close to those observed for genistein and daidzein. Hence, our study could not demonstrate any significant effect of loss of the 2,3-double bond alone on the antioxidant activities of these compounds.

In summary, the protective effects of isoflavonoids in a liposomal system were dependent on the chemical structures of these compounds and the system used to initiate peroxidation. The isoflavonoids were more potent inhibitors of metal-ion-induced peroxidation than of the peroxidation induced by peroxyl radicals. For the structural criteria, the number and position of hydroxyl groups was found to be an important determinant of antioxidant activity (Figure 4. 9). Hydroxyl groups were found to be of critical importance at the C-4' position, of moderate importance at the C-5 position and of negligible importance at the C-7 position. Loss of the 2,3-double bond coupled with the absence of the 4-oxo group conferred the greatest antioxidant activities to these compounds.

As consumer interest in the use of plant-derived antioxidants in food products grows, a systematic approach will be required to rapidly evaluate plant extracts and purified compounds for antioxidant activity. The simplicity, sensitivity, and rapidity of the fluorescence assay developed in our laboratory combine to make this an attractive screening method to identify compounds or extracts for subsequent comprehensive evaluations in food products. In addition, the results of this study indicate that this method has the ability to discriminate between the antioxidant abilities of a class of structurally related compounds.

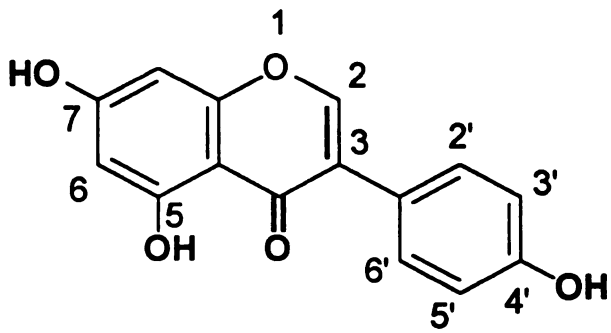


Figure 4.9. Structural criteria that enhance the antioxidant activity of isoflavonoids.

CHAPTER 5

MODULATION OF LIPOSOMAL MEMBRANE FLUIDITY BY FLAVONOIDS AND ISOFLAVONOIDS

5.1 ABSTRACT

The polyphenolic structures of flavonoids and isoflavonoids confer them with the ability to scavenge free radicals and to chelate transition metals, a basis for their potent antioxidant abilities. Another contributory mechanism towards their antioxidant activities could be their ability to stabilize membranes by decreasing membrane fluidity. In this study, the effects of representative flavonoids, isoflavonoids and their metabolites on membrane fluidity, and their preferential localization in the membrane were investigated using large unilamellar vesicles (LUVs) as the membrane models. These results were compared with cholesterol and α-tocopherol. Changes in fluorescence anisotropy values for the probes 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) and a series of *n*-(9-anthroyloxy) fatty acids (n = 6, 12, 16) upon addition of the test compounds were used to monitor alterations in membrane fluidity at graded depths in lipid bilayer. The results of the study suggested that the flavonoids and isoflavonoids, similar to cholesterol and α-tocopherol, partitioned into the hydrophobic core of the membrane and caused a dramatic decrease in lipid

fluidity in this region of the membrane. Localization of flavonoids and isoflavonoids into the membrane interiors and their restrictions on the fluidity of membrane components would limit accessibility of free radicals and thus decrease the kinetics of free radical reactions.

5.2 INTRODUCTION

Free radical peroxidation of unsaturated lipids in biomembranes disrupts the various important structural and protective functions associated with biomembranes, and various *in vivo* pathological events are implicated as a result of this oxidation (Barclay, 1993; Choe *et al.*, 1995; Niki *et al.*, 1991). The deleterious consequences of membrane peroxidation have stimulated numerous studies on the efficacies and mechanisms of action of biologically relevant antioxidants. A common structural criteria shared by the most potent membrane antioxidants is the presence of an aromatic nucleus with at least one phenolic hydroxyl group (Kagan *et al.*, 1990). It is the presence of an easily donatable hydrogen atom coupled with the stability of the resulting phenoxyl radical due to electron delocalization that contributes to the effectiveness of these phenolic compounds as prototypic chain-breaking, free radical scavenging antioxidants. Recent research, however, suggests that another factor contributing to the effectiveness of certain phenolic compounds as antioxidants is their degree of incorporation, uniformity of distribution and orientation in the membrane bilayer (Barclay *et al.*, 1990; Kaneko *et al.*, 1994; Niki *et al.*, 1985; Serbinova *et al.*, 1991).

Flavonoids, a class of naturally-occurring benzo-γ-pyrone compounds, are increasingly gaining recognition for their ability to inhibit lipid peroxidation in biological membranes (Chen *et al.*, 1996; Ioku *et al.*, 1995; Ramanathan and Das, 1992; Ratty and

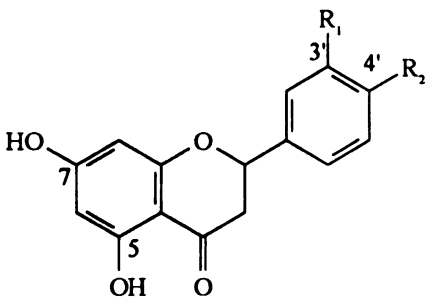
Das, 1988; Terao *et al.*, 1994). The mechanism of antioxidant action for these compounds has not yet been fully elucidated and is still a matter of considerable debate. As a consequence of their polyphenolic structure, these compounds are considered effective scavengers of peroxyl (Ioku *et al.*, 1995; Montesinos *et al.*, 1995; Torel *et al.*, 1986), hydroxyl (Hanasaki *et al.*, 1994; Rekka and Kourounakis, 1991; Sanz *et al.*, 1994) and superoxide (Cotelle *et al.*, 1996; Cotelle *et al.*, 1992 Hu *et al.*, 1995) radicals. This free radical scavenging mechanism has generally been ascribed as the basis of their antioxidant activity. In addition, flavonoids are known to chelate metal ions (Afanas'ev *et al.*, 1995; Afanas'ev *et al.*, 1989; Thompson and Williams, 1976), another possible contributory mechanism towards their antioxidant action. However, the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and fluidity of membranes has not been investigated as a possible antioxidant mechanism.

In order to better understand the antioxidant activities of flavonoids in biomembranes, it is important to determine the precise location of these compounds in membranes and to examine their effects on membrane fluidity and packing order. α-Tocopherol, the major lipid-soluble, chain-breaking antioxidant in biological membranes, is known to act as an antioxidant partly through stabilization of biological membranes by restricting the molecular mobility of their components (Kagan *et al.*, 1990; Niki *et al.*, 1985; Urano *et al.*, 1990; Urano *et al.*, 1988). Similar observations have also been reported for the potent anticancer drug tamoxifen and for cholesterol (Clarke *et al.*, 1990; Wiseman *et al.*, 1993; Wiseman *et al.*, 1990).

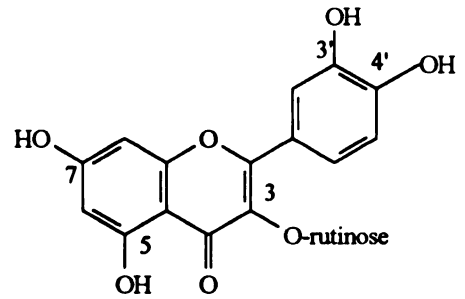
The aim of the present study was to investigate the effects of a range of structurally representative flavonoids and isoflavonoids on the fluidity of liposomal membranes and to

ascertain their precise location in the lipid bilayer. Of the different types of liposomes available, large unilamellar vesicles (LUVs) were selected as the model system in the study since they are the most representative of real biological membranes. Representative flavonoid and isoflavonoid samples exhibiting a broad multiplicity in structure were included in the study. The flavanones naringenin and hesperetin and the flavone glycoside rutin were selected to represent the flavonoids (Figure 5.1). For the subgroup of isoflavonoids, the isoflavone aglycones genistein and biochanin A and the glucosides genistin and daidzin were examined for their effects on membrane fluidity (Figure 5.2). As these compounds are rapidly degraded to other compounds in the human body, it is meaningful to include their metabolites in studies on biomembrane stabilization. Genistein metabolite dihydrogenistein and daidzein metabolites dihydrodaidzein, equol and 4-hydroxy equol were included in the study. 5-hydroxy equol, a possible genistein metabolite that has not been detected in human urine to date, was also examined for its effects on membrane fluidity (Figure 5.2). The effects of flavonoids and isoflavonoids on membrane fluidity were compared against α-tocopherol and cholesterol (Figure 5.1).

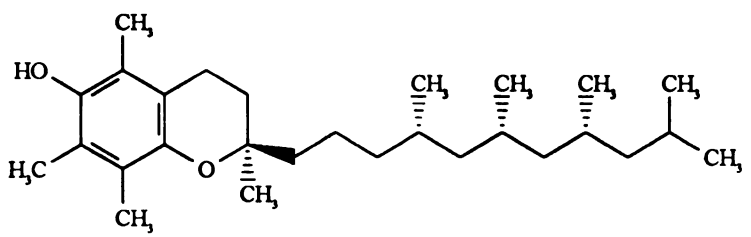
The localization of flavonoids and isoflavonoids in membranes and their effects on membrane fluidity were studied by fluorescence anisotropy. Fluorescence anisotropy measurement of fluorescent probes is one of the most widely used techniques in study of the influence of different molecules on membrane biophysical properties (Jemiola-Rzeminska *et al.*, 1996). A series of *n*-(9-anthroyloxy) fatty acids with the anthroyloxy moiety covalently linked at various positions along the alkyl chains were selected as the fluorescent probes in this study. As the position of the fluorophore is fixed along the fatty acid acyl chain for these



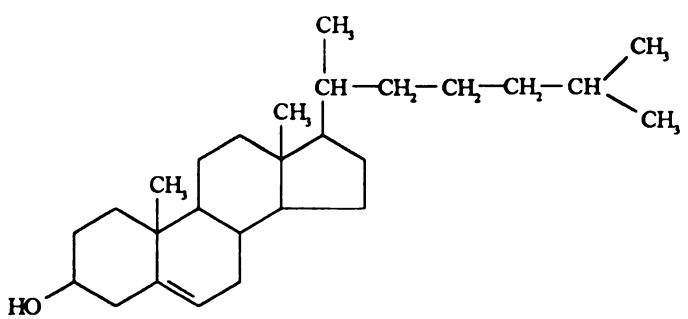
R_1 = H, R_2 = OH Naringenin
R_1 = OH, R_2 = OCH_3 Hesperetin



Rutin

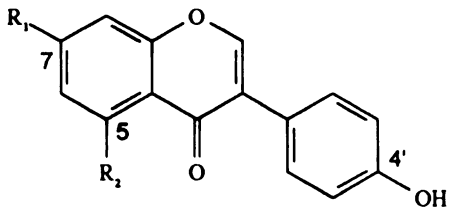


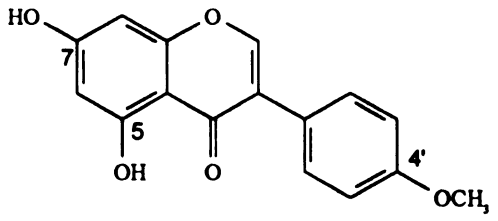
α-Tocopherol



Cholesterol

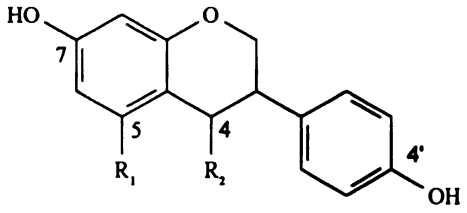
Figure 5.1. Structures of naringenin, hesperetin, rutin, α-tocopherol and cholesterol.



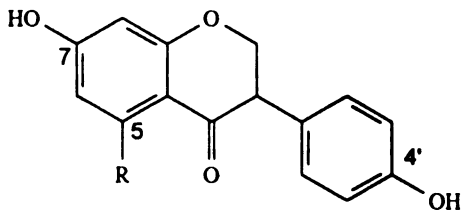


R_1 = OH, R_2 = OH Genistein
R_1 = O-glu, R_2 = OH Genistin
R_1 = O-glu, R_2 = H Daidzin

Biochanin A



R_1 = H, R_2 = H Equol
R_1 = H, R_2 = OH 4-Hydroxy Equol
R_1 = OH, R_2 = H 5-Hydroxy Equol



R = H Dihydrodaidzein
R = OH Dihydrogenistein

Figure 5.2. Structures of the isoflavonoids and their metabolites included in the study.

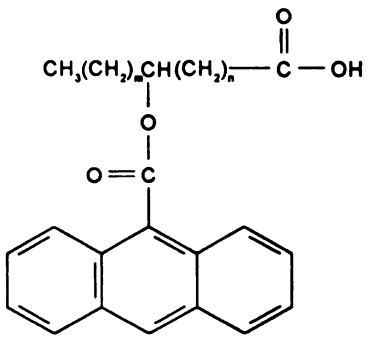
probes, their location in the lipid bilayer is well defined (Garrison *et al.*, 1994). Previous studies have confirmed that the *n*-(9-anthroyloxy) fatty acids fit into the bilayer with the acyl chains parallel to those of the phospholipids and with their anthroyloxyl moieties located at a graded series of depths in the bilayer, as revealed by energy transfer, NMR and fluorescence quenching studies (Mason, 1994; Tricerri *et al.*, 1994).

In the study, the fluorescent probes employed for monitoring changes in membrane fluidity at graded depths in the phospholipid bilayer were the 6-(9-anthroyloxy)stearic acid (6-AS), 12-(9-anthroyloxy)stearic acid (12-AS), 16-(9-anthroyloxy)palmitic acid (16-AP) and 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA) (Figure 5.3). The anionic charge of DPH-PA ensures that this probe labels the lipid-water interfacial region of the membrane bilayer and reports on fluidity changes near the membrane surface; whereas the anthroyloxyl moieties included in the study are linked to carbons 6, 12 and 16 of the base fatty acids and report on fluidity changes at defined depths within the membrane bilayer. As each of the four fluorescence probes labels a different region of the membrane, the information obtained with them is complementary.

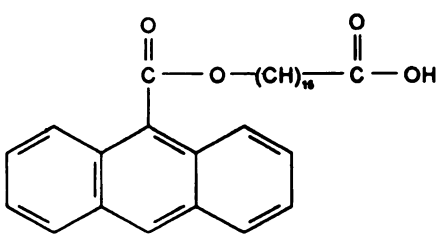
5.3 EXPERIMENTAL PROCEDURES

5.3.1 Materials

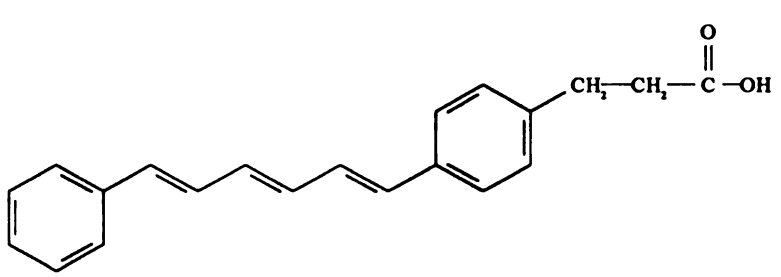
The fluorescent probes 3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid (DPH-PA), 16-(9-anthroyloxy)palmitic acid (16-AP), 6-(9-anthroyloxy)stearic acid (6-AS) and 12-(9-anthroyloxy)stearic acid (12-AS) were purchased from Molecular Probes (Eugene, OR).



n = 4, m = 11 6-(9-anthroyloxy)stearic acid
n = 10, m = 5 12-(9-anthroyloxy)stearic acid



16-(9-anthroyloxy)palmitic acid



3-(*p*-(6-phenyl)-1,3,5-hexatrienyl)phenylpropionic acid

Figure 5.3. Structures of the fluorescent probes employed in the study.

The purity of the *n*-(9-anthroyloxy) fatty acids was confirmed by silica gel thin-layer chromatography using ethanol/water (95:5, v/v) as solvent followed by visualization with iodine (Mason, 1994). Synthetic 1-stearoyl-2-linoleoyl-*sn*-glycero-3-phosphocholine (SLPC) was obtained from Avanti Polar Lipids (Alabaster, AL) and its purity checked by silica gel thin-layer chromatography employing two different solvent systems (chloroform / methanol / water : 65/25/4 and chloroform / methanol / water : 65/25/4) followed by sulfuric acid charring.

The isoflavone glycosides were purified from soy molasses, and the aglycones and their metabolites were obtained by synthesis (Chang *et al.*, 1994; Chang *et al.*, 1995). Rutin was purchased from Sigma Chemical Co. (St. Louis, MO) and the other flavonoids were from Indofine Chemical Co., Inc. (Somerville, NJ). Cholesterol (99%) was from Sigma Chemical Co. (St. Louis, MO) and α-tocopherol (99%) was donated by Henkel Corporation (La Grange, IL).

The lipid stock solutions, dissolved in chloroform, were maintained at -20^{0}C in amber glass vials that were layered with nitrogen. The fluorescent probe stock solutions were prepared in N,N-dimethylformamide and the flavonoid and isoflavonoid stocks were prepared in dimethyl sulfoxide (DMSO). Cholesterol and α-tocopherol stock solutions were in ethanol and methanol respectively. All the stock solutions were stored under nitrogen at -20^{0}C.

5.3.2 Preparation of Large Unilamellar Vesicles

LUVs containing the probe DPH-PA were prepared fresh for each day of experimentation using the extrusion procedure of MacDonald *et al.* (1991). Briefly, a mixture

containing 10 μmol of SLPC and 40 nmol of the fluorescent probe to be used was dried under vacuum onto the wall of a 15 mL round-bottomed flask to yield a probe to lipid molar ratio of 1:250. At this mole ratio, the fluorescence intensity for the four probes was well within their linear concentration ranges and no inner filter effects were displayed. The dried lipid and probe mixture was dispersed in 1 mL of a solution containing 0.15 M NaCl, 0.01 M MOPS (pH 7.0) and 0.1 mM EDTA. After 10 freeze-thaw cycles using a dry ice/ethanol bath, the suspension was passed 29 times through two stacked polycarbonate filters (pore size 100 nm) using a Liposofast extruder apparatus (Avestin, Ottawa, Canada). During the preparation steps, the lipid and probe were shielded from light and oxygen as much as possible. As the fluorescent probes were present in the dried lipid films when they were hydrated, it can be assumed they were symmetrically incorporated into both leaflets of the phospholipid bilayers.

5.3.3 Fluorescence Anisotropy Measurements

Steady-state fluorescence emission anisotropy measurements were obtained with a SLM Instruments, Model 4800, spectrofluorometer (Urbana, IL) equipped with a xenon light source, a thermostated cuvette holder and rotatable polarizers. The samples were excited with vertically polarized light at 384 nm (slit width 2 nm), and vertical and horizontal components of the sample fluorescence, $I_{\parallel}$ and $I_{\perp}$, emitted through optical filters (KV 418, Schott, Duryea, PA), were detected in the T-format. The steady-state anisotropy, r_s, was calculated as:

$$r_s = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescent intensities of the vertically ($\parallel$) and horizontally ($\perp$) polarized emission when the sample is excited with vertically polarized light (Lakowicz, 1983). The contribution of scattered light to the fluorescent emission was determined using an unlabeled reference solution of the same composition excited with vertically polarized light and detection of the emitted light with the polarizer at 55^0. The ratio of this signal to that determined for membranes containing the probe gave the fraction of signal resulting from light scattering. At the lipid concentrations employed, the contribution of scattered light to the total fluorescence emission was negligible and no corrections were made to the anisotropy values.

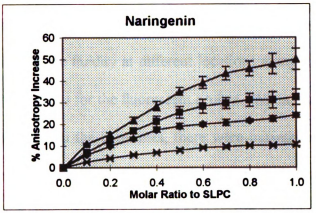
For fluorescence anisotropy measurements, a 20 μL aliquot of the liposome suspension was diluted to 2 mL in buffer containing 100 mM NaCl and 50 mM Tris-HEPES (pH 7.0) to achieve final concentrations of 100 μM lipid and 400 nM fluorescent probe. Cuvette temperature was maintained at 22^0C with a circulating water bath. For each compound to be tested, different volumes of the stock solutions were sequentially titrated into the cuvette and changes in anisotropy values for each probe as a function of concentration of the test compound were determined. During the titrations, there was a 5 min incubation period following each addition of test sample to allow the compound to partition into the membrane. Control experiments were conducted with addition of equivalent volumes of each of the blank solvents and found to have no effect on the anisotropy values.

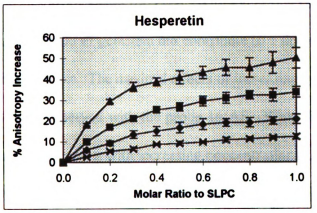
5.4 RESULTS

5.4.1 Effects of Flavonoids on Membrane Fluidity

The effects of the flavonoids naringenin, hesperetin and rutin on membrane fluidity along graded depths in the phospholipid bilayer are presented in Figure 5.4. Data are reported as increases in the steady-state anisotropy parameters for the four probes used in the study; an increase in the anisotropy parameter of a probe being indicative of a decrease in the fluidity of the membrane. The two flavanones naringenin and hesperetin displayed similar effects on the fluidity of the membrane. In both cases, only a marginal increase in the anisotropy parameter for the probe DPH-PA was observed with increasing concentrations of these flavanones. This suggested that naringenin and hesperetin were not influencing membrane fluidity properties in the exterior region of the membrane. As the anthroyloxy fluorophore was shifted along the acyl chain towards the interior of the membrane, the influence of naringenin and hesperetin on membrane fluidity increased. The greatest restriction of membrane mobility with naringenin and hesperetin was noted in the interior regions of the membrane labeled by the probe 16-AP. For the flavone rutin, a linear increase in anisotropy values of 16-AP as a function of rutin concentration was observed, also demonstrating the strong influence of flavonoids on membrane fluidity in the interior of the membrane. The influence of rutin on membrane fluidity diminished considerably as the fluorophore position shifted to the exterior regions of the membrane.

Figure 5.4. Effects of flavonoids on membrane fluidity as studied by increases in anisotropy values of the fluorescent probes DPH-PA (x), 6-AS (◆), 12-AS (■) and 16-AS (▲) at 22^{0}C. Measurements were carried out as described under Experimental Procedures. Values represent the means of triplicate measurements.


Naringenin
% Anisotropy Increase
60
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
Molar Ratio to SLPC


Hesperetin
% Anisotropy Increase
60
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
Molar Ratio to SLPC

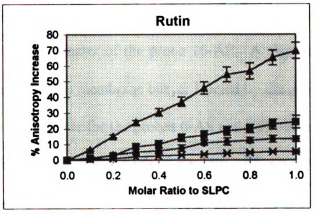

Rutin
% Anisotropy Increase
80
70
60
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
Molar Ratio to SLPC

Figure 5.4

5.4.2 Effects of Isoflavones on Membrane Fluidity

Figure 5.5 illustrates the effects of the isoflavones genistein, genistin, daidzin and biochanin A on membrane fluidity at different locations along the bilayer, as revealed by changes in anisotropy values for the fluorescent probes. When increasing concentrations of genistein were titrated into the liposomes, there were minimal changes in the anisotropy values of the probes DPH-PA and 6-AS. This indicated that incorporation of genistein had little effect on the dynamics of the external regions of the membrane bilayer. There was a moderate increase in anisotropy values for 12-AS with increasing concentrations of genistein suggesting that incorporation of genistein did contribute to a decrease in membrane fluidity in this region of the membrane. The maximal effects of increasing genistein concentration on anisotropy values were observed with the probe 16-AS. With 16-AS, there was almost a linear increase in anisotropy values with increasing concentrations of genistein; evidence that genistein was causing a strong membrane rigidifying effect in the interior of the bilayer.

Introduction of increasing amounts of genistin, the 7-β-glucoside of genistein, also resulted in a maximal reduction in fluidity in the membrane interior, as indicated by the increase in anisotropy parameter of the probe 16-AP. A slight reduction in fluidity was observed in the region of the membrane bilayer labeled by the probe 12-AS. On the other hand, the anisotropy parameters for the probes 6-AS and DPH-PA showed a slight decrease in value when increasing amounts of genistin were titrated into the liposomes.

The glycoside daidzin showed similar patterns to genistin. The incorporation of daidzin into the membrane bilayer resulted in a linear increase in anisotropy values of 16-AP, though the increase was somewhat smaller than that observed with genistin. Again, the

Figure 5.5. Effects of isoflavonoids on membrane fluidity as studied by increases in anisotropy values of the fluorescent probes DPH-PA (x), 6-AS (◆), 12-AS (■) and 16-AS (▲) at 22°C. Measurements were carried out as described under Experimental Procedures. Values represent the means of triplicate measurements.

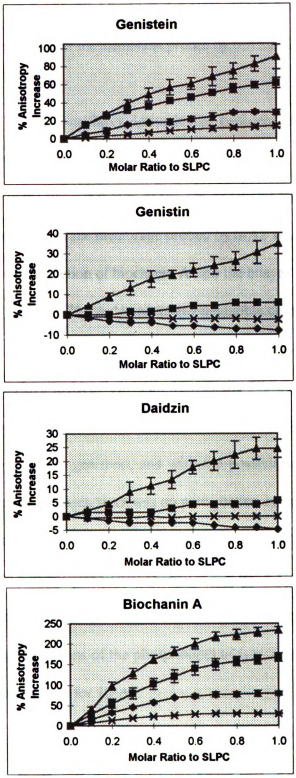

Genistein
% Anisotropy Increase
100
80
60
40
20
0
0.0
0.2
0.4
0.6
0.8
1.0
Molar Ratio to SLPC
Genistin
% Anisotropy Increase
40
30
20
10
0
-10
0.0
0.2
0.4
0.6
0.8
1.0
Molar Ratio to SLPC
Daidzin
% Anisotropy Increase
30
25
20
15
10
5
0
-5
0.0
0.2
0.4
0.6
0.8
1.0
Molar Ratio to SLPC
Biochanin A
% Anisotropy Increase
250
200
150
100
50
0
0.0
0.2
0.4
0.6
0.8
1.0
Molar Ratio to SLPC

Figure 5.5

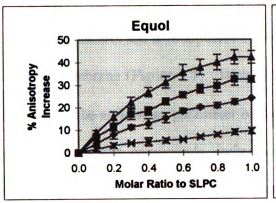
region probed by the probe 12-AS showed a negligible reduction in membrane fluidity, whereas the external regions of the membrane probed by 6-AS and DPH-PA showed no effect or a slight increase in fluidity.

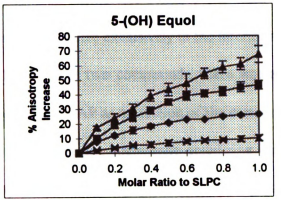
Biochanin A, the 4'-methoxy derivative of genistein, demonstrated the strongest influence on membrane fluidity. Incorporation of biochanin A into the liposomes led to strong decreases in fluidity in the interior region of the bilayer labeled by the probes 16-AP and 12-AS. In contrast, the region of the membrane probed by 6-AS was affected to a considerably lower degree by the introduction of biochanin A into the bilayer. The superficial regions of the membrane probed by DPH-PA demonstrated negligible changes in membrane fluidity.

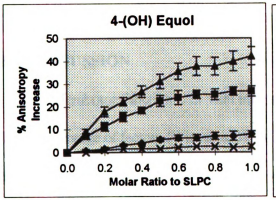
5.4.3 Effects of Isoflavone Metabolites on Membrane Fluidity

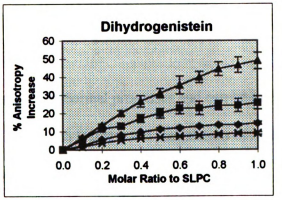
Similar trends were obtained with the isoflavone metabolites equol, 4-hydroxy equol, dihydrodaidzein and dihydrogenistein, and with the possible metabolite 5-hydroxy equol (Figure 5.6). In each of the cases, there was no perturbation or very little perturbation to the superficial region of the membrane labeled by the probe DPH-PA. As the fluorophore was moved from position 6 to position 16 along the fatty acid chain, the effects of these isoflavone derivatives on membrane fluidity increased accordingly. Maximal reduction in fluidity was observed in the interior regions of the phospholipid bilayer, as evidenced by the substantial increase in anisotropy values for 16-AP.

Figure 5.6. Effects of isoflavonoid metabolites on membrane fluidity as studied by increases in anisotropy values of the fluorescent probes DPH-PA (x), 6-AS (◆), 12-AS (■) and 16-AS (▲) at 22°C. Measurements were carried out as described under Experimental Procedures. Values represent the means of triplicate measurements.


Equol
% Anisotropy Increase
50
40
30
20
10
0
0.0 0.2 0.4 0.6 0.8 1.0
Molar Ratio to SLPC


5-(OH) Equol
% Anisotropy Increase
80
70
60
50
40
30
20
10
0
0.0 0.2 0.4 0.6 0.8 1.0
Molar Ratio to SLPC


4-(OH) Equol
% Anisotropy Increase
50
40
30
20
10
0
0.0 0.2 0.4 0.6 0.8 1.0
Molar Ratio to SLPC


Dihydrogenistein
% Anisotropy Increase
60
50
40
30
20
10
0
0.0 0.2 0.4 0.6 0.8 1.0
Molar Ratio to SLPC

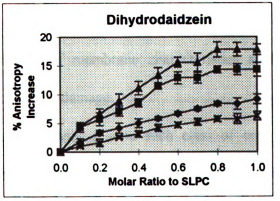

Dihydrodaidzein
% Anisotropy Increase
20
15
10
5
0
0.0 0.2 0.4 0.6 0.8 1.0
Molar Ratio to SLPC

Figure 5.6

5.4.4 Effects of Cholesterol and α-Tocopherol on Membrane Fluidity

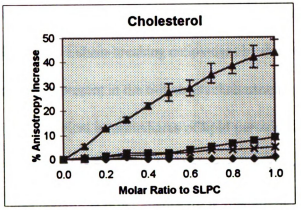
Cholesterol and α-tocopherol appeared to localize exclusively in the interior regions of the membrane (Figure 5.7). With either of these two compounds, there was a linear increase in the anisotropy parameter of the probe 16-AP as a function of the concentration of these two test compounds, indicative of the strong membrane stabilizing effect of cholesterol and α-tocopherol in the hydrophobic core of the membrane. In strong contrast, both of the compounds trivial effects on the fluidity of the membrane in the domains probed by 12-AS, 6-AS and DPH-PA.

5.5 DISCUSSION

Membrane function is of vital importance to normal processes and can be influenced by a wide range of factors (Wiseman, 1996). One such factor is the modulation of membrane function by dietary components such as vitamin E (Bisby, 1990; Morrissey *et al.*, 1994), vitamin D (Wiseman, 1993), vitamin C (Halliwell and Gutteridge, 1989; Thurnham, 1994), β-carotene (Halliwell and Gutteridge, 1989; Thurnham, 1994), flavonoids (Chen et al., 1996; Saija *et al.*, 1995) and isoflavonoid-type phytoestrogens (Jha et al., 1985; Messina *et al.*, 1994) through alteration of membrane characteristics such as fluidity, stability, and susceptibility to peroxidative damage.

The antioxidant activity of one such class of compounds, the flavonoids and isoflavonoids, in membranes is well established. In contrast, there are no available data on the precise location of these compounds in membranes and on their consequent effect on membrane integrity. An understanding of the effects of flavonoids and isoflavonoids on

Figure 5.7. Effects of cholesterol and α-tocopherol on membrane fluidity as studied by increases in anisotropy values of the fluorescent probes DPH-PA (x), 6-AS (◆), 12-AS (■) and 16-AS (▲) at 22°C. Measurements were carried out as described under Experimental Procedures. Values represent the means of triplicate measurements.


Cholesterol
% Anisotropy Increase
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
Molar Ratio to SLPC

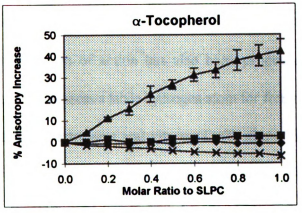

α-Tocopherol
% Anisotropy Increase
50
40
30
20
10
0
-10
0.0
0.2
0.4
0.6
0.8
1.0
Molar Ratio to SLPC

Figure 5.7

biophysical properties of membranes may help to better elucidate their mechanism of action as antioxidants. Other prototypic free radical scavenging antioxidants have been shown to act as membrane stabilizers partly through a restriction of membrane fluidity. α-tocopherol is generally believed to act as a chain-breaking antioxidant by donating a hydrogen atom from the phenolic hydroxyl group present in the 6-hydroxychromane ring to the chain-propagating lipid peroxyl and alkoxyl radical intermediates of lipid peroxidation, thereby producing the stable tocopheroxyl radical and terminating the chain reaction (Liebler, 1993). In addition to its chain-breaking antioxidant action, a structural membrane antioxidant action for α-tocopherol mediated by the hydrocarbon chain through decreased membrane fluidity has also been demonstrated (Kagan *et al.*, 1990; Urano *et al.*, 1992; Urano *et al.*, 1990; Urano *et al.*, 1988).

A similar mechanism of action has also been proposed for the anticancer drug tamoxifen, which does not possess a labile hydrogen atom for free radical scavenging, and for 4-hydroxy tamoxifen and estrogens and vitamin D (Clarke *et al.*, 1990; Wiseman *et al.*, 1993). Though the rest of these compounds possess a potentially donatable hydrogen atom, time-course data indicate that they are not predominantly chain-breaking antioxidants (Lacort *et al.*, 1995; Ruiz-Larrea *et al.*, 1994; Wiseman *et al.*, 1990). For all these compounds, a structural antioxidant action has been proposed based on their ability to mimic the membrane stabilization against lipid peroxidation demonstrated by the structurally similar endogenous membrane sterol cholesterol (Wiseman, 1994).

The purpose of the present study was to investigate the effects of flavonoids and isoflavonoids on lipid fluidity in model membranes. The term "lipid fluidity" refers to the

relative motional freedom of the lipid molecules in the membrane bilayer (Jourd'heuil *et al.*, 1993). Probes such as DPH-PA possess a relatively high limiting hindered anisotropy, and it is hypothesized that membrane order plays an important role in determining the motional freedom of these probes. Hence, the use of such probes allows an estimation of the static component of membrane fluidity (Schachter, 1984). In contrast, probes such as the anthroyloxy fatty acid derivatives possess a relatively low value for hindered anisotropy, and the anisotropy parameter for these probes reflects primarily their rotational movement, i.e., a dynamic component of membrane fluidity (Thulborn and Sawyer, 1978).

By the use of DPH-PA, 6-AS, 12-AS and 16-AP as the fluorescent probes in our study, it was possible to determine fluidity of the membrane as a function of depth within the bilayer. Our results suggest that all the flavonoids, isoflavonoids and their metabolites examined in the study partitioned preferentially into the hydrophobic core of the model membrane, where they modified the lipid packing order. These compounds decreased membrane fluidity in a concentration dependent manner in the interior region of the membrane labeled by the probe 16-AP. As the fluorophore position was moved from position 16 to position 6 on the fatty acyl chain, these molecules appeared to have a smaller effect on the lipid packing order. Similarly, the superficial regions of the membrane probed by DPH-PA were not influenced by introduction of flavonoids and isoflavonoids into the bilayer.

The ability of flavonoids and isoflavonoids to influence membrane fluidity and their preferential localization in the membrane were contrasted against the known membrane stabilizers cholesterol and α-tocopherol. Like the flavonoids and isoflavonoids, cholesterol and α-tocopherol had a strong, dose-dependent effect on the fluidity of the hydrophobic core

of the membrane, as revealed by the increase in anisotropy values for 16-AP. However, while most of the flavonoids and isoflavonoids examined did exert some influence on lipid packing order in other regions of the bilayer, cholesterol and α-tocopherol exclusively partitioned into the internal regions of the membrane.

Other studies have indicated that, for α-tocopherol, the chromanol moiety fits into the "space" formed by the polyunsaturated phospholipids of the membrane bilayer and the isoprenoid side chain is embedded in the membrane interior to retain the molecule in the lipid core (Urano *et al.*, 1993; Urano *et al.*, 1992; Urano *et al.*, 1990). From our data, we can conclude that α-tocopherol does not have much impact on the membrane mobility properties in the exterior regions of the bilayer, but the isoprenoid side chain considerably restricts mobility in the core of the lipid bilayer. Similarly, cholesterol is believed to act as a membrane stabilizer via interactions between its hydrophobic rings and the saturated, monounsaturated and polyunsaturated residues of phospholipid fatty acids (Wiseman, 1996; Wiseman, 1994), and would thereby be expected to exert its strongest influence in the interior regions of the membrane as was observed in this study.

Since the flavonoids and isoflavonoids mimicked the membrane stabilizing effects of cholesterol and α-tocopherol, this mechanism may partly account for their antioxidant activities, as has been previously confirmed for cholesterol and α-tocopherol. By imposing a greater degree of structural order and rigidity to the membrane, the flavonoids and isoflavonoids could reduce the mobility of free radicals in the lipid bilayer. Consequently, the decreased membrane fluidity would result in inhibition of lipid peroxidation due to a slow-down of free radical reactions.

A previous study indicated that the ability of flavonoids to interact with and penetrate the lipid bilayer influenced their antioxidant capabilities in biomembranes (Saija *et al.*, 1995). Comparing the flavonoids quercetin, rutin, hesperetin and naringenin, these researchers demonstrated that the ability of these compounds to shift the main transition peak temperature for dipalmitoylphosphatidylcholine (DPPC) liposomes to lower values was related to their ability to inhibit peroxidation in rat cerebral membranes. The results obtained by these researchers were in direct contrast to our study. These researchers observed that the introduction of flavonoids into the liposomes caused a membrane fluidifying effect. The different results for the two studies are probably a consequence of differences in the lipid substrates employed. The authors of the previous study had used the highly saturated DPPC as their lipid substrate, whereas we employed the more unsaturated SLPC that has a composition that is truly representative of phospholipids in biological membranes, with a saturated fatty acid at the *sn*-1 position, an unsaturated fatty acid at the *sn*-2, and a phosphate-containing polar group at the *sn*-3 position (Stanley, 1991).

Another flavonoid, diosmetin, has previously been shown to exert a protective effect against *in vitro* cell membrane damage and oxidative stress in cultured rat hepatocytes (Villa *et al.*, 1992). This protective effect of diosmetin towards cell membranes was attributed in part to its ability to stabilize the structure of biological membranes by modifying fluidity of the lipid bilayer.

In most of these studies, it has been assumed that flavonoids and isoflavonoids are localized near the lipid-water interface of the membrane. The precise location of these compounds in the membrane has not been examined in any of these studies and would be

crucial for fully understanding the role of flavonoids and isoflavonoids in stabilizing the membrane, both through chemical and physical means. In contrast to the generally held belief, our results suggest that despite the presence of polar substituents, flavonoids and isoflavonoids partition preferentially into the hydrophobic core of the membrane, where they exert a membrane stabilizing effect by modifying the lipid packing order.

On the surface, such a conclusion appears highly improbable since it would involve the unfavorable localization of polar hydroxyl groups in a very hydrophobic environment. However, this may not be the case as the final conformation of these compounds could be influenced by various other factors such as the strength of the hydrophobic interactions between these compounds and the phospholipids, and also their ability to form intramolecular and intermolecular hydrogen bonds.

Summarizing, our results demonstrate that flavonoids and isoflavonoids partition preferentially into the hydrophobic core of membranes. They stabilize the membrane through a decrease in lipid fluidity, and this may be a contributory mechanism towards their known ability to inhibit membrane peroxidation.

CHAPTER 6

OXIDATION PRODUCTS OF GENISTEIN FORMED BY REACTION WITH ALKYLPEROXYL RADICALS

6.1 ABSTRACT

To facilitate a better understanding of genistein antioxidant chemistry, oxidation studies were conducted with genistein and alkylperoxyl radicals generated by thermal decomposition of an azo compound in a homogenous system. The oxidation products of genistein were separated and analyzed by reversed-phase HPLC. Successive analyses of the reaction mixture over a 24 h time period indicated a gradual disappearance of genistein, and the appearance of two major oxidation products. By ^{1}H-NMR and MS analyses, one of the products was assigned the structure 2-dehydro-*o*-demethyl angolensol, a C-ring-cleavage product of genistein. Although an unambiguous chemical structure could not be assigned to the second oxidation product, ^{1}H-NMR analysis indicated that genistein was undergoing opening at the C-ring to yield this oxidation product. The structural identification of these two products could provide biochemical markers of oxidation of genistein by peroxyl radicals in biological systems.

6.2 INTRODUCTION

In recent years, the potential role of soybeans in cancer prevention has received a lot of attention. Epidemiological data indicate that consumption of soybean-containing diets is associated with the lower incidence of certain human cancers in Asian compared to Caucasian populations (King and Locke, 1980; Locke and King, 1980; Setchell *et al.*, 1984). It is hypothesized that many of the anticancer properties of soy could be attributable to their isoflavone content (Messina *et al.*, 1994; Messina and Barnes, 1991; Messina and Messina, 1991).

Genistein, the most abundant soy isoflavone, possesses a wide range of biological properties that could contribute to the possible anticancer abilities of soybeans. Some of these properties include its potent inhibition of protein tyrosine kinases (Akiyama *et al.*, 1987), DNA topoisomerases I and II (Okura *et al.*, 1988), and ribosomal S6 kinase (Linassier *et al.*, 1990). The antioxidant ability of genistein is also believed to be linked to its anticancer properties. Genistein has been reported to inhibit tumor promoter-induced formations of hydrogen peroxide *in vivo* and *in vitro* in mouse skin (Wei *et al.*, 1993). Other studies have demonstrated the antioxidant abilities of genistein in a coupled linoleic acid/β-carotene system (Pratt and Birac, 1979), in a liposomal system against UVA and UVB or peroxyl-radical induced peroxidation (Record *et al.*, 1995), and in a microsomal system using Fe(II)/ADP/NADPH (Jha *et al.*, 1985).

Genistein is thought to act as a chain-breaking antioxidant through its demonstrated ability to scavenge hydroxyl, superoxide and peroxyl radicals (Record *et al.*, 1995; Wei *et al.*,

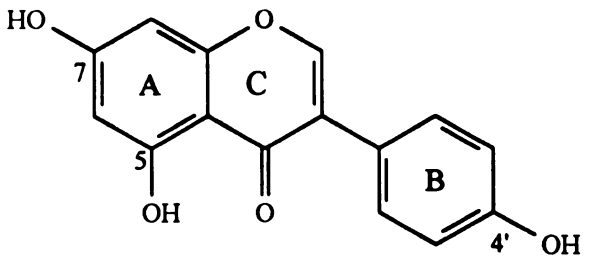
1995; Wei *et al.*, 1993). Since kinetic, spin-trapping and product analysis studies indicate that the principal chain carrier of lipid peroxidation is the peroxyl radical (Matsuo *et al.*, 1989), the ability of genistein to scavenge peroxyl radicals would be critical towards its biological relevance as an antioxidant. The structure of genistein (Gen-H) confers it with three potentially donatable hydrogen atoms for radical scavenging (Figure 6.1). Lipid peroxyl radicals ($ROO^{\cdot}$) can react with genistein via abstraction of a hydrogen radical to yield lipid hydroperoxides (ROOH) and the resonance-stabilized genistein phenoxyl radical ($Gen^{\cdot}$) (Equation 1).

$$\text{Gen-H} + ROO^{\cdot} \rightarrow \text{ROOH} + Gen^{\cdot} \quad (1)$$

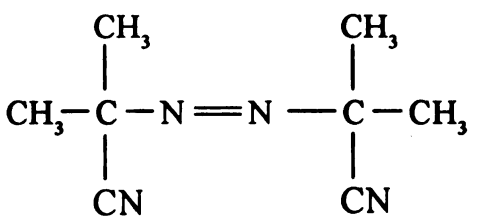
The genistein phenoxyl radical may react further with other peroxyl radical to yield a variety of non-radical products (Equation 2).

$$\text{Gen.} + ROO^{\cdot} \rightarrow \text{non-radical products} \quad (2)$$

Further understanding of this chain-breaking antioxidant mechanism for genistein requires a knowledge of the products formed by the reaction between genistein and peroxyl radicals. Characterization of these products could provide useful markers for genistein antioxidant chemistry. So far, however, no reports have been published on the structural determination of genistein oxidation products.



Genistein



2,2'-Azobis(isobutyronitrile)

Figure 6.1. Structures for genistein and 2,2'-azobis(isobutyronitrile).

The present investigation was undertaken to profile the products formed by reaction of genistein with alkylperoxyl radicals obtained by thermal decomposition of the lipophilic azo initiator 2,2'-azobis(isobutyronitrile) (Figure 6.1). Use of the azo initiator enabled us to generate initiating radicals at a known and constant rate.

6.3 EXPERIMENTAL PROCEDURES

6.3.1 Materials

Genistein was obtained by synthesis (Chang *et al.*, 1994). 2,2'-azobis(isobutyronitrile) (AIBN) was obtained from Wako Chemical Company (Richmond, VA). All the solvents used in the study were HPLC grade.

6.3.2 HPLC

Analytical reverse-phase HPLC experiments were performed on a Supelcosil LC-18 (5 μm, 250 x 4.6 mm) column with a Supelguard LC-18 guard column (Supelco, Bellefonte, PA) eluted with methanol/water (50:50, v/v) at a flow rate of 1.4 mL/min with continuous helium sparging. A Waters 991 photodiode array detector (Milford, MA) set at a range of 200-300 nm was used to detect the formation of oxidation products. Preparative HPLC was conducted on and LC-20 Recycling Preparative HPLC (Japan Analytical Instruments, Japan) with a C-18 reversed-phase column (Jaigel, S-343-15, 15 μm, 250 x 20 mm) using a mobile phase of methanol/water (70:30, v/v) at a flow rate of 4 mL/min and UV detection at 260 nm.

6.3.3 Spectroscopy

^{1}H-NMR spectra were recorded on a Varian VXR 300 spectrometer in CD_3OD solution at ambient temperature. Mass spectra were acquired on a JEOL HX-110 double focusing mass spectrometer (JEOL, Tokyo, Japan).

6.3.4 Oxidation Studies with Genistein

In experiments studying oxidative fate of genistein over time, 250 μg (925 nmol) of genistein and 20.5 mg (125 μmol) of AIBN were dissolved in 1 mL of oxygen-saturated acetonitrile and the mixture was heated in screw-capped vials at 50°C. At various time-points, 50 μL aliquots of the reaction mixture were removed to micro-centrifuge tubes for product analysis, and rapidly chilled by immersion in ice. The resulting AIBN crystals were removed by filtration, the supernatant dried under nitrogen and redissolved in the mobile phase for analytical HPLC analysis. Larger-scale oxidations contained 75 mg (278 μmol) genistein and 6.15 g (37.5 mmol) AIBN and were purified by preparative HPLC for collection of oxidation products for structural analysis.

6.4 RESULTS

6.4.1 Formation of Genistein Oxidation Products

Successive analyses of the genistein-AIBN oxidation products over time indicated a gradual disappearance of the genistein peak eluting at 12 min (peak G), concomitant with the appearance of other fractions. As indicated in the representative chromatograms in Figure

6.2, two major reaction products were observed by 8 h of oxidation. One of these two products eluted around 19 min (peak B) on the C-18 column, indicating that it was more non-polar than the reactants. The second product at a retention time of 3.5 min (peak A), eluted very close to the solvent front which indicated that it had higher polarity than genistein. Virtually all of the genistein was oxidized by the end of 20 h. Photo diode array detection revealed that both genistein and its two major oxidation products detected by HPLC displayed UV absorption maxima at 210 and 260 nm.

6.4.2 Chemical Structures of Oxidation Products

^{1}H NMR spectrum for compound **1** (CD_3OD) δ 8.20 (1H, s, H-1), 7.40 (2H, d, J = 9 Hz, H-2', H-6'), 6.40 (2H, d, J = 9 Hz, H-3', H-5'), 6.30 (1H, d, J = 1.5 Hz, H-6), 6.20 (1H, d, J = 1.5 Hz, H-7) and 3.40 (3H, s, OCH_3). A structure for compound **1** is given in Figure 6.3. It appears that ring C in genistein is cleaved with methoxylation of the 4'-hydroxyl of genistein to yield 2-dehydro-*o*-demethyl angolensol. This indicates that, during oxidation reactions with peroxyl radicals, genistein forms a monophenoxyl radical at the 4'-position. During work up of the reaction mixture, the phenoxyl radical may have reacted with methanol to yield the stable methoxy derivative **1**. The MS of Compound **1** did not give the molecular ion at m/z 302. However, a peak was observed at m/z 286 with 60% intensity which would result from cyclization to form a C ring with the concomitant loss of an oxygen atom (shown in the scheme in Figure 6.3). This is not unusual for compounds with this type of a structure. It is well known that chalcones undergo rapid cyclization to form flavones during MS

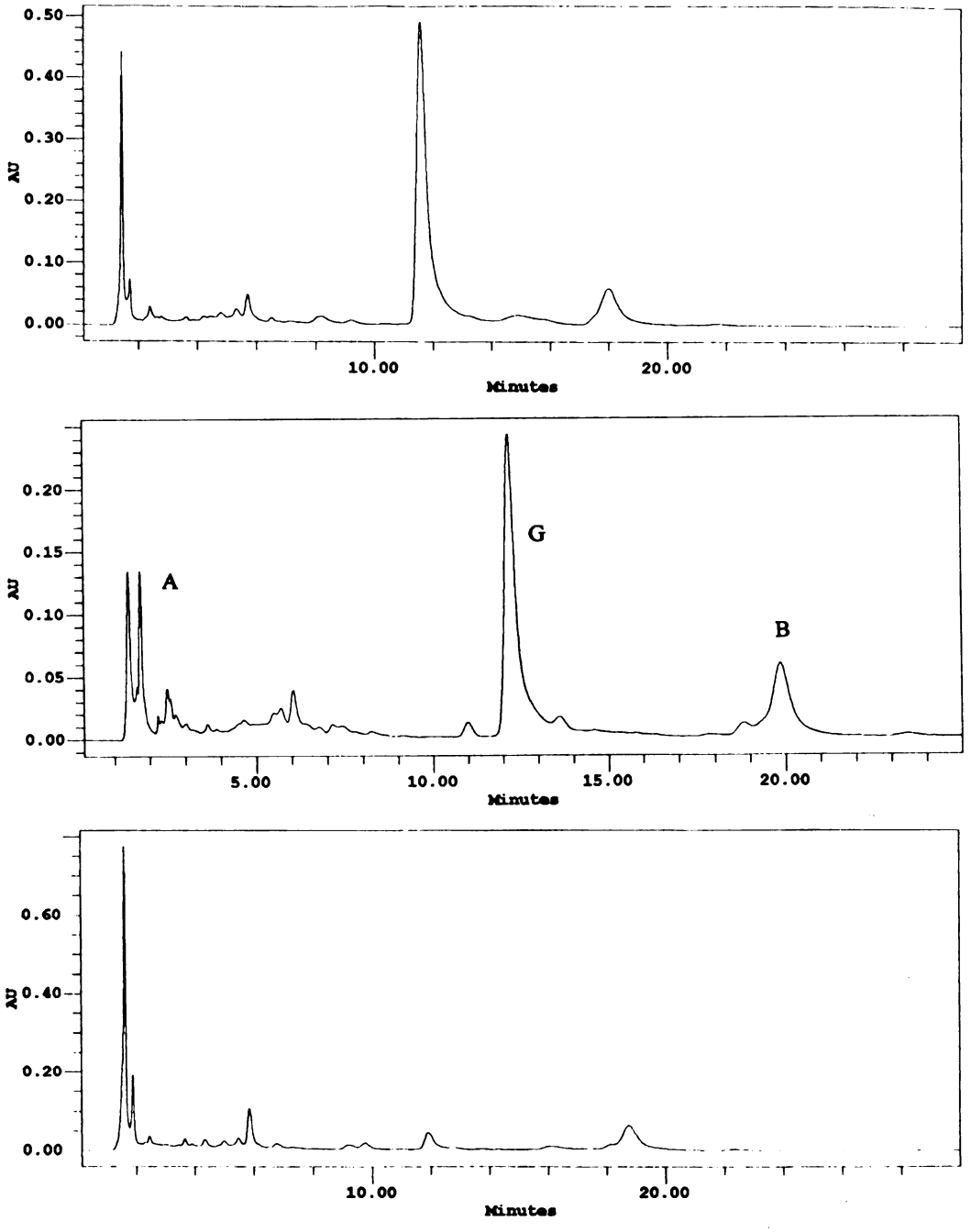
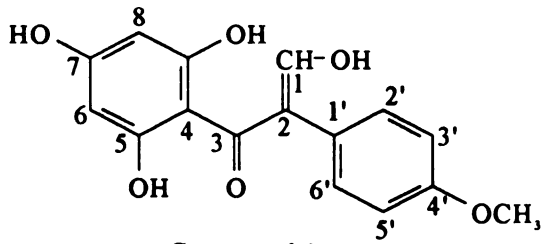
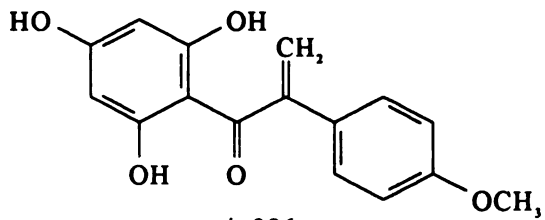


Figure 6.2. Reversed-phase HPLC analysis of products formed by reaction between genistein and AIBN-derived peroxyl radicals during incubation at 50°C for 3 h (top), 8 h (middle) and 20 h (bottom).

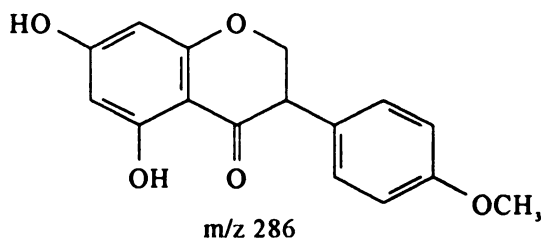


Compound 1
2-dehydro-*O*-demethyl angolensol
(m/z 302, not detected)

?

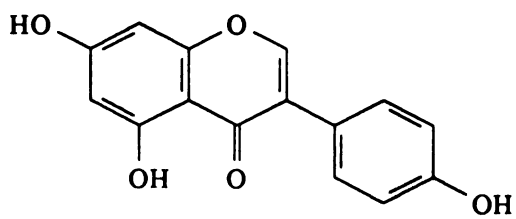


m/z 286
60%



m/z 286

?



m/z 270
100 %

Figure 6.3. Structure for Compound 1.

analyses. The base peak seen at m/z 270 was identical to genistein. The rest of the fragmentation pattern was similar to the MS fragmentation of genistein.

The second oxidation product was not collected in a sufficiently pure form to yield unambiguous structural identification. Preliminary NMR data indicates that oxidation of genistein resulted in the opening of ring C of genistein to yield this product, while rings A and B appeared to remain intact. Additional NMR experiments are necessary to determine the structure of this product.

6.5 DISCUSSION

Azo initiators such as AIBN decompose thermally to yield alkyl radicals ($R^{\cdot}$) which, in turn, react with molecular oxygen to form alkylperoxyl radicals ($ROO^{\cdot}$) (Equations 3 and 4).

$$R\text{-}N=N\text{-}R \rightarrow 2R^{\cdot} + N_2 \quad (3)$$

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \quad (4)$$

By use of such initiators, the generation of free radicals occurs at a known and specific rate, and at a specific site (Niki, 1990). Admittedly, such initiators are not representative of biological conditions. However, use of these chemically well-defined compounds can serve as kinetically reproducible oxidative challenges (Ham and Liebler, 1995).

The fate of genistein during reaction with peroxyl radicals has not been examined previously. As peroxyl radicals are recognized as being the primary chain carriers of

membrane peroxidation, profiling the stable end-products formed during the oxidation reactions of genistein with peroxyl radicals would lead to a better understanding of genistein antioxidant chemistry. Similar studies examining the oxidation products of α-tocopherol have greatly clarified the reaction pathways involved in α-tocopherol antioxidant chemistry (Liebler *et al.*, 1996; Liebler and Burr, 1995; Liebler and Burr, 1992; Liebler *et al.*, 1991; Winterle *et al.*, 1984; Yamauchi *et al.*, 1990; Yamauchi *et al.*, 1989).

The results from this study indicate that oxidation of genistein with azo derived alkylperoxyl radicals results in the formation of two major reaction products. It appears that ring C of genistein undergoes cleavage in both of these products, whereas rings A and B remain intact. Further advanced level NMR experiments are needed to yield unequivocal chemical structures for the genistein oxidation product detected by HPLC as peak A.

SUMMARY AND CONCLUSIONS

As consumer interest in the use of plant-derived antioxidants in food products is increasing, a systematic approach for rapid evaluation of plant extracts and purified compounds for antioxidant activity is required. This study was primarily aimed at utilizing the technique of fluorescence spectroscopy for development of assays to monitor lipid peroxidation and to evaluate the efficacy of antioxidants.

Two fluorescence spectroscopic assays were developed for following lipid peroxidation in membranes. The assays involved incorporation of the fluorescent probe diphenylhexatriene-propionic acid into unilamellar vesicles and monitoring changes in its intensity and anisotropy parameters as a function of time. The fluorescence intensity assay was based on the quenching of the intensity of the probe due to the free radicals generated during lipid peroxidation, whereas the fluorescence anisotropy assay reflected the decrease in membrane fluidity that occurred as a result of cross-linking reactions in lipid peroxidation. Validation studies using conjugated diene and hydroperoxide measurements confirmed that the fluorescence spectroscopic assays were accurately reflecting the progress of initial stages of peroxidation.

These new assays were used to evaluate the efficacies of metal chelating and free radical scavenging antioxidants. With the metal chelators, markedly different effects on rates

of lipid peroxidation were observed depending on the type of metal ion used to initiate lipid peroxidation and on the molar ratio of chelator to metal ions employed.

Flavonoids and isoflavonoids were selected as the representative class of prototypic, phenolic, free radical scavenging compounds. The abilities of these compounds to inhibit metal-ion-induced and free-radical-induced peroxidations were compared. Flavonoids and isoflavonoids exhibited superior antioxidant activities against metal-ion-induced peroxidations, suggesting that the metal chelation abilities of these compounds are a major contributor to their antioxidative abilities.

The results from our study confirmed earlier findings that the chemical structure of these compounds is a large determinant of their antioxidant activities. The B-ring substitution pattern was especially important, with hydroxyl group substitutions increasing activity and methoxy substitutions diminishing it. Studies with isoflavonoids revealed that in addition to the number of hydroxyls, the position of substitution was also important. Comparing antioxidant activities of plant-derived isoflavonoids with their biological metabolites, it was demonstrated that the activities for the metabolites were similar to or higher than the parent compounds. This suggests that these compounds may maintain their antioxidant activities under *in vivo* conditions. Additionally, the results of our studies with flavonoids and isoflavonoids indicated that the fluorescence assays developed were useful tools for characterization of structure-activity relationships within a class of structurally related compounds.

Experiments to determine the localization of flavonoids and isoflavonoids in membranes revealed that these compounds partitioned preferentially into the hydrophobic

core of the membrane, the major site of most peroxidation reactions. The flavonoids and isoflavonoids substantially reduced fluidity in the inner regions of the membrane. This would limit mobility of free radical species in the lipid bilayer, a possible structural explanation of their antioxidant activities in membranes.

An oxidation product of the reaction between genistein and alkylperoxyl radicals in homogenous system was characterized. This reaction product could be used as a marker of genistein antioxidant chemistry.

FUTURE RESEARCH

Some suggestions for future research are listed below:

1. Experiments with chelators demonstrated that their effects on metal-ion-induced peroxidation vary depending on the type of metal ions used to initiate peroxidation. Measuring the redox potential of the free and complexed metals would be important for a full understanding of the effects of metal chelators on rates of metal-catalyzed lipid peroxidation.

2. The studies conducted with flavonoids and isoflavonoids suggest that metal chelation is a significant contributor towards the antioxidant activities of these compounds. Conducting experiments that can specifically measure their metal chelating capacity and can determine the sites of chelation on the flavonoids and isoflavonoids will help in further clarification of their mechanisms of antioxidant action.

3. The flavonoids and isoflavonoids tested in this study show great potential for use as antioxidants. However, the fluorescence assays used for evaluating the efficacy of these compounds were only following the initial stages of oxidation. To ensure that these compounds maintain their antioxidant activities at later stages of lipid peroxidation, they

should be tested using an assay that measures some secondary products of peroxidation such as hexanal or malondialdehyde.

4. To conclusively demonstrate the ability of these compounds to prolong the shelf-life of foods, their activity should be tested in representative food lipid systems such as emulsions, bulk oils and phospholipid membranes.

5. An oxidation product of the reaction between genistein and peroxyl radicals in homogenous system was characterized in our study, a first important step in clarifying isoflavone antioxidant chemistry. Additional experiments need to be conducted in membranes using lipid-derived free radicals as this system would be more representative of biological conditions.

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