

# The Effects of Plant Flavonoids on Mammalian Cells: Implications for Inflammation, Heart Disease, and Cancer

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**Abstract**—Flavonoids are nearly ubiquitous in plants and are recognized as the pigments responsible for the colors of leaves, especially in autumn. They are rich in seeds, citrus fruits, olive oil, tea, and red wine. They are low molecular weight compounds composed of a three-ring structure with various substitutions. This basic structure is shared by tocopherols (vitamin E). Flavonoids can be subdivided according to the presence of an oxy group at position 4, a double bond between carbon atoms 2 and 3, or a hydroxyl group in position 3 of the C (middle) ring. These characteristics appear to

also be required for best activity, especially antioxidant and antiproliferative, in the systems studied. The particular hydroxylation pattern of the B ring of the flavonoles increases their activities, especially in inhibition of mast cell secretion. Certain plants and spices containing flavonoids have been used for thousands of years in traditional Eastern medicine. In spite of the voluminous literature available, however, Western medicine has not yet used flavonoids therapeutically, even though their safety record is exceptional. Suggestions are made where such possibilities may be worth pursuing.

## I. General Aspects

### A. Introduction

Over 4000 structurally unique flavonoids have been identified in plant sources (Harborne et al., 1975; Harborne, 1985a,b, 1986). Primarily recognized as the pigments responsible for the autumnal burst of hues and the many shades of yellow, orange, and red in flowers and food (Timberlake and Henry, 1986; Brouillard and Cheminant, 1988), the flavonoids are found in fruits, vegetables, nuts, seeds, herbs, spices, stems, flowers, as well as tea and red wine. They are prominent components of citrus fruits (Kefford and Chandler, 1970) and other food sources (Herrmann, 1976) and are consumed regularly with the human diet. These low molecular weight substances, found in all vascular plants, are phenylbenzo-pyrone (phenylchromones) with an assortment of structures based on a common three-ring nucleus. They are usually subdivided according to their substituents into flavanols (a), anthocyanidins (b), and flavones, flavanones, and chalcones (c) (Table 1 and Fig. 1). This basic structure is comprised of two benzene rings (A and B) linked through a heterocyclic pyran or pyrone (with a double bond) ring (c) in the middle (Fig. 1). This subdivision is primarily based on the presence (or absence) of a double bond on position 4 of the C (middle) ring, the presence (or absence) of a double bond between carbon atoms 2 and 3 of the C ring, and the presence of hydroxyl groups in the B ring (Fig. 1). In the flavonoid structure, a phenyl group is usually substituted at the 2-position of the pyrone ring. In isoflavonoids, the substitution is at the 3-position. Flavonoids and tocopherols (vitamin E) share a common structure, i.e., the chromane ring. There have been several efforts to quantitate the amounts of different flavonoids in assorted food plants (Bilyk and Sapers, 1985; Hertog et al., 1992; Rice-Evans and Packer, 1998). Establishing these

kinds of data will help nutrition scientists, for example, with studies of flavonoid pharmacodynamic effects and may lead to a better understanding of whether there is an optimal consumption level for flavonoids. On average, the daily USA diet was estimated to contain approximately 1 g of mixed flavonoids expressed as glycosides (Kühnau, 1976). However, according to Hertog et al. (1992), the average intake of mixed flavonoids was only 23 mg/day based on data from the 1987–88 Dutch National Food Consumption Survey (Hertog et al., 1993b). The flavonoid consumed most was quercetin, and the richest sources of flavonoids consumed in general were tea (48% of total), onions, and apples (Hertog et al., 1993b). The amount of 23 mg/day was mostly flavonols and flavones measured as aglycones (Hertog et al., 1993b). The corresponding amount of daily aglycones consumed in the USA would be about 650 mg/day, since Kühnau had estimated 1 g/day to be the daily flavonoid-glycoside consumption. Although there is a 5-fold difference between the estimates of Kühnau and Hertog, it should be stressed that recent evidence indicates that flavonoid-glycosides are much more readily absorbed (than the aglycones) by humans (Hollman and Katan, 1998). Moreover, both the amount and the source could vary appreciably in different countries. For instance, the amount consumed could be considerably higher in the Mediterranean diet, which is rich in olive oil, citrus fruits, and greens. These quantities could provide pharmacologically significant concentrations in body fluids and tissues. Nevertheless, flavonoid dietary intake far exceeds that of vitamin E, a monophenolic antioxidant, and that of  $\beta$ -carotene on a milligram per day basis (Hertog et al., 1993b). A resurgence of interest in traditional Eastern medicine during the past two decades, together with an expanded effort in pharmacognosy, has rekindled interest in the flavonoids and the need to

TABLE 1  
Some examples of subclasses of naturally occurring flavonoids

Class	Flavonoids	Substituents					
		3	5	7	3'	4'	5'
Flavan-3-ols	(+)-Catechin	OH	OH	OH	OH	OH	H
Anthocyanidins	Cyanidin	OH	OH	OH	OH	OH	H
	Pelargonidin	OH	OH	OH	OH	H	H
Flavones	Apigenin	H	OH	OH	H	OH	H
	Diosmin	H	OH	Oru	OH	Ome	H
	Luteolin	H	OH	OH	OH	OH	H
Flavanones	Naringenin	H	OH	OH	H	OH	H
	Naringin	H	OH	Oru	H	OH	H
	Hesperetin	H	OH	OH	OH	Ome	H
	Hesperedin	H	OH	Oru	OH	Ome	H
Chalcones	Phloretin	OH (2) <sup>a</sup>	OH (4)	OH (6)	H	H	OH (6')
	Phloridzin	Ogl (2)	H (4)	OH (6)	H	H	OH (6')
Flavon-3-ols	Quercetin	OH	OH	OH	H	OH	H
	Kaempferol	OH	OH	OH	H	OH	H
	Myricetin	OH	OH	OH	OH	OH	OH
	Fisetin	OH	H	OH	OH	OH	H
	Morin <sup>b</sup>	OH	OH	OH	H	OH	H

ru, rutinose.

<sup>a</sup> Number in parentheses denotes additional similar substituent at the position indicated by the number.

<sup>b</sup> Morin has one more OH group at position 2'.

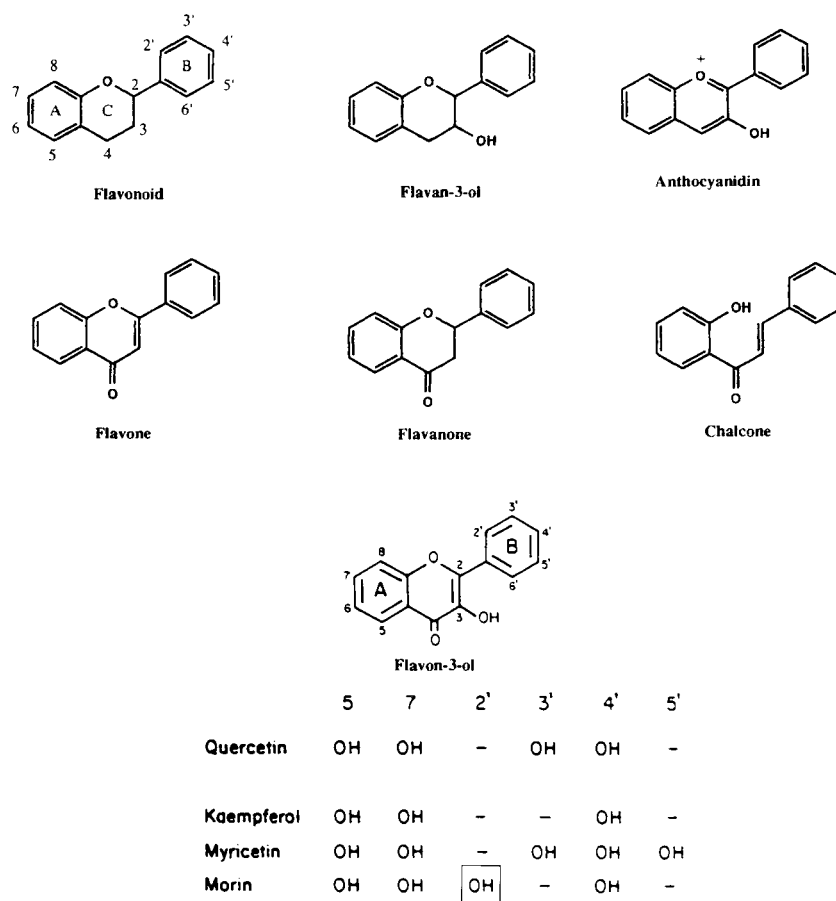


FIG. 1. Chemical structures of the most common flavonoid subclasses. The lower part of the figure shows the generic structure of flavon-3-ols and some representative compounds where the hydroxyl groups of ring B are shown.

understand their interaction with mammalian cells and tissues.

Flavonoids may have existed in nature for over one billion years (Swain, 1975) and thus have interacted with evolving organisms over the eons. Clearly, the flavonoids possess some important purposes in nature, having survived in vascular plants throughout evolution (Swain, 1975). The very long association of plant flavonoids with various animal species and other organisms throughout evolution may account for the extraordinary range of biochemical and pharmacological activities of these chemicals in mammalian and other biological systems. Unique examples are the inhibition of gamete membrane fusion in sea urchins caused by quercetin during egg fertilization (Eckberg and Perotti, 1983) and modulation of mammalian sperm motility by quercetin (Nass-Arden and Breitbart, 1990). Also, prenatal exposure to genistein does indeed influence sexual differentiation in rats (Levy et al., 1995) and thus raises the question of analogous effects in humans.

Flavonoids have important effects in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors, precursors of toxic substances, and pigments and light screens (Harborne et al., 1975; McClure, 1986). In addition, these compounds are involved in photosen-

sitization and energy transfer, the actions of plant growth hormones and growth regulators, the control of respiration, photosynthesis, morphogenesis, and sex determination, as well as defense against infection (Smith and Banks, 1986). Reports indicate that plant flavonoids cause the activation of bacterial (*Rhizobium*) modulation genes involved in control of nitrogen fixation, which suggests important relationships between particular flavonoids and the activation and expression of mammalian genes (Firmin et al., 1986; Peters et al., 1986; Djordjevic et al., 1987; Zaat et al., 1987).

The flavonoids have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral, and anticarcinogenic activities, discussed below separately (Gabor, 1979, 1986; Havsteen, 1984; Cody et al., 1986; Farkas et al., 1986; Selway, 1986; Cody et al., 1988; Welton et al., 1988; Das, 1989; Middleton and Kandaswami, 1993; Carroll et al., 1998; Hertog and Katan, 1998). The flavonoids are typical phenolic compounds and, therefore, act as potent metal chelators and free radical scavengers (Hughes and Wilson, 1977; Torel et al., 1986; Clemetson, 1989; Pratt, 1992; Kandaswami and Middleton, 1994). They are powerful chain-breaking antioxidants. The flavonoids display a remarkable array of biochemical and pharmacological

actions, some of which suggest that certain members of this group of compounds may significantly affect the function of various mammalian cellular systems. Anti-inflammatory flavonoids were discussed by Lewis (1989), and their potential utility as therapeutic agents was emphasized. In 1955, the New York Academy of Medicine published a series of papers discussing bioflavonoids and the capillary (Miner, 1955). As early as 1950, there was evidence of antiallergic activity, including information on vitamin C-flavonoid interaction. In 1952, Schoenkerman and Justice suggested that treatment with rutin plus an antihistamine conferred a clinical benefit to patients with allergic disease.

Of historical importance is the observation that a mixture of two flavonoids called citrin and hesperidin were considered to possess vitamin-like activity (Scarborough and Bacharach, 1949; Kühnau, 1976; Hughes and Wilson, 1977). The term vitamin P was coined to indicate that this material had the property of decreasing capillary permeability (and fragility), prolonging the life of marginally scorbutic guinea pigs, and reducing the signs of hypovitaminosis C in experimental animals. Although so-called vitamin P was shown ultimately not to fulfill the definition of a vitamin and the term was appropriately abandoned, there was nonetheless a strong indication that the flavonoids had potent antioxidant-dependent and vitamin C-sparing activity (Clemetson, 1989). This will be discussed in detail later. At present, flavonoids are considered to be secondary, nonessential dietary factors without any documented relevance to human health and/or disease. As the contents of this review will indicate, however, this position may need to be modified in view of the pleiotropic, potentially health-promoting, and disease-preventing activities of the flavonoids that have come to be appreciated, at least in experimental situations. Moreover, some flavonoids also have anticarcinogenic properties (Hertog et al., 1992, 1993b, 1995). The flavonoids do not have carcinogenic potential in experimental animals (Aeschbacher et al., 1982).

Alcoholism is a prevalent human disorder, and the search for effective remedies continues. For about 2000 years, the Chinese have recognized the antidipsotropic effect of *Radix puerariae*, an herb used in Chinese traditional medicine for the treatment of alcohol abuse. Keung and Vallee (1993) took advantage of the propensity for alcohol of the Syrian golden hamster to study the effect of extracts of *R. puerariae* and of daidzin and daidzein, two isoflavones found in the extracts. The isoflavone compounds effectively reduced ethanol consumption in the Syrian golden hamsters by approximately 50%, thus pointing the way to the development of a new class of therapeutic agents for alcoholism.

Another briefly reported observation of potentially great significance is the finding of quercetin in bovine retinal tissue (Pautler et al., 1986). Do ingested flavonoids accumulate in various tissues and modulate

their functions? An excellent review of flavonoids in health and disease has been published recently (Rice-Evans and Packer, 1998).

Das et al. (1994) conducted a careful structure-system-activity-relationship study of flavonoids with special respect to carcinogenicity, mutagenicity, and cancer-preventing activities. They concluded, in spite of some ongoing controversy, that not only are the "vast majority of flavonoids and isoflavonoids completely innocuous, but may be beneficial in a variety of human disorders". The naturally occurring flavonoids will be the primary focus of this review, with occasional reference to synthetic compounds. The review is not exhaustive; it is intended to acquaint the reader with this interesting group of natural plant compounds. There has been, in recent years, a major rekindling of interest in pharmacognosy. Flavonoids turn out to be present in many natural therapeutically utilized products. For example, a drug profile on *Ginkgo biloba* shows that flavonoids are a major component (Kleinjnen and Knipschild, 1992).

### B. Synthesis

The flavonoids are formed in plants and participate in the light-dependent phase of photosynthesis during which they catalyze electron transport (Das, 1994). They are synthesized from the aromatic amino acids, phenylalanine and tyrosine, together with acetate units (Heller and Forkmann, 1993). Phenylalanine and tyrosine are converted to cinnamic acid and parahydroxycinnamic acid, respectively, by the action of phenylalanine and tyrosine ammonia lyases (Wagner and Farkas, 1975). Cinnamic acid (or parahydroxycinnamic acid) condenses with acetate units to form the cinnamoyl structure of the flavonoids (Fries rearrangement). A variety of phenolic acids, such as caffeic acid, ferulic acid, and chlorogenic acid, are cinnamic acid derivatives. There is then alkali-catalyzed condensation of an *ortho*-hydroxyacetophenone with a benzaldehyde derivative generating chalcones and flavonones (Fig. 1), as well as a similar condensation of an *ortho*-hydroxyacetophenone with a benzoic acid derivative (acid chloride or anhydride), leading to 2-hydroxyflavanones and flavones (Heller and Forkman, 1993). The synthesis of chalcones and anthocyanidins has been described in detail by Dhar (1994). Biotransformation of flavonoids in the gut can release these cinnamic acid (phenolic acids) derivatives (Scheline, 1991). Flavonoids are complex and highly evolved molecules with intricate structural variation. In plants, they generally occur as glycosylated and sulfated derivatives.

### C. Metabolism and Disposition

The fate of orally and parenterally administered flavonoids in mammals and the significance of biliary excretion was reviewed by Griffiths and Barrow in 1972. Since then, progress in understanding flavonoid phar-

macokinetics has been slow. Published studies of flavonoid metabolism are not extensive, and were reviewed again recently (Hollman and Katan, 1998). Such studies are essential to enhance our understanding of the possible importance of flavonoids in human health and disease. The subject has been reviewed by Griffiths and Barrow (1972), Hackett (1986), and Scheline (1991) and will not be exhaustively reviewed here. Considerable information is available regarding the metabolism of flavonoids in animals and to a very limited extent in humans (Hackett, 1986; Scheline, 1991).

Ring scission occurs under the influence of intestinal microorganisms, which also account for the subsequent demethylation and dehydroxylation of the resulting phenolic acids (cinnamic acid derivatives and simple phenols). Intestinal bacteria also possess glycosidases capable of cleaving sugar residues from flavonoid glycosides. Such glycosidases do not appear to exist in mammalian tissues. Flavonoids can undergo oxidation and reduction reactions, as well as methylation, glucuronidation, and sulfation in animal species. An early evaluation of the absorption and metabolism of (+)-catechin in humans was presented by Das (1971). Oral administration (83 mg/kg) resulted in rapid absorption, metabolism, and excretion of the flavonoid within 24 h. Eleven metabolites were detected in urine. No quercetin could be found in plasma after oral administration of up to 4 g in humans (Gugler et al., 1975; Shali et al., 1991). Hepatic metabolism of quercetin and catechin by isolated perfused rat liver has been demonstrated in studies by Shah et al. (1991). The flavonoids were converted into sulfated and/or glucuronidated metabolites, which were excreted in the bile. Recent improvements in analytical techniques have made possible the determination of baicalin and baicalin (the glycoside of baicalein) in rat plasma by high pressure liquid chromatography with electrochemical detection (Wakui et al., 1992). Oral administration of these flavonoids to rats led to readily measurable concentrations of the compound in plasma (100–10,000 ng/ml). This assay would be suitable for

clinical pharmacokinetic studies. More recently, Ferry and coworkers (1996) performed a phase I clinical trial of quercetin; pharmacokinetic patterns were established following i.v. bolus administration. The plasma concentrations achieved inhibited lymphocyte protein tyrosine phosphorylation, and there was some evidence of anti-tumor activity.

Silibinin (two diastereomers), the principal component in extracts of *Silybum marianum*, can be measured in plasma by refined chromatographic assays (Rickling et al., 1995), permitting pharmacokinetic studies. Silibinin is absorbed following oral administration of silymarin. The several plasma concentration peaks detected could be caused by enterohepatic circulation of the compound. The significant biliary route of excretion of baicalin and baicalein was also noted by Abe et al. (1990). Chronic exposure to soya (soy milk) in the diet did not modify the metabolic pathways of the isoflavones daidzein and genistein but did alter the time courses of their excretion (Lu et al., 1995).

In long overdue studies, Hertog et al. (1993a) in The Netherlands measured the flavonoid content of several foods, their consumption by elderly males, and the relationship to the development of coronary artery disease. The flavonoids measured were quercetin, kaempferol, myricetin, apigenin, and luteolin. The principal sources of dietary flavonoids were tea, onions, and apples. Flavonoid consumption was significantly inversely related to mortality from coronary artery disease (after adjustment for multiple variables). The authors concluded that the regular ingestion of flavonoid-containing foods may protect against death from coronary artery disease in elderly men. The same group measured the content of potentially anticarcinogenic flavonoids of 28 vegetables, wine, and fruits frequently consumed in The Netherlands (Hertog et al., 1992). Again, the measured flavonoids were quercetin, kaempferol, myricetin, apigenin, and luteolin. The mean daily intake of these five antioxidant flavonoids was 23 mg/day, which exceeds the intake of other familiar antioxidants such as  $\beta$ -car-

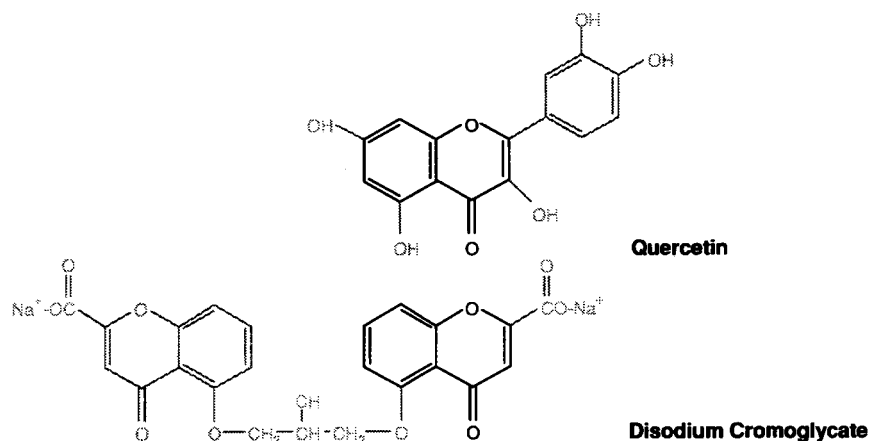


FIG. 2. Structures of quercetin and disodium cromoglycate. Those substituents that are different are shown in light print.

otene (2–3 mg/day) and vitamin E (7–10 mg/day) and is about one-third the average intake of vitamin C (70–100 mg/day) (Hertog et al., 1993b). If The Netherlands investigators had measured total flavonoid intake, including all sources of these chemicals, and had estimated the flavonoid glycoside content (Kühnau, 1976), the daily intake could have been considerably higher. The total aglycone consumption according to Kühnau (1976) was 650 mg/day in the USA. It would be useful to have comparable data for other countries. On the other hand, Rimm and coworkers (1996) did not find a strong inverse association between intake of flavonoids and total coronary heart disease. The authors suggested that flavonoids may exert a protective effect in men with established coronary artery disease.

One of the few recent pharmacokinetic studies of flavonoids in humans was conducted by Cova et al. (1992) using diosmin, the 7-rhamnoglucoside of diosmetin, 5,7,3'-trihydroxy-4'-methoxyflavone. Five healthy volunteers received 10 mg/kg of body weight of diosmin. Diosmin and diosmetin were measured in blood and urine by high performance liquid chromatography and liquid chromatography-mass spectrometry techniques. Only diosmetin (the aglycone) could be detected in plasma. The time course of diosmetin plasma concentrations indicated rapid initial distribution and prolonged final elimination half-life of 31.5 h. Neither diosmin nor diosmetin could be detected in urine. The metabolites in urine were *m*-hydroxyphenylpropionic acid and several other phenolic acids. The prolonged presence of diosmetin in blood suggests an enterohepatic circulation. The apparent volume of distribution of approximately 62.1 liters points to an extensive uptake of diosmetin by tissues. Using more recent analytical techniques, some Netherlands investigators (Hollman et al., 1996) measured plasma quercetin concentrations following ingestion of fried onions containing quercetin glycosides equivalent to 64 mg of quercetin aglycone. Peak plasma levels of 196  $\mu\text{g/ml}$  were achieved after 2.9 h with a half-life of absorption of 0.87 h. The distribution phase half-life was 3.8 h and the elimination phase half-life was 16.8 h. Thus, oral dietary (cooked vegetable) quercetin can be absorbed and reach tissues and plasma where antioxidant and other activities could be exerted. What is true for quercetin is very likely true also for other flavonoids in other vegetable sources. Thus, the cumulative concentration of quercetin plus other flavonoids could be substantially greater than that shown for quercetin alone. The possible importance of quercetin metabolites and their antioxidant properties has been discussed by Morand et al. (1998). Rats fed quercetin in the diet (0.2%) generated measurable quantities of metabolites with antioxidant properties. Rats adapted to this diet also had a total "antioxidant status" much greater than the control animals. In studies of absorption of quercetin and kaempferol from the diet of human subjects, de Vries and coworkers (1998) found that these

flavonols (from tea and onions) could be used as biomarkers for dietary intake.

Hollman and Katan (1998) reviewed the bioavailability and health effects of dietary flavonols in humans. They found that quercetin glycosides from onions were more readily absorbed than the pure aglycone; absorbed quercetin was eliminated slowly from the blood, suggesting that the enterohepatic circulation may be operative. In related studies, Hollman et al. (1995) concluded that quercetin-glucose conjugates were more readily absorbable; the suggestion was made that the glycosides may be absorbed via the intestinal sugar uptake route.

Determination of the urinary metabolites of deuterated rutin was performed by Baba et al. (1981) following oral administration of 10 mg/kg rutin-d or 50 mg/kg unlabeled rutin. Several metabolites appeared (consistent with scission of the C ring), but no unchanged rutin (or quercetin) was detected in the urine.

Isoflavonoid phytoestrogens and mammalian lignans, occurring in animal and human biological fluids and in feces, are diphenolic compounds with molecular weights similar to those of steroid estrogens. The mammalian compounds are produced from plant sources and isoflavonoids by intestinal microflora (Axelson and Setchell, 1981; Setchell et al., 1981; Borriello et al., 1985). Bannwart et al. (1984) described the presence of the phytoestrogenic isoflavone daidzein in human urine by GC-MS.<sup>2</sup> The isoflavonoids have been shown to bind with relatively high affinities to the estrogen receptors of human mammary tumor cells (Martin et al., 1978). They

<sup>2</sup> Abbreviations: GC-MS, gas chromatography-mass spectrometry; EGF, endothelial growth factor; PKC, protein kinase C; PLC, phospholipase C; MAP, mitogen-activated protein; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; MLC, myosin light chain; MLCK, MLC kinase; PTK, protein tyrosine kinase; NK, natural killer; PLA<sub>2</sub>, phospholipase A<sub>2</sub>; CO, cyclooxygenase; LO, lipoxygenase; LT, leukotriene; IP<sub>3</sub>, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PDE, phosphodiesterase; RT, reverse transcriptase; MMLV, Moloney murine leukemia virus; ODC, ornithine decarboxylase; GST, glutathione *S*-transferase; GSH, glutathione; MFO, multifunction oxidase; CD, cluster determinant; EGFR, epidermal growth factor receptor; PAH, polynuclear aromatic hydrocarbon; BP, benzo[a]pyrene; COMT, catechol-*O*-methyltransferase; TNF, tumor necrosis factor; LPS, lipopolysaccharide; NO, nitric oxide; iNOS, inducible NO synthase; TCR, T cell receptor; PI, phosphatidylinositol; PIP<sub>2</sub>, PI biphosphate; mAb, monoclonal antibody; PMA, phorbol 12-myristate 13-acetate; Pgp, P-glycoprotein; DMBA, 7,12-dimethylbenzo[*a*]anthracene; SOD, superoxide dismutase; EBV, Epstein Barr virus; EA, early antigen; LDL, low density lipoproteins; RBL, rat basophil leukemia; MPO, myeloperoxidase; PAF, platelet activating factor; ICAM-1, intercellular adhesion molecule-1; HUVEC, umbilical vein endothelial cells; IFN, interferon; PGE, prostaglandin E; EGCG, (-)-epigallocatechin gallate; HSV, herpes simplex virus; MDA, malondialdehyde; ROS, reactive oxygen species; DPPH, 1,1-diphenyl-2-picrylhydrazyl; HETE, hydroxyeicosatetraenoic acid; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; CAD, coronary artery disease; DCFH, 2,7'-dichlorofluorescein; IL, interleukin; EH, epoxide hydrolase; MCF, human mammary cancer cells; HS, heat shock; HSP, HS protein; bFGF, basic fibroblast growth factor; EBS, estrogen binding sites; GJIC, gap junctional intercellular communication; PA, plasminogen activator; MDR1, multidrug resistance gene-1; UDPGT, UDP-glucuronyltransferase.

may, therefore, be implicated in the inhibition of breast carcinoma cell growth mediated by estrogen.

Wheat fiber is recognized to be a potentially important anticancer food material, as is the case with soy isoflavones, such as genistein. Interestingly, therefore, Tew et al. (1996) found that a fiber-rich diet produced a marked decrease in plasma genistein concentrations after 24 h following soy dosing and reduced total urinary genistein excretion. Urinary daidzein was not related to fiber intake. The significance of this observation in relationship to the future design of flavonoid-rich diets must be taken into consideration. When human volunteers consumed soya flour, the urinary excretion of genistein, daidzein, and glycitein increased after 24 h as did the isoflavonoid metabolites equol and *O*-desmethylangolensin. The experiments also indicated that individual subjects exhibited preferred metabolic pathways (Kelly et al., 1995).

The plasma concentrations of four isoflavonoids, daidzein, genistein, *O*-desmethylangolensin, and equol, were very high in Japanese men consuming a low fat diet with a high content of soy products (Adlercreutz et al., 1993). The geometric mean plasma total and individual isoflavonoid levels were 7 to 110 times higher in the Japanese men than in the Finnish men. These phytoestrogen levels may inhibit the growth of prostate cancer in Japanese men, which may explain the low mortality from prostatic cancer in that country. Genistein concentrations in urine of subjects consuming a traditional soy-rich Japanese diet were in the micromolar range, while these concentrations were 1/30th or less of those in urine of omnivores (Adlercreutz et al., 1991).

The most important information derived from recent studies is the fact that most flavonoids, except catechins, exist in nature as glycosides. Moreover, at least quercetin glucosides were absorbed better than the aglycone quercetin- $\beta$ -glucoside (Hollman and Katan, 1998). Consequently, the amount of flavonoid glycosides consumed is a better indication than the amount of aglycones, thus raising the lower level estimated for the flavonoid aglycones. Finally, supplementation of the diet should more appropriately use flavonoid glycosides instead of aglycones.

#### D. Adverse Reactions

Adverse reactions to flavonoids in humans appear to be rare. Studies of Salama and Mueller-Eckhardt (1987) indicated that (+)-catechin and its metabolites can bind tightly to erythrocyte membranes and that this generates new antigenic sites with consequent development of autoantibodies presumed to be the cause of hemolytic anemia in six patients who had taken the catechin. The hemolytic anemia disappeared after discontinuation of catechin ingestion although the subjects continued to ingest cross-reactive dietary flavonoids.

Some flavonoids are capable of quinone formation, a familiar pathway leading to contact sensitization. However, as reviewed by Schmalle et al. (1986), the fla-

vonoids are not potent contact allergens and are not distinguished as contact sensitizers in the dermatologic literature, even though essentially all human beings have daily physical contact with flavonoid-containing foods and plants. Hausen et al. (1990) have described the development of contact allergy to the Australian blackwood, which is known to be an important cause of contact dermatitis in this region; several hydroxyflavans proved to be allergenic. Some flavonoids and their related phenolic compounds could have toxic effects. However, such flavonoids are not found in our food supply.

While there is a popular impression that flavonoids have "antiaging" properties, possibly through their antioxidant activity, note that quercetin may significantly reduce the life span of mice, (an effect was noted mainly in the "shorter-living" males (Jones and Hughes, 1982).

On balance, the flavonoids appear to be remarkably safe nutrients with a wide range of biochemical and pharmacologic activities that strongly suggest their possible role as health-promoting, disease-preventing dietary supplements.

## II. Effects on Mammalian Enzyme Systems

Flavonoids have been demonstrated to affect the activity of many mammalian enzyme systems *in vitro*. Some evidence indicates that they can also do so *in vivo*; however, the question remains how flavonoids enter the cells and whether they could accumulate in certain organ cells. Notable structure-activity relationships have been detected in many cases and are mentioned. The following listing is not exhaustive and aims to familiarize the reader with the extent of enzyme modulatory activities recorded.

### A. Kinases

Protein kinase C (PKC), the ubiquitous, largely  $\text{Ca}^{2+}$ - and phospholipid-dependent, multifunctional serine- and threonine-phosphorylating enzyme, is involved in a wide range of cellular activities, including tumor promotion, mitogenesis, secretory processes, inflammatory cell function, and T lymphocyte function, among others (Nishizuka, 1986, 1988, 1995). PKC has been shown to be inhibitable *in vitro* by certain flavonoids (Graziani et al., 1981; Gschwendt et al., 1983; End et al., 1987; Hagiwara et al., 1988; Ferriola et al., 1989; Picq et al., 1989). Graziani et al. (1983) demonstrated that quercetin inhibited the phosphorylating activity of the Rous sarcoma virus transforming gene product both *in vitro* and *in vivo*. In addition, quercetin was competitive toward the nucleotide substrates ATP and GTP. Mitogen activated protein (MAP) kinase in human epidermal carcinoma cells was strongly inhibited by quercetin (30  $\mu\text{M}$ ) (Bird et al., 1992).

Ferriola et al. (1989) used a partially purified rat brain PKC preparation and found that fisetin, quercetin, and luteolin were the most active flavonoid inhibitors of

this enzyme. Experiments utilizing different protein substrates (histone and protamine) and different activators [diacylglycerol and tetradecanoylphorbol acetate (TPA)] showed that fisetin (and luteolin) competitively blocked the ATP binding site on the catalytic unit of PKC. Several other ATP-utilizing enzymes inhibited by flavonoids were affected by competitive binding of the flavonoid to the ATP binding site (vide infra). Structure activity studies suggested that addition of one hydroxyl group at position 3 largely eliminated inhibitory activity (Alexandrakis et al., 1999).

Myosin light chain kinase (MLCK) catalyzes the phosphorylation of MLCs in many cell types. It is essential for the development of active tension in smooth muscle and for movement or migration of other cells. It is of interest, therefore, that kaempferol was an active and relatively specific inhibitor ( $IC_{50}$ ,  $0.45 \mu\text{M}$ ) of purified bovine aorta MLCK (Rogers and Williams, 1989). Kaempferol was specific for MLCK by a factor of 30 or greater as compared with several other kinases. As in other systems with different flavonoids, kaempferol acted competitively with ATP. Avian MLCK was also inhibited by several flavonoids, maximally with compounds with C2-C3 unsaturation and polyhydroxylation of two of the three ring structures (Jinsart et al., 1991). Methoxylation or glycosylation markedly reduced or abolished activity.

A large number of protein tyrosine kinases (PTK) have been described. They are found in many different types of cells and are implicated in the regulation of cell transformation and cell growth, gene expression, cell-cell adhesion interactions, cell motility, and shape (cf. Huang, 1989; Taniguchi et al., 1995; Qian and Weiss, 1997). PTK was inhibited by genistein (Akiyama et al., 1987). In addition to affecting PTK and PKC activity, quercetin was also capable of inhibiting nuclear kinase II-catalyzed phosphorylation of isolated nuclear proteins in HeLa cells using GTP as phosphate donor (Friedman et al., 1985). This result is of interest because it shows that quercetin could inhibit a GTP-dependent phosphorylation reaction and raised the question whether intact cell nuclear protein phosphorylation could be affected by flavonoids and thus affect many non-ATP-dependent aspects of cell function.

Another flavonoid-sensitive kinase is rabbit muscle phosphorylase kinase. Kyriakidis et al. (1986) found quercetin and fisetin to be effective inhibitors of nonactivated phosphorylase kinase, while the flavanone hesperetin stimulated the enzyme. Quercetin acted as a competitive inhibitor of ATP binding and was more effective as an inhibitor of the enzyme when stimulated by ethanol or alkaline pH. Cochet et al. (1982) examined the effect of quercetin and several other flavonoids on inhibition of cyclic nucleotide-independent protein kinase (G type casein kinase) and two other kinases. The G type kinase, which utilizes GTP as well as ATP, was selectively inhibited by several flavonoids. Kinetic eval-

uation showed that quercetin behaved as a competitive antagonist. Fisetin, chrysin, and kaempferol were also active. The importance of the pattern of A and B ring hydroxylation, C2-C3 unsaturation, and C4 keto were again recognized as strongly affecting inhibitory activity. Srivastava (1985) showed quercetin to be an effective inhibitor of phosphorylase kinase and also of protein tyrosine kinase. ATP competitively blocked quercetin's inhibitory activity with protein tyrosine kinase, but not with phosphorylase kinase. The data suggested once more that quercetin competed for the ATP binding site of the tyrosine kinase. It is currently unknown how the flavonoids enter the cell and react in the compartment where the kinases are localized. One possibility is that the flavonoids have no effect on kinases in quiescent cells and only interfere with the ATP binding site when the enzyme *trans*-locates upon activation.

Takeya et al. (1993) isolated a unique substrate-competitive tyrosine kinase inhibitor from the plant *Desmos chinensis*; they named it "desmal" and determined its structure to be 8-formyl-2',5,7-trihydroxy-6-methylflavanone. Desmal showed competitive inhibition of phosphorylation with respect to histone and noncompetitive inhibition with respect to ATP (in contrast to some other flavonoid inhibitors of phosphorylation noted above). Desmal also inhibited EGF-induced inositol phosphate formation. Moreover, desmal inhibited intracellular tyrosine phosphorylation in EGF receptor-overexpressing NIH 3T3 (ER12) cells.

Human cytomegalovirus DNA can induce a serine-threonine protein kinase with a molecular mass of 68 kDa in human diploid lung fibroblasts. This p68 kinase catalytic activity was inhibitable by quercetin acting competitively with respect to the nucleotide substrate (Michelson et al., 1985).

In studies of NK cell-mediated cytotoxicity, Nishio et al. (1994) found that genistein decreased the affinity of the tyrosine kinase  $p56^{\text{ck}}$  to the  $\beta$ -chain of the interleukin (IL)-2 receptor, a crucial event in IL-2-stimulated signaling events. In addition, genistein decreased the fast  $\text{Na}^+$  current in a concentration-dependent manner with an  $IC_{50}$  of  $9 \mu\text{M}$  in human uterine leiomyosarcoma cells (Kusaka and Sperelakis, 1996). These investigators also studied the effect of genistein and daidzein on regulation of L-type  $\text{Ca}^{2+}$  channels in freshly isolated uterine smooth muscle cells. Genistein decreased L-type  $\text{Ca}^{2+}$  current concentration dependently, while daidzein had no effect (Kusaka and Sperelakis, 1995).

Rat liver cyclic AMP-dependent protein kinase catalytic subunit could be inhibited by a variety of flavonoids (Jinsart et al., 1992). Again, C2-C3 unsaturation and polyhydroxylation of two or more flavonoid rings favored the development of inhibitory activity. Methoxylated and glycosylated agents were much less active. Several flavonoids inactive against MLCK were good inhibitors of cyclic AMP-dependent protein kinase catalytic subunit.

Recent evidence indicates that flavonoids can *induce* the phosphorylation of a 78-kDa protein, which was recently shown to be homologous to moesin (Theoharides et al., 2000). Further work showed that this phosphorylation was caused by a  $\text{Ca}^{2+}$ - and phorbol ester-independent PKC isozyme " $\zeta$ " (Wang et al., 1999). The possibility that the increase in phosphate incorporation may be due to inhibition of a phosphatase is unlikely because there has not been any such evidence. Preliminary data from our studies suggest that flavonoids *reduce* intracellular calcium ion levels, thus reducing secretion and activating a  $\text{Ca}^{2+}$ -independent PKC isozyme. The combined effect is regulation of secretion.

### B. Phospholipase $A_2$

Phospholipase  $A_2$  ( $\text{PLA}_2$ ), an enzyme involved in many cell activation processes, catalyzes the hydrolysis of phospholipids esterified at the second carbon in the glycerol backbone. Arachidonic acid is commonly esterified in this position, and the action of  $\text{PLA}_2$  releases arachidonic acid for subsequent metabolism via the cyclooxygenase (CO) and lipoxygenase (LO) pathways.  $\text{PLA}_2$  is likely an important intra- and extracellular mediator of inflammation (Pruszanski and Vadas, 1991). Quercetin was found to be an effective inhibitor of  $\text{PLA}_2$  from human (Lee et al., 1982) and rabbit (Lanni and Becker, 1985) leukocytes. Quercetagenin, kaempferol-3-O-galactoside, and scutellarein inhibited human recombinant synovial  $\text{PLA}_2$  with  $\text{IC}_{50}$  values ranging from 12.2 to 17.6  $\mu\text{M}$  (Gil et al., 1994).

### C. ATPases

Flavonoids can affect the function of plasma membrane transport  $\text{Na}^+$ - and  $\text{K}^+$ -ATPases (Rodney et al., 1950; Carpenedo et al., 1969; Lang and Racker, 1974), mitochondrial ATPase, and  $\text{Ca}^{2+}$ -ATPase (Deters et al., 1975; Cantley and Hammes, 1976). The  $\text{Mg}^{2+}$ -ectoATPase of human leukocytes was inhibited by quercetin (Long et al., 1981). Rabbit muscle sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase was effectively inhibited by several flavonoids that were also active inhibitors of antigen-induced rat mast cell histamine release (Fewtrell and Gomperts, 1977a). Inhibition of  $\text{Ca}^{2+}$ -ATPases by flavonoids such as quercetin was demonstrated (Shoshan et al., 1980; Shoshan and MacLennan, 1981), and quercetin acted as a competitive inhibitor of ATP binding to the enzyme. Others have described quercetin inhibition of hog gastric  $\text{H}^+$ ,  $\text{K}^+$ -ATPase where the inhibition was competitive with respect to ATP (Murakami et al., 1992). In studies of contractile proteins of rabbit skeletal muscle, Zyma et al. (1988) found quercetin to cause conformational changes in the structure of myosin with a coincident increase in ATPase activity. At higher concentrations, quercetin inhibited actomyosin superprecipitation as well as ATPase activity. Inhibition of  $\text{Ca}^{2+}$  transport across erythrocyte membranes by quercetin has also been described (Wuthrich and Schatzmann,

1980). Fischer et al. (1987) showed that quercetin inhibited platelet and sarcoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase activities in a concentration-dependent manner. Quercetin proved to be a competitive inhibitor of the calcium pump ATPase with respect to ATP. Inhibition of  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase apparently was not related to the cardiac glycoside-specific (ouabain) binding site(s) of this enzyme (Hirano et al., 1989a).

### D. Lipoxygenases and Cyclooxygenases

Arachidonic acid released from membrane phospholipids or other sources is metabolized by the LO pathway to the smooth muscle contractile and vasoactive leukotrienes (LT),  $\text{LTC}_4$ ,  $\text{LTD}_4$ , and  $\text{LTE}_4$ , as well as to the potent chemoattractant,  $\text{LTB}_4$  (Lewis and Austen, 1984). These molecules are intimately involved in inflammation, asthma, and allergy, as well as in multiple other physiologic and pathologic processes. Yamamoto and coworkers (1984) studied the effect of several benzoquinone and flavonoid compounds on various enzymes of the LT biosynthetic pathway. For instance, cirsiol (3',4',5-trihydroxy-6,7-dimethoxyflavone) proved to be a potent inhibitor of 5-LO ( $\text{IC}_{50}$ , 0.1  $\mu\text{M}$ ) derived from rat basophilic leukemia cells and guinea pig peritoneal polymorphonuclear leukocytes. The partially purified 5-LO of rat basophilic leukemia cells was also strongly inhibited by cirsiol (Furukawa et al., 1984). Hoult et al. (1994) studied the effects of flavonoids on 5-LO and CO in rat peritoneal leukocytes and human polymorphonuclear leukocytes stimulated with the nonphysiological cation ionophore A23187. 5-LO was best inhibited by polyhydroxylated compounds. The authors considered that 5-LO, but not CO, inhibition could be caused by a combination of iron ion-reducing/iron ion-chelating abilities and was not dependent on lipid peroxyl scavenging. Laughton et al. (1991) had also indicated that a combination of iron-chelating and iron ion-reducing properties was required for selective peritoneal leukocyte 5-LO inhibition by phenolic compounds.

Differential inhibition of LT biosynthetic enzymes was further documented when cirsiol was shown to have approximately 10-fold less activity against the 12-LO enzyme and negligible effect on CO of bovine vesicular gland. Partially purified mouse epidermal cell LO was inhibited potently by flavone derivatives bearing appropriate patterns of hydroxylation, but not by flavone itself (Wheeler and Berry, 1986). Baicalein was reported to selectively inhibit platelet 5-LO (Sekiya and Okuda, 1982). Artonin E (5'-hydroxymorusin) was a potent and fairly selective inhibitor of porcine leukocyte 5-LO (Reddy et al., 1991). Hypolaetin (a catecholic flavonoid), but not its 8-glucoside, proved to be a good inhibitor of stimulated rat peritoneal leukocyte 5-LO, although it was inactive as a CO inhibitor (Moroney et al., 1988). Interestingly, these investigators found more CO inhibition and less LO inhibition with flavone compounds

containing few hydroxyl substituents, including absence of the 3',4'-dihydroxy pattern in the B ring.

In contrast, Kalkbrenner et al. (1992) found that non-planar flavans were more potent inhibitors of rat seminal vesicle LO than planar flavones and flavonols. No flavanones caused inhibition except silibinin, a flavanon-3-ol. Kinetics of inhibition varied with the class of flavonoid. On the other hand, Swies et al. (1984) found that ram seminal vesicle CO was stimulated by quercetin and several other flavonoids at high substrate arachidonic acid concentrations, whereas at low substrate concentration quercetin was inhibitory.

Baumann et al. (1980a) also examined the effect of several flavonoids on arachidonic acid peroxidation. Luteolin (3',4'-dihydroxyflavone), morin, galangin, and (+)-catechin were moderately active inhibitors of rat renal medulla CO. Landolfi et al. (1984) found that flavone, chrysin, apigenin, and phloretin depressed CO activity and inhibited platelet aggregation. In early experiments, Fiebrich and Koch (1979) showed that the three pharmacologically active compounds of silymarin, namely, silybin, silydianin, and silychristin, inhibited CO.

Ferrandiz et al. (1990) studied some unusual flavonoids for their effect on arachidonic acid metabolism via the LO (5-HETE and LTB<sub>4</sub>) and CO (TxB<sub>2</sub>, PGE<sub>2</sub>, 6-keto-PGF<sub>1α</sub>) pathways in rat peritoneal leukocytes. IC<sub>50</sub> of less than 10 μM was found for sideretoflavone, oroxinidin, quercetagenin-7-glucoside, and tambuletin against both pathways. Also, eight naturally occurring isoprenylated flavones were studied for their effect on 5-LO activity purified from porcine leukocytes. Artonin E (5'-hydroxymorusin) was the most potent inhibitor, with an IC<sub>50</sub> of 0.36 μM. Butenko et al. (1993) also showed baicalein to be an inhibitor of LTC<sub>4</sub> production via inhibition of 5-LO; the resultant anti-inflammatory activity was greater in the rat adjuvant arthritis model than in the rat carrageenan-induced paw edema model.

Rao and coworkers (1985) found differential effects of the inhibitors on membrane- and cytosol-associated LO activity. Quercetin was an effective inhibitor of 12-LO activity in human platelets. Inhibitory activity of some chalcone derivatives on mouse epidermal 12-LO and CO was studied by Nakadate et al. (1985b). Effects of chalcones on 12-LO were much greater than on CO. Inhibitory activity was related to the chalcone's having a cinnamoyl or 4-hydroxycinnamoyl residue in the molecule. Skin tumor formation and TPA-induced ornithine decarboxylase activation were also strongly inhibited by some LO inhibitors (Aizu et al., 1986).

### E. Phospholipase C

No direct measurements of the effect of flavonoids on PLC have been reported. However, as reviewed in a later section, evidence strongly suggests that PTK-dependent phosphorylation of PLC-γ is required for activation of the enzyme; consequently, inhibition of PTK with such

flavonoids as genistein blocks PLC activation and formation of inositol trisphosphate (IP<sub>3</sub>) and diacylglycerol (DAG). Earlier work of Cockcroft (1982) indirectly indicated quercetin inhibition of PLC activity in stimulated rat mast cells, but the mechanism of action was not established.

### F. Cyclic Nucleotide Phosphodiesterase

The cyclic nucleotides, cAMP and cGMP, mediate many biological processes through their ability to stimulate cyclic nucleotide-dependent protein kinases, which in turn phosphorylate cellular protein substrates and evoke specific responses. cAMP and cGMP are formed from ATP and GTP by the catalytic activity of adenylate and guanylate cyclases stimulated by various agonists. Their activity is terminated by the cyclic nucleotide phosphodiesterases (PDE). The cyclic nucleotides are involved in regulation of many cellular processes, such as cell division, smooth muscle contractility, secretory functions, immunological processes, and platelet aggregation, to name a few. Flavonoid inhibition of PDEs from many cellular sources has been described (Ruckstuhl and Landry, 1981; Beretz et al., 1986). The minimal structural requirements for PDE inhibitor activity include a flavone, flavonol, or flavylum skeleton (Beretz et al., 1979). Ferrell et al. (1979) proposed that the flavonoid inhibitory activity on PDE could be ascribed to the structural mimicry of the pyrimidine ring in cAMP and the pyranone ring of active flavonoids.

### G. Adenylate Cyclase

Landolfi et al. (1984) reported that flavone, chrysin, and apigenin decreased the platelet cyclic AMP response to prostacyclin, an effect attributed to inhibition of adenylate cyclase. The isoflavone prunetin was also active, while the flavones 7-hydroxyflavone, apigenin, galangin, and kaempferol were less active.

### H. Reverse Transcriptase

Selected naturally occurring flavonoids have been shown (Spedding et al., 1989) to inhibit three reverse transcriptases (RT) [avian myeloblastosis RT, Rous-associated virus-2 RT, and Moloney murine leukemia virus (MMLV) RT] when poly(rA)oligo(dT) 12–18 or rabbit globin mRNA were used as template. Amentoflavone, scutellarein and quercetin were the most active compounds, and their effect was concentration-dependent. The enzymes exhibited differential sensitivity to the inhibitory effects of the flavonoids. These flavonoids also inhibited rabbit globin mRNA-directed MMLV RT-catalyzed DNA synthesis. Amentoflavone and scutellarein inhibited ongoing new DNA synthesis catalyzed by Rous-associated virus-2 RT. Kinetic studies were performed in an attempt to elucidate the mechanism of action of amentoflavone and scutellarein (Spedding et al., 1989). Inhibition of Moloney murine leukemia strains of RT by baicalein (5,6,7-trihydroxyflavone) was

described by Ono et al. (1989). Baicalein inhibition of RT was competitive with respect to the template primer (rA)n (dT) 12–18 and noncompetitive with respect to the substrate dTTP. In other experiments, Ono et al. (1990) found that baicalein, quercetin, quercetagenin, and myricetin were potent inhibitors (there was significant activity at 1–2  $\mu\text{g/ml}$ ) of RTs from Rauscher murine leukemia virus and HIV. The inhibition noted with baicalein was very specific, whereas quercetin and quercetagenin proved also to be potent inhibitors of DNA polymerase  $\beta$  and DNA polymerase I, respectively. Moloney murine and Rous associated virus-2 RT were also inhibited by baicalin (Baylor et al., 1992). This flavone caused a concentration-dependent inhibition of human T cell leukemia virus type 1 (HTLV-1) replication in infected T and B cells and selectively inhibited the HTLV-1 p19 gag protein without otherwise adversely affecting the cells. Inoue and coworkers (1989) found inhibitory activity against avian myeloblastosis RT with fisetin, quercetin, myricetin, and baicalein. The effect of flavonoids on MMLV RT was studied by Chu et al. (1992), who found that flavononols and flavonols were active, while flavones and flavanones were not. There was no requirement for a double bond at C2-C3.

Nakane and Ono (1990) found two components of green tea, namely (–)-epigallocatechin gallate and (–)-epicatechin gallate, to differentially inhibit the activities of RT and cellular DNA and RNA polymerases. RT was most strongly inhibited, as were DNA polymerases  $\alpha$  and  $\beta$ . The authors suggested the possibility that these compounds might exert selective inhibition of HIV RT at appropriate concentrations.

#### I. HIV-1 Proteinase

This enzyme is a necessary component for the processing and replication of HIV-1. Brinkworth et al. (1992) suggested that certain flavones may be potential non-peptidic inhibitors of the enzyme. Gardenin A, myricetin, morin, quercetin, and fisetin exhibited activity with  $\text{IC}_{50}$  values in the 10 to 50  $\mu\text{M}$  range. Lineweaver-Burk analysis indicated competitive inhibition for fisetin and quercetin.

#### J. HIV-1 Integrase

Yet another enzyme involved in HIV replication could be inhibited by quercetin, namely the integrase (Fesen et al., 1993). This inhibition required at least one ortho pair of phenolic hydroxyl groups and at least one or two additional hydroxyl groups (Fesen et al., 1994).

#### K. Ornithine Decarboxylase

The effects of flavonoids on ornithine decarboxylase (ODC) have not been studied in depth. ODC catalyzes the transformation of ornithine to the polycationic bases, putresine, spermine, and spermidine; these compounds exert regulatory effects on cell growth. Studies by Kato et al. (1983) showed that quercetin (10–30  $\mu\text{mol/}$

mouse) markedly suppressed the stimulatory effect of TPA on ODC activity and on skin tumor formation in mice initiated with dimethylbenzanthracene. Such inhibition may be related to the activation of the catalytic site, which is under nonconventional regulation by small molecules (Theoharides and Canellakis, 1975). Also, the synthetic flavonoid, flavone acetic acid, was shown to inhibit the activity of ODC in stimulated human peripheral blood lymphocytes and human colonic lamina propria lymphocytes (Elitsur et al., 1990). Nakadate et al. (1985a) reported that quercetin suppressed ODC induction by teleocidin. Topical application of the flavonoid silymarin to mice inhibited TPA-induced epidermal ODC activity and TPA-induced ODC mRNA expression (Agarwal et al., 1994). Topical application of apigenin, a close chemical relative of quercetin, also proved to be an effective, dose-dependent inhibitor of ODC activity and papilloma formation (Wei et al., 1990).

#### L. Topoisomerase

DNA topoisomerases are enzymes that introduce transient breaks in linear DNA sequences. They participate in several genetically related processes, including replication, transcription, recombination, integration, and transposition (Okura et al., 1988). DNA topoisomerase II is an important cellular target for several anti-neoplastic DNA intercalators and nonintercalators. Flavonoids had apparently different effects on these enzymes. Markovits et al. (1989) found that genistein inhibited mammalian DNA topoisomerase II as well as protein tyrosine kinase. Two flavones, fisetin and quercetin, also showed the same activity (Yamashita et al., 1990). Okura and coworkers (1988) showed that both topoisomerase I and II were sensitive to genistein by increasing the DNA-enzyme complex in L1210 cells and interfering with enzyme-induced DNA relaxation (pBR22 DNA). Genistein selectively suppressed the growth of the ras-transformed NIH 3T3 cells, but not the normal NIH 3T3 cells, and inhibited topoisomerase II-catalyzed ATP hydrolysis (Robinson et al., 1993). In contrast, baicalein, quercetin, quercetagenin, and myricetin, known inhibitors of RT, unwound DNA and appeared to promote mammalian DNA topoisomerase-mediated site-specific DNA cleavage (Austin et al., 1992).

#### M. Glutathione S-Transferase

Glutathione S-transferase (GST) isozymes participate in detoxification processes by catalyzing the formation of xenobiotic-glutathione (GSH) conjugates. Anionic and cationic GST isozymes were differentially inhibited to varying degrees by quercetin in vitro (Das and Ratty, 1986). Flavonoid administration in vivo, however, induced this activity (Trela and Carlson, 1987). Rat liver GST was effectively inhibited in vitro by several other flavonoids. This activity was again closely related to the pattern of hydroxylation and presence of a C2-C3 double bond (Merlos et al., 1991).

### N. Epoxide Hydrolase

Epoxide hydrolase catalyzes the hydration of arene oxides (generated by cytochrome P450 enzymes) to yield dihydrodiols, which can be converted to diol epoxides by cytochrome P450-dependent multifunction oxidases (MFOs). Diol epoxides generated from polynuclear aromatic hydrocarbons (PAHs), such as benzo[a]pyrene (BP), may function as ultimate carcinogens (Dipple et al., 1984). Flavone and 7,8-benzoflavone both stimulated epoxide hydrase activity, and flavone fed to rats increased the activity of the enzyme in liver microsomes (Alworth et al., 1980).

### O. Glyoxalase

Glyoxalase substrates may be important in the regulation of cell division. Glyoxalases detoxify  $\alpha$ -ketoaldehydes (glyoxalase I) by facilitating their oxidation to inert  $\alpha$ -hydroxy acids (glyoxalase II). Quercetin, fisetin, myricetin, and several other flavonoids were potent inhibitors of glyoxalase I (Klopman and Dimayuga, 1988).

### P. Xanthine Oxidase

Xanthine oxidase catalyzes the formation of urate and superoxide anion from xanthine. Bindoli et al. (1985), in early experiments, demonstrated the inhibitory action of quercetin on both xanthine oxidase and xanthine dehydrogenase activity. Hayashi et al. (1988) also found several flavonoids to be effective inhibitors of cow milk xanthine oxidase. Quercetin and several other flavonoids were weak (100  $\mu$ M) inhibitors of the enzyme; inhibitory activity did not correlate consistently with flavonoid-induced cytochrome *c* reduction (Iio et al., 1986). Chang et al. (1993) also found that baicalein and quercetin were potent inhibitors of xanthine oxidase. These authors also noted that xanthine oxidase serum levels were increased in patients with hepatitis and brain tumor; they suggested that selected flavonoids might be useful in treating these disorders.

### Q. Aromatase

The conversion of androstenedione to estrone is catalyzed by aromatase. Inhibition of aromatase (human estrogen synthetase) by several naturally occurring flavonoids (including quercetin, chrysin, apigenin, and others) was described by Kellis and Vickery (1984). The synthetic flavone 7,8-benzoflavone was most active. Aromatization of androstenedione was affected by several flavonoids, of which 7-hydroxyflavone and 7,4-dihydroxyflavone were the most potent (Ibrahim and Abul-Hajj, 1990). Inhibition by 7-hydroxyflavone was competitive with respect to the substrate androstenedione. According to Moochhala et al. (1988), flavonoids of the 5,7-dihydroxyflavone series could bind to the active site human cytochrome P450 aromatase with affinity. The flavonoid kaempferol inhibited aromatase enzyme activity competitively in a human Glyoxalase cell culture

system (Wang et al., 1994). Such results suggest that diets rich in these compounds could contribute to the control of estrogen-dependent conditions, such as breast cancer.

### R. 11- $\beta$ -Hydroxysteroid Dehydrogenase

This enzyme oxidizes hydrocortisone to inactive cortisone. It is also a key regulator of renal  $K^+$  clearance. Slight inhibition of enzyme activity was noted with morin and quercetin (Song et al., 1992).

### S. Catechol-O-methyltransferase

Early studies demonstrated that certain flavonoids have an epinephrine-sparing action (Clark and Geissman, 1949) that is probably attributable to inhibition of the catecholamine-metabolizing enzyme catechol-O-methyltransferase (COMT) (Gugler and Dengler, 1973; Borchardt and Huber, 1975). Three isoflavone inhibitors of COMT were isolated from a streptomyces culture filtrate (Chimura et al., 1975).

### T. Aldose Reductase

Lens aldose reductase has been implicated in the pathogenesis of cataracts in diabetic and galactosemic animals. The enzyme catalyzes the reduction of glucose and galactose to their polyols, which accumulate in large quantities in the lens and ultimately lead to mature lens opacities. Several key bioflavones have activity against this enzyme (Iwu et al., 1989). In 1977, Varma et al. found that oral administration of quercitrin decreased the accumulation of sorbitol in the lens of the rodent *Ocrodon degus*; a similar effect was seen with quercetin in the galactosemic neonatal rat. The accumulation of lens opacities could be partially abrogated by certain flavonoids. In a study of 30 flavones, 4 isoflavones and 13 coumarins, many potent inhibitors were found, but 5,7,3',4'-tetrahydroxy-3,6-dimethoxyflavone and 6,3',4'-trihydroxy-5,7,8-trimethoxyflavone were especially active (Varma, 1986). In a subsequent study (Okuda et al., 1984) of 3',4'-dihydroxyflavones, another potent inhibitor was discovered: 3',4'-dihydroxy-5,6,7,8-tetramethoxyflavone (Okuda et al., 1982). Aldose reductase inhibition by the compounds was noncompetitive with respect to both DL-glyceraldehyde and the reduced form of NADP. Hypoglycemia-inducing effects (rabbits) and inhibition of rat lens aldose reductase activity of a mixture of biflavanones were reported by Iwu et al. (1989).

### U. Monoamine Oxidase (FAD-Containing)

Flavones, coumarins (neoflavonoids), and other oxygen-containing compounds were found to inhibit monoamine oxidases A and B in a reversible and time-independent manner (Thull and Testa, 1994).

### V. Aldo-Keto-Reductase Family of Enzymes

Carbonyl reduction is a metabolic pathway widely distributed in nature. Many endogenous substances,

such as prostaglandins, biogenic amines, and steroids, together with xenobiotic chemicals of several varieties, are transformed to the corresponding alcohols before further metabolism and elimination. Carbonyl reduction in several continuous cell lines was investigated using metyrapone as a substrate ketone. Quercitrin was reported to inhibit carbonyl reductase (Maser and Netter, 1991).

#### W. *Hyaluronidase*

Hyaluronidases depolymerize hyaluronic acid to oligosaccharides by breaking glucosaminidic bonds, have been referred to as "spreading factor", and are possibly involved in tumor cell invasiveness. Rodney and coworkers (1950) described the inhibitory effect of a series of flavonoids on hyaluronidase and some other related enzymes. More recently, Kuppusamy et al. (1990) re-examined the effects of 31 flavonoids representing several chemical classes on the activity of bovine testis hyaluronidase. Kaempferol and silybin were most active. Kinetic analysis revealed that these compounds acted competitively.

#### X. *Histidine Decarboxylase and DOPA Decarboxylase*

Early experiments (Martin et al., 1949) suggested that histidine decarboxylase was inhibited by selected flavonoids such as quercetin and (+)-catechin, whereas the flavonoid glycosides were inactive. Histamine stimulates gastric acid secretion, making the reported inhibition of histamine-induced gastric secretion by the synthetic flavone-6-carboxylic acid of interest (Pfister et al., 1980). Parmar et al. (1984) described the gastric antisecretory activity of the flavan derivative 3-methoxy-5,7,3',4'-tetrahydroxyflavan, a compound that appears to be a specific histidine decarboxylase inhibitor in rats and is as effective as cimetidine in reducing gastric acid secretion. This flavan also reduced gastric tissue histamine content in rats (Parmar and Hennings, 1984; Parmar et al., 1984). Naringenin, the aglycone of naringin, was a weak inhibitor of histidine decarboxylase and also exhibited some gastric antiulcer activity (Parmar, 1983).

Umezawa et al. (1975) reported orobol and 3',4',5,7-tetrahydroxy-8-methoxy isoflavone from culture filtrates of fungi and streptomyces were effective inhibitors of DOPA decarboxylase, and orobol had a significant hypotensive effect in spontaneously hypertensive rats.

#### Y. *Malate Dehydrogenase*

Malate dehydrogenase was inhibited by quercetin, which Seddon and Douglas (1981) also showed could produce photo-induced covalent labeling of the enzyme.

#### Z. *Lactic Dehydrogenase and Pyruvate Kinase*

Grisiola and coworkers (1975) found that these enzymes were quite effectively inhibited by quercetin.

#### AA. *Aldehyde and Alcohol Dehydrogenases*

An extract of *R. puerariae*, an herb long-used in traditional Chinese medicine for alcohol addiction and intoxication, suppressed the free-choice ethanol intake of ethanol-preferring Syrian golden hamsters (Keung and Vallee, 1994). The isoflavonoids daidzein (4',7-dihydroxyisoflavone) and daidzin (7-glucoside of daidzein) isolated from the extract (Keung, 1993) were shown to account for this effect by inhibiting human alcohol dehydrogenase. Daidzin and daidzein, at doses that suppressed ethanol intake, exhibited no effect on overall acetaldehyde and ethanol metabolism in hamsters, although they inhibited human mitochondrial aldehyde dehydrogenase and gamma-gamma alcohol dehydrogenase in vitro. These observations clearly distinguish the action(s) of these isoflavones from those of the classic, broadly acting inhibitors of aldehyde dehydrogenase and of class 1 alcohol dehydrogenase enzymes. Consequently, daidzin and daidzein represent a new class of compounds offering promise as safe and effective therapeutic agents for alcohol abuse.

#### BB. *Amylase*

Rat pancreatic acinar cell amylase secretion stimulated by cholecystokinin octapeptide, carbachol, or TPA was inhibited by quercetin; however, vasoactive intestinal polypeptide-induced secretion was unaffected (Lee et al., 1988).

#### CC. *RNA and DNA Polymerases*

The experiments of Nose (1984) demonstrated that quercetin, kaempferol, and fisetin inhibited transcription with RNA polymerase II in permeabilized normal human fibroblasts (WI-38 cells); flavone and chrysin exhibited weak activity. Addition of quercetin to an ongoing transcription reaction arrested it promptly, suggesting that quercetin was inhibiting the elongation step. The effects of several flavonoids (quercetin, quercetagenin, myricetin, and baicalein) exhibited complex interactions with DNA and RNA polymerases, depending on the particular flavonoid and the enzyme species (Ono and Nakane, 1990).

#### DD. *Human DNA Ligase I*

In an ongoing effort to identify clinically useful anticancer drugs, Tan et al. (1996) examined the effect of several natural products for their ability to disrupt the function of human DNA ligase I, which catalyzes the covalent joining of single-stranded breaks in double-stranded DNA. Interestingly, a flavonoxanthone glucoside, swertifrancheside (isolated from *Swerua franchetiana*), inhibited enzyme function with  $IC_{50}$  of 11  $\mu$ M.

#### EE. *Ribonuclease*

Mori and Noguchi (1970) studied the effects of flavonoids on bovine pancreatic ribonuclease 1. They found

that flavones and flavonols with hydroxy substitutions at positions 7, 3', and 4 dramatically inhibited the activity of ribonuclease 1. A keto group at position 4 was also important.

#### FF. Sialidase

Sialidase (neuraminidase) catalyzes the hydrolysis of sialic acid residues from sialoglycoconjugates and may have an effect on biological functions such as antigen presentation and receptor function. Mouse liver sialidase was noncompetitively inhibited by isoscutellarein-8-*O*-glucuronide (IC<sub>50</sub>, 40 μM), while influenza virus sialidase was only weakly inhibited (Nagai et al., 1989). Flavanone and chalcone structures essentially lacked activity against the liver enzyme. In studies of influenza sialidase, Nagai and coworkers (1990, 1992) examined the effect of other flavonoids derived from *Scutellana baicalensis*. 5,7,4'-Trihydroxy-8-methoxyflavone proved to be a moderately active compound among 103 tested. Since binding of influenza virus to target cells takes place via sialic acid residues in the viral envelope glycoprotein, it is of interest that 5,7,4'-trihydroxy-8-methoxyflavone also inhibited infection by influenza virus A/PR/8/34 of Madin-Darby canine kidney cells and replication of virus in embryonated egg allantoic sacs.

#### GG. Cytochrome P450 Systems

Studies on the influence of flavonoids on cytochrome P450 enzymes are discussed elsewhere. A recent study has examined the relationship between the electrochemical properties of flavonoids and the influence on phenol hydroxylase of rat liver microsomes. The effect of flavonoids on this P450-dependent hydroxylase activity was found to correlate well with the oxidation potential for flavonoid aglycones (Hendrickson et al., 1994). Easily oxidizable flavonoids inhibited microsomal phenol hydroxylase activity in a dose-dependent manner, with the extent of inhibition correlating with the ease of oxidation. In contrast, flavonoids with high oxidation potentials stimulated the hydroxylase activity in a dose-independent manner. No correlation was apparent between electrochemical properties and effects on microsomal benzene hydroxylase activity.

#### HH. Elastase

A unique flavonoid, 3'-hydroxyfarrerol (6,8-dimethyl-5,7,3',4'-tetrahydroxyflavanone (also known as IdB1031), inhibited human neutrophil elastase, but only weakly (IC<sub>50</sub>, approximately 200 μM), acting with a reversible, noncompetitive mode of inhibition (Meloni et al., 1995). Moreover, this compound significantly reduced tumor necrosis factor (TNF)-α and IL-8 generation in lipopolysaccharide (LPS)-stimulated peripheral blood mononuclear cells (at 10 μM) (Meloni et al., 1995). These properties, together with its ability to inhibit human neutrophil elastase, make it a possible candidate for

pharmacotherapy of chronic lung disorders characterized by leukocytic infiltration.

#### II. Nitric-Oxide Synthase

The recently recognized and intriguing chemical mediator, nitric oxide (NO), possesses many important physiological activities, e.g., smooth muscle relaxation, tumor cell lysis and destruction of microorganisms, among many others (Lowenstein and Snyder, 1992; Nathan, 1992; Moncada and Higgs, 1993). Its synthesis from arginine is catalyzed by an inducible enzyme, nitric oxide synthase (iNOS). Of great interest is the observation that genistein and two other PTK inhibitors (herbimycin and tyrphostin) inhibited the generation of NO and the induction of iNOS in murine macrophages (Dong et al., 1993). Both LPS- and cytokine-dependent inducible NO synthase were blocked by genistein in C6 glioma cells (Feinstein et al., 1994). Several dietary polyphenolic compounds were shown to attenuate NO production in C6 astrocyte cell cultures. Active flavonoid compounds included quercetin, epigallocatechin gallate, morin, apigenin, taxifolin, fisetin, and catechin (Soliman and Mazzi, 1998). Chiesi and Schwaller (1995) found tannin and quercetin to inhibit NO synthase activity of three isoforms of the enzyme.

It is hard to speculate on the broad ability of flavonoids to inhibit the activity of so many different enzyme systems. The apparent requirement of a C2-C3 double bond and hydroxylation of the B ring points toward some stereospecific interaction, especially as it concerns the competitive interferences with the ATP binding site of kinases. Yet it is unlikely that the same three-dimensional orientation would be required by widely different enzymes.

Another possibility is that flavonoids bind to proteins, thus changing their orientations and making their active site inaccessible. For instance, about 98% of quercetin in human plasma was protein-bound (Gugler et al., 1975). Moreover, there has been a recent report of a *stable* flavonoid-protein complex in vivo (Manach et al., 1998).

#### III. Modulation of the Functions of Inflammatory Cells

The immune system is a highly complex, intricately regulated group of cells whose integrated function is essential to health. Cells of the immune system may interact in a cell-cell manner and may also respond to intercellular messages including hormones, cytokines, and autacoids elaborated by various cells. Autacoids usually include histamine, kinins, leukotrienes, prostaglandins, and serotonin. The immune system can be modified by diet, pharmacologic agents, environmental pollutants, and naturally occurring food chemicals, such as vitamins and flavonoids. Some effects of the flavonoids on the function of T cells, B cells, macrophages,

NK cells, basophils, mast cells, neutrophils, eosinophils, and platelets are described below.

It is evident that the flavonoids display, to a variable extent, a remarkable array of biochemical and pharmacological actions which suggest that certain members of this group of compounds significantly affect the function of the immune system and inflammatory cells (Middleton and Kandaswami, 1992). Several flavonoids specifically affect the function of enzyme systems critically involved in the generation of inflammatory processes, especially tyrosine (Nishizuka, 1988; Hunter, 1995) and serine-threonine protein kinases, reviewed above. Recently, it has become evident that these enzymes are intimately involved in signal transduction and cell activation processes involving cells of the immune system, as well as other cells activated by hormones, autoids, neurotransmitters, and growth factors. Weber et al. (1997) reviewed the broad subject of the regulation of signal transduction by drugs. The complexity of the signal transduction process was illustrated in the review by Gomez et al. (1998) on IL-2-induced cellular events. The possible effects of flavonoids on the various components of the signal-transduction pathway were reviewed recently, and the various relevant studies were summarized in a nice table (Packer et al., 1998). The potential importance of such actions on cell proliferation and cancer growth is discussed in later sections.

#### A. *T Lymphocytes*

Recent work on the nature of T cell antigen recognition and investigations of signal transduction in T and B cells has led to new fundamental concepts. T cell proliferation follows the cooperative interaction of cluster determinant 4 (CD4), CD8, and the T cell receptor (TCR)-CD3 complex upon exposure to foreign antigen and in association with appropriate molecules of the major histocompatibility complex. It is now understood that the proliferative signal is generated by members of a family of PTKs that catalyze the phosphorylation of cellular substrates, which in turn leads to T cell proliferation (Rudd, 1990). Tyrosine phosphatases dephosphorylate the phosphoproteins, returning the cell toward baseline conditions (Fisher et al., 1991; Hunter, 1995). Certain flavonoids affect the activity of PTKs, but PTKs of different cellular sources are not uniformly affected by various flavonoids (Geahlen et al., 1989). Little is known about their possible effect on tyrosine phosphatases (Van Wart-Hood et al., 1989).

T lymphocyte stimulation through the antigen receptor causes early activation of a tyrosine kinase (Samelson et al., 1986; Patel et al., 1987; Trevillyan et al., 1990) and the generation of phosphatidylinositol (PI) biphosphate (PIP<sub>2</sub>)-derived second messengers, namely IP<sub>3</sub> and DAG, via activation of phospholipase C (Koretzky et al., 1990; Ledbetter et al., 1991). Several cellular substrates are phosphorylated, including TCR- $\alpha$  through the activation of PTK p56<sup>lck</sup>. Trevillyan et al. (1990)

showed that the isoflavone genistein, a selective PTK inhibitor (Akiyama et al., 1987), blocked the activity of p56<sup>lck</sup> in a concentration-dependent manner (IC<sub>50</sub>, 40  $\mu$ M). Inhibition of enzyme activity correlated with reduced IL-2 secretion and IL-2R expression, but not with TCR-mediated PI hydrolysis. Studies with the PTK inhibitors known as tyrphostins support the contention that tyrosine phosphorylation is an obligatory event in IL-2 secretion (Stanley et al., 1990).

Rao et al. (1995) found that the rapid induction of phosphatidylcholine hydrolysis in transfected NIH 3T3 cells, stimulated by human IL-3, was inhibited by genistein, but not by PKC inhibitors.

Atluru and Atluru (1991) compared the immunosuppressive effects of genistein with cyclosporin A on anti-CD28 monoclonal antibody stimulation of T cell proliferation, IL-2 formation, and the expression of IL-2 receptors. Genistein inhibited T cell proliferation, IL-2 synthesis, and IL-2 receptor expression without toxic effects on T cells at the concentrations studied (1–100  $\mu$ M). The potential use of genistein as an immunosuppressive agent together with cyclosporin in allograft rejection was suggested.

Namgoong et al. (1993) found generally similar results in studies of con canavalin A and LPS-induced murine lymphocyte proliferation and mixed lymphocyte culture, although flavonoid sensitivity of the three mitogenic stimuli did vary considerably. This latter point strongly suggested that the flavonoid sensitivity reflects utilization of different pathways of cell activation. As described by Dibirdik et al. (1991), engagement of the IL-7 receptor by recombinant human IL-7 leads to markedly enhanced tyrosine phosphorylation associated with a rapid increase in inositol trisphosphate generation in acute lymphoblastic leukemia blasts. These changes were blocked by genistein, but not by H-7, a PKC inhibitor. IL-7 may thus play an important role in regulation of acute lymphoblastic leukemia, and genistein's effect may indicate potential therapeutic applications.

Recently, it has been demonstrated that CD45 tyrosine phosphatase is essential for coupling the T cell antigen receptor to the PI pathway (Koretzky et al., 1990). Experiments by Ledbetter et al. (1991) and others demonstrated that CD45 tyrosine phosphatase can serve as a regulator of TCR complex-mediated phospholipase C activation in human peripheral blood lymphocytes. CD45 inhibited the increase in cytoplasmic Ca<sup>2+</sup> concentration, suggesting that PI hydrolysis is regulated by CD45. Also, ligation of CD45 inhibited phosphorylation of tyrosine on specific substrates during T cell activation. It will be important to determine the effects of flavonoids on CD45 tyrosine phosphatase. Protein tyrosine phosphorylation and calcium mobilization are strongly augmented by cross-linking CD4 or CD8 with CD3; this finding has implications for positive and negative thymic selection (Turka et al., 1991). Quercetin-inhibitable Rous pp60<sup>src</sup> tyrosine kinase has also

been found in human plasma (Haas et al., 1986). Since protein tyrosine phosphorylation is known to be affected by at least two flavonoids, genistein (Akiyama et al., 1987) and quercetin (Glossmann et al., 1981; Levy et al., 1984), it seems likely that this fundamental process determining thymic selection is a flavonoid-sensitive mechanism.

Phosphatidylinositol turnover is a central phenomenon in intracellular signal transduction, occurring in response to neurotransmitters, growth factors, and hormones (Berridge and Irvine, 1984, 1989; Bradford, 1998). Oncogene-induced transformation by ras, src, erb, fms, and fes also augments cellular PI turnover (Nishioka et al., 1989). An important enzyme in PI turnover is PI kinase, which phosphorylates the inositol moiety of PI on the 4-position and is referred to as phosphatidylinositol 4-kinase. Interestingly, Nishioka and coworkers (1989) found that the isoflavone orobol was a potent inhibitor of PI kinase from streptomyces with an  $IC_{50}$  of 0.25  $\mu\text{g/ml}$ ; quercetin had an  $IC_{50}$  value of 1.8 and fisetin of 2.0  $\mu\text{g/ml}$ . Kinetic analysis revealed that orobol is competitive with respect to ATP and uncompetitive with respect to PI. Another isoflavonoid related to genistein,  $\psi$ -tectorigenin and orobol, proved to be a potent inhibitor of EGF-induced PI turnover in A431 cells with an  $IC_{50}$  of approximately 1  $\mu\text{g/ml}$  (Imoto et al., 1988). This compound inhibited PI turnover without affecting EGF receptor tyrosine kinase activity. Flavonoids with these biochemical properties should be useful probes in the functional analysis of PI turnover and its relationship to immune cell function. A structure-activity study of flavonoid inhibition of phosphatidylinositol 3-kinase was conducted by Agullo et al. (1997), including comparisons with PTK and PKC inhibition. Myricetin, luteolin, apigenin, quercetin, and fisetin were active compounds. B ring hydroxylation patterns and state of saturation of the C2-C3 bond proved to be important determinants of activity, as shown for inhibition of other cellular processes.

In addition to PTK, the ubiquitous generally  $\text{Ca}^{2+}$ - and phospholipid-dependent, multifunctional serine-threonine phosphorylating enzyme PKC, which is involved in a wide range of cellular activities including tumor promotion and T lymphocyte function (Nishizuka, 1986, 1995; Patel et al., 1987), is also inhibited by certain flavonoids in vitro (Graziani et al., 1981; Gschwendt et al., 1983; Ferriola et al., 1989). Fisetin, quercetin, and luteolin were the most active compounds in the study of Ferriola et al. (1989), while an isoflavone congener of genistein, formononetin, was inactive. Fisetin was shown to competitively block the ATP binding site on the catalytic unit of PKC (Ferriola et al., 1989). Huang et al. (1996) demonstrated that apigenin suppresses TPA-induced tumor promotion in mouse epidermis by competing with ATP, yet another example of an ATP-dependent system being inhibited by selected flavonoids (e.g., Ferriola et al., 1989). The differential effects and structure-

activity relationships of flavonoids as inhibitors of tyrosine kinases and serine-threonine protein kinases have been discussed by Hagiwara et al. (1988).

Bagmasco et al. (1989) studied transmembrane signaling by both CD3 and CD2 human T cell surface molecules and the involvement of PKC translocation. T cell activation by monoclonal antibodies (mAbs) directed against both the CD3/TCR complex and the CD2 molecule resulted in the rapid increase of intracellular ionized  $\text{Ca}^{2+}$ . Moreover, it was demonstrated in the Jurkat human leukemic T cell line that triggering with appropriate anti-CD2 mAbs induced the generation of  $\text{IP}_3$  and DAG from the breakdown of  $\text{PIP}_2$ . The appearance of such second messengers suggested that the CD2 molecule, like the CD3/TCR complex, may be linked to PLC. These investigators demonstrated that activation of Jurkat cells by anti-CD2 mAbs was also accompanied by translocation of PKC activity to the cell membrane in association with increased intracellular  $\text{Ca}^{2+}$ . By analogy with the effects of flavonoids on PTK, each of the steps in these experiments is potentially flavonoid-sensitive.

An important question is whether PTK activation is a prerequisite for PLC activation or whether these two pathways of signal transduction are independently regulated. It appears from experiments by June et al. (1990a,b) that rapidly increased PTK activity is measurable before PLC activation (as determined by appearance of  $\text{IP}_3$ ) after T cell receptor complex ligation with anti-CD3 mAb. This PTK activity is sensitive to the effects of herbimycin, a benzoquinonoid ansamycin antibiotic that blocks oncogenic transformation by  $\text{pp60}^{\text{v-src}}$ . Mustelin and coworkers (1990) obtained similar results, but they used the isoflavone genistein as an inhibitor of PTK. At concentrations that inhibited tyrosine phosphorylation of the TCR-x subunit, but not PLC activity ( $\text{IP}_3$  increase), genistein blocked TCR-CD3-mediated activation of PLC, T cell proliferation, and expression of IL-2 receptors. The effects were not related to genistein toxicity. Nishibe and coworkers (1990) demonstrated that PLC- $\gamma$  1, an isozyme of the phosphoinositide-specific PLC family, was an excellent substrate for EGF receptor tyrosine kinase and that EGF elicited tyrosine phosphorylation of PLC- $\gamma$  1 accompanied by  $\text{PIP}_2$  hydrolysis in several cell lines. Supportive data were provided by Uckun et al. (1991b), who observed genistein abrogation of PTK activity and PLC stimulation in human B cells exposed to a monoclonal antibody directed against the pan-B-cell receptor CD40/Bp50.

PLC- $\gamma$  1 has also been detected in human Jurkat leukemia T cells as a phosphoprotein (Granja et al., 1991). CD3 activation of T cells causes tyrosine phosphorylation of PLC- $\gamma$  1, associated with a marked increase in PLC activity. Genistein inhibited both the tyrosine phosphorylation and increased PLC activity. On balance, all of these observations support the notion that

PLC activation is a genistein-sensitive, PTK-dependent process.

Traganos and coworkers (1992) studied the effects of genistein on the growth and cell cycle progression of normal human lymphocytes and human leukemic MOLT-4 and HL-60 cells. Short-term exposure of the leukemic cells to genistein (5–20  $\mu\text{g/ml}$ ) suppressed cell progression through S or S and G<sub>2</sub> phases, while similar treatment had no effect on proliferating lymphocytes. Mitogen-induced transition of lymphocytes from G<sub>0</sub> to G<sub>1</sub> phase was extremely sensitive to genistein (IC<sub>50</sub>, 1.6  $\mu\text{g/ml}$ ). Luton et al. (1994) demonstrated a genistein-sensitive PTK activity that appeared to control ligand-induced TCR/CD3 complex redistribution and internalization in a CD8 5-cyano-2,3-ditoyltetrazolium chloride clone, another indication that leukocyte function can be affected by this isoflavone.

Development of the immune repertoire depends on selective cell death and the elimination of cells expressing foreign antigens. Ligation of Fas antigen induces rapid (1-min) phosphorylation of multiple cellular proteins in Jurkat T cell leukemia, U937 human histiocytic lymphoma, and K562 human myelogenous leukemia cells with a decline to baseline after 30 min, presumably due to tyrosine phosphatase activity. Genistein blocked Fas-induced DNA fragmentation and prolonged cell survival. The results support the contention that PTK activation is an early obligatory event in Fas-induced apoptosis (Eischen et al., 1994). The growth of T-lymphoid leukemia cells was inhibited by baicalein, as was PTK activity. PKC activity, stimulated by PMA, was also reduced by this flavonoid (Huang et al., 1994a).

The inhibition of PTKs by genistein may not be universal, however, since purified bovine thymocyte PTK (designated p40) was unaffected (Geahlen et al., 1989). Synthetic PTK-reactive flavonoid analogs have been prepared (Ogawara et al., 1989; Cushman et al., 1991) and like genistein could be potent immunosuppressants, especially on actively dividing leukocytes.

While these results clearly demonstrate that both PTK and PKC, as well as PI kinase, can be inhibited *in vitro* by certain flavonoids, more *in vivo* experiments are required to clearly show an effect on some facet of immune function.

T lymphocyte cytotoxic effector function is, at least in part, dependent on the activity of the multidrug resistance gene 1 product, P-glycoprotein (Pgp). The action of Pgp, which is an efflux pump active in multidrug-resistant cancer cell lines, can be circumvented in certain drug-resistant cancer cells in tissue culture by the flavonoid luteolin and is accompanied by inhibition of cell proliferation (Gupta et al., 1992).

Mookerjee and coworkers (1986) demonstrated that both quercetin and tangeretin, a polymethoxylated flavonoid, could depress the expression of class II histocompatibility (DR) antigens in human peripheral blood monocytes processing streptolysin O as antigen. Class II

antigen expression was measured by determining the binding of OK-la-1 antibody by solid phase radioimmunoassay. The flavonoid effect was reversible. These investigators also observed that certain flavonoids reversibly inhibited lymphocyte proliferative responses to phytomitogens, soluble antigens, and phorbol esters by blocking an event(s) that follows exposure to the stimulus. Furthermore, quercetin and tangeretin were found to inhibit thymidine transport in stimulated lymphocytes. These findings are consistent with the results of earlier investigations (Hume et al., 1979) demonstrating quercetin inhibition of lymphocyte glucose uptake in mitogen-stimulated cells. Quercetin also inhibited 2-deoxyglucose and 3-O-methylglucose uptake in a cultured human diploid fibroblast preparation (Salter et al., 1978). Quercetin was also reported to inhibit the incorporation of [<sup>3</sup>H]thymidine into DNA of cultured lymphocytes from C3H/HCJ mice and in human lymphoid (Daudi and Bristol-8) cell lines (Jung et al., 1983). The observed inhibition appeared to be partially reversed by the addition of divalent cations. The finding that a flavonoid such as quercetin inhibited lymphocyte uptake of thymidine confirmed earlier reports by Graziani and Chayoth (1979).

Okada et al. (1990) studied the possible involvement of quercetin in tumor cell immunity. After exposure of the metastatic tumor BMT-11 I-9 cells (a clone of BMT-11, a transplantable mouse fibrosarcoma) to quercetin, clones were obtained that spontaneously regressed in normal syngeneic hosts. Possible mechanisms of regression of these clones were studied by measuring cytotoxic T lymphocyte activity generated during mixed lymphocyte/tumor cell culture of spleen cells obtained from tumor-bearing mice. These studies showed the potential ability of flavonoids to cause enzymatic alterations that may result in the production of tumor variants exhibiting modified immunological responses.

Rutin-derivatized bovine serum albumin stimulates an IgE response to bovine serum albumin but without hemagglutinating antibodies. The data suggested that rutin exerts a regulatory effect on isotype expression. Subsequently, it was shown that the tobacco polyphenol-containing glycoprotein stimulated IL-4 production by murine Th2 cells, thus accounting for the augmented IgE formation (Baum et al., 1990). In mice, intradermal prostate transglutaminase stimulates a prolonged IgE response (Francus et al., 1983).

In other experiments, Schwartz et al. (1982) and Schwartz and Middleton (1984) described the effect of quercetin and several other flavonoids on the generation and effector function of cytotoxic lymphocytes. Certain flavonoids inhibited in a concentration-dependent manner the generation of cytotoxic lymphocytes in murine mixed spleen cell cultures and depressed their cytotoxic activity against P815 murine mastocytoma target cells. The addition of Cu<sup>2+</sup> blocked the inhibition observed only by certain flavonoids, thus demonstrating that che-

lation of divalent cations such as  $\text{Cu}^{2+}$  cannot explain the action of all flavonoids in these systems.

Yamada et al. (1989) found that the flavanone glucoside, plantagoside, inhibited the in vitro immune response of mouse spleen cells to sheep red blood cells in a concentration-dependent manner. Plantagoside also inhibited the proliferative response of BALB/c spleen cells to the T cell mitogen concanavalin A but had no effect on the mitogenic activity of lipopolysaccharide or phytohemagglutinin, showing that the latter two mitogens probably use activation pathways that are insensitive to this particular flavonoid. Plantagoside is an  $\alpha$ -mannosidase inhibitor, and it is of interest that another mannosidase inhibitor, swainsonine, could restore immune function in immunosuppressed mice (Hino et al., 1985; Kino et al., 1985).

The immunopharmacological profile of a unique flavonoid has been described by Li et al. (1991). Baohuoside-1 (3,5,7-trihydroxy-4'-methoxy-8-prenylflavone-3-O- $\alpha$ -L-rhamnopyranoside) significantly suppressed human neutrophil chemotaxis, mitogen-induced lymphocyte transformation, mixed lymphocyte culture, NK cell cytotoxic activity, and IL-2 synthesis (Gibbon leukemic MLA-144 cell line); this effect was concentration-dependent and was not caused by direct cytotoxicity of the compound. Further work by Li and coworkers (1990) revealed that baohuoside also had cytotoxic and cytostatic effects on six cancer cell lines accompanied by inhibition of DNA and RNA synthesis but not protein synthesis.

In mice treated with the flavonol glycosides, mauritianin and myricitrin, delayed type hypersensitivity reactions to dinitrofluorobenzene, but not sheep red blood cells, were reduced in mice undergoing two-stage carcinogenesis initiated with 7,12-dimethylbenz[a]anthracene (DMBA) followed by promotion with TPA (Takeuchi et al., 1986; Yasukawa et al., 1990). Interestingly, the effects of flavonoid derivatives on TPA-induced inflammation (Yasukawa et al., 1989) were roughly parallel to their inhibitory activities on tumor promotion in mice (Yasukawa et al., 1990). Gerritsen et al. (1995) described the inhibitory effect of apigenin on delayed type hypersensitivity responses in mice and in carrageenin-induced rat paw edema.

Silymarin significantly increased the response of peripheral blood lymphocytes in patients with alcoholic cirrhosis to stimulation with concanavalin A and phytohemagglutinin A, while it decreased antibody-dependent cellular cytotoxicity, NK cell activity, and reduced the percentage of T8+ cells in the peripheral blood (Lang et al., 1988). This group of investigators also examined the effect of silymarin on superoxide dismutase (SOD) activity of erythrocytes and lymphocytes of patients with cirrhosis (Feher et al., 1986). SOD activity of both lymphocytes and erythrocytes increased significantly upon in vitro exposure to silymarin, as well as following oral administration of 210 mg daily.

McCabe and Orrenius (1993) reported that genistein induced apoptosis in a subset of human thymocytes ( $\text{CD3}^-$ ,  $\text{CD4}^+$ ,  $\text{CD8}^+$ ), sensitive to glucocorticoid-induced apoptosis. Herbimycin, a PTK inhibitor like genistein, failed to induce apoptosis in these cells, leading the investigators to conclude that the inhibitory effect of genistein on PTK could not account for its apoptotic action. Rather, genistein's activity as a topoisomerase II inhibitor could possibly account for its apoptosis-inducing effect.

It is apparent from the findings summarized above that flavonoids could have primarily inhibitory, but also some stimulatory, effects on T lymphocytes. These findings require further clarification and may derive from different mechanisms of action such as protein binding, active site interference, or antioxidant effects.

### B. B Lymphocytes

Cross-linking of B cell membrane immunoglobulin (J), the B cell antigen receptor, initiates the signal for B cell activation and maturation. B lymphocyte activation, like T cell activation, is accompanied by phosphorylation of tyrosine on particular B cell proteins (Campbell and Sefton, 1990; Gold et al., 1990; Lane et al., 1991; Yamashita et al., 1991). B cell aggregation induced by MHC class II ligands is accompanied by tyrosine phosphorylation (Fuleihan et al., 1992). To study the possibility that I cross-linking on B cells is coupled to PLC activation and  $\text{Ca}^{2+}$  mobilization secondary to activation of a PTK, Cambier et al. (1991) examined the ability of the PTK inhibitors genistein and herbimycin to inhibit activation of these responses. Each inhibitor reduced the I-dependent  $\text{Ca}^{2+}$  response, but the genistein concentration used was high (60  $\mu\text{g}/\text{ml}$ ). Carter et al. (1991b) also showed that genistein inhibited the rise in B lymphocyte intracellular  $\text{Ca}^{2+}$  and inositol trisphosphate generation by activated PLC in CD19/CR2 complex-activated cells.

Cumella et al. (1987) found that quercetin, but not taxifolin (dihydroquercetin), inhibited mitogen-stimulated immunoglobulin secretion of IgG, IgM and IgA isotypes in vitro with an  $\text{IC}_{50}$  of approximately 30  $\mu\text{M}$  for each isotype. In studies of human B cell precursors, Uckun et al. (1991a) found that IL-7 receptor ligation with recombinant human IL-7 caused increased phosphorylation on tyrosine of multiple substrate proteins, stimulated phosphatidylinositol turnover with increased  $\text{IP}_3$  generation (PLC activation), and also DNA synthesis. Genistein effectively abrogated the tyrosine kinase activity and the accompanying increase in  $\text{IP}_3$ . Interestingly, the protein tyrosine phosphatase inhibitor, sodium orthovanadate, permitted sustained protein tyrosine phosphorylation products upon exposure of cells to the IL-7. Also noteworthy is the finding that quercetin acted synergistically with orthovanadate to markedly increase the extent of protein tyrosine phosphorylation in normal chick embryo fibroblasts and in chick embryo

fibroblasts transformed by Rous sarcoma virus (Van Wart-Hood et al., 1989).

An example of ongoing, concurrent phosphorylation and dephosphorylation is seen in the experiments of Carter et al. (1991a), who studied tyrosine phosphorylation of PLC- $\gamma$  1 in L4B-lymphoblastoid cells. From 0 to 30 min, there was clear-cut evidence of phosphorylation followed by dephosphorylation of several cellular proteins. These investigators also studied the PTK inhibitors genistein, tyrphostin, and herbimycin. They found that genistein reduced the rise in cytosolic  $\text{Ca}^{2+}$  in B lymphocytes following ligation of membrane IgM and also observed the PTK-dependence of PLC activation. PI turnover increased cytosolic  $\text{Ca}^{2+}$  and proliferation as observed by Lane et al. (1990). At noncytotoxic concentrations, genistein inhibited Epstein Barr virus (EBV) activation, as determined by the induction of EBV early antigen (EA) and EBV early BZF1 mRNA and its protein product ZEBRA, in the Burkitt's lymphoma cell line Akata stimulated with anti-IgG (Daibata et al., 1991). Tumor promoter-stimulated induction of EA expression in EBV genome-carrying lymphoblastoid cells (Raji cells) and the effects of flavonoids were studied by Okamoto et al. (1983). Quercetin (and retinol) effectively inhibited EA expression while  $\alpha$ -naphthoflavone, a synthetic flavonoid, had a weaker effect. Several other naturally occurring flavonoids were inactive. As described by Polke et al. (1986), and in keeping with the observations of Trevillyan et al. (1990) with T cells, certain flavonoids inhibited the enhanced expression of IL-2 receptors and immunoglobulin secretion stimulated by TPA from sublines of an EBV-immortalized human B cell line.

In studies of PAF activation of an EBV-positive, human B lymphoblastoid cell line, Kuruvilla et al. (1993) observed that genistein inhibited PAF-induced incorporation of  $^{32}\text{P}$  into PI and decreased the generation of inositol phosphates and intracellular  $\text{Ca}^{2+}$ . Furthermore, induction of expression of the protooncogene, *c-fos*, was substantially reduced.

### C. Natural Killer Cells

Flavone acetic acid, a synthetic flavonoid, exhibited dose-dependent in vivo antitumor activity against certain solid tumors in mice. This compound augmented murine NK cell activity in vivo through induction of interferon- $\alpha$  synthesis (Hornung et al., 1988a,b). Spleen cells of flavone acetic acid-treated mice demonstrated rapid expression of interferon- $\alpha$  mRNA (Hornung et al., 1988b). The flavone acetic acid effect was selective since no up-regulation of splenic mRNA for interferon- $\beta$ , IL-1, or IL-2 was detected after administration of flavone acetic acid (Mace et al., 1990). Flavone acetic acid also exhibited antitumor activity through its ability to cause vascular shutdown in tumors. This effect was attributed to the rapid induction of TNF; pretreatment with anti-TNF antibody abrogated the effect on TNF expression

(Mahadevan et al., 1990; Pratesi et al., 1990). A brief report (Wleklík et al., 1987) suggested that mice treated with amentoflavone or quercetin developed measurable serum content of interferon. The antitumor (Verma et al., 1988) and antiviral (Selway, 1986) activity of naturally occurring flavonoids could be attributable to the immunomodulatory properties of induced interferons with associated augmentation of NK cell function.

NK cell cytotoxic activity against NK-sensitive K562 and U937 tumor target cells was accompanied by early increased incorporation of  $^{32}\text{P}$  into PI, suggesting activation of phospholipase C (Steele and Brahmi, 1988). Quercetin (100  $\mu\text{M}$ ) profoundly inhibited the increased PI metabolism and also inhibited killing activity. Ng et al. (1987) studied the  $\text{Ca}^{2+}$ -dependence of T lymphocyte and NK cell cytotoxic activity using quercetin and  $\text{Ca}^{2+}$  channel antagonists. Cytolysis could be induced by simultaneous stimulation with TPA and ionophore A23187, suggesting that PKC activation is involved. Quercetin inhibited  $\text{Ca}^{2+}$ -dependent killing possibly through its action on PKC (Graziani et al., 1981; Gschwendt et al., 1983; Ferriola et al., 1989).

Here, again, flavonoids appeared to have opposing actions. However, a stimulatory action indirectly via interferon synthesis could be distinguished from an inhibitory action on NK cell cytotoxic activity. Different flavonoid concentrations and/or different conditions could explain the seemingly opposite results.

### D. Macrophages and Monocytes

Relatively few studies on the effect of flavonoids on macrophage function have appeared. Oxyradical generation by peripheral blood monocytes was suppressed by catechin as noted by Berg and Daniel (1988). A synthetic lipophilic derivative, 3-palmitoyl-(+)-catechin, enhanced the phagocytic activity of guinea pig Kupfer cells in vivo according to Piazza et al. (1985).

The synthesis of IL-2 and  $\text{LTB}_4$  by human peripheral blood mononuclear cells was studied by Atluru et al. (1991). At a noncytotoxic concentration, genistein inhibited phytohemagglutinin A-induced cell proliferation and IL-2 formation. This isoflavone also blocked  $\text{LTB}_4$  generation in A23187-stimulated cells, while H-7, a protein kinase C inhibitor, had no effect.  $\text{LTB}_4$  formation in carrageenin-induced intrapleural exudates in rats was reduced by intraperitoneal injection of quercetin and quercitrin, but not by apigenin or luteolin, both of which lack a 3-position hydroxyl group (present in quercetin). Baicalein, the principal component of the traditional Chinese remedy Quing-Fe-Tang (Seihai-to), was also a fairly potent inhibitor of ionophore-induced human alveolar macrophage  $\text{LTB}_4$  synthesis and lucigenin-dependent chemiluminescence (Tanno et al., 1988). Shapira et al. (1994) showed that both PKC and PTK are involved in LPS-induced production of TNF- $\alpha$  and IL-1 $\beta$  by human monocytes. Preliminary experiments showed that TNF- $\alpha$  gene expression in normal human peripheral

blood monocytes was inhibited by quercetin (Nair et al., 1997).

Protein tyrosine phosphorylation and  $\text{Ca}^{2+}$  mobilization by Fc receptor cross-linking in the monocytic leukemia cell line THP-1 were reduced in a concentration-dependent fashion by the PTK inhibitors genistein, herbimycin, and erbstatin (Rankin et al., 1993). However, the concentration of genistein used was very high ( $370 \mu\text{M}$ ). Mitogen stimulation of bovine mixed mononuclear cell proliferation, IL-2 synthesis, and  $\text{LTB}_4$  production were all inhibited by genistein (Atluru and Gudapaty, 1993). The phosphorylation of PTK  $\text{p56}^{\text{lck}}$  was also inhibited, and genistein overcame the mitogenesis-augmenting effect of added IL-2, implicating an effect of the flavonoid on the outcome of the IL-2-IL-2R interaction.

As shown by Geng and coworkers (1993), PTK activation is required for LPS induction and release of cytokines such as IL- $1\beta$ , IL-6, and TNF- $\alpha$  from human blood monocytes. The over 10-fold increase in mRNA of these cytokines was blocked by >80% by genistein ( $37 \mu\text{M}$ ); IL-6 protein synthesis and bioactivity were likewise inhibited. Significantly, genistein also reduced the LPS-induced activation of nuclear factor  $\chi\text{B}$ , a transcription factor involved in the expression of cytokine genes including IL-6 and TNF- $\alpha$ , illustrating once again a potentially very important flavonoid-gene interaction.

De Whalley and coworkers (1990) demonstrated that fisetin and quercetin were potent inhibitors ( $\text{IC}_{50}$ , 1–2  $\mu\text{M}$ ) of macrophage modification of low density lipoproteins (LDL). The flavonoids apparently modulated macrophage-stimulated LDL oxidation, possibly through inhibition of generation of lipid hydroperoxides. Interestingly, the flavonoid compounds were also very active in conserving the  $\alpha$ -tocopherol content of LDL, and they delayed the onset of measurable lipid peroxidation. Diluted wine phenolics were as active antioxidants as 10  $\mu\text{M}$  quercetin (Frankel et al., 1993). The precise mechanism of action of the flavonoids to inhibit LDL oxidation is uncertain, but they may reduce the formation or release of free radicals in the macrophages or protect the  $\alpha$ -tocopherol in LDL from oxidation by metal complexation and radical scavenging. The protection of lymphoid cell lines against peroxidative stress induced by oxidized LDL has been demonstrated using a combination of  $\alpha$ -tocopherol, ascorbic acid, and the quercetin glycoside, rutin (Negre-Salvayre et al., 1991a,b). More recently, these investigators (Negre-Salvayre and Salvayre, 1992) concluded that quercetin and rutin at low concentrations were effective in preventing the cytotoxic action of oxidized LDL on UV-irradiated lymphoid cell lines. Flavonoids with antioxidant properties might also protect against lymphotoxicity from oxidized plasma lipoproteins (Cathcart et al., 1985). Flavonoids may also act like ascorbic acid, which has been shown to react with tocopheryl radicals and regenerate tocopherol (Bendich, 1990).

Quercetin significantly inhibited phorbol 12,13-dibutyrate-induced cell aggregation/adhesion of human mononuclear leukocytes (Patarroyo and Jondal, 1985). The authors attributed the quercetin effect to inhibition of cellular ATPases, but it is alternatively possible that the effect of quercetin could be due to its activity as an inhibitor of LO and/or PKC.

Endocytosis in the human promonocytic cell line THP-1 was inhibited by genistein which concurrently inhibited tyrosine phosphorylation of several cellular proteins (Ghazizadeh and Fleit, 1994).

#### *E. Mast Cells and Basophils*

Mast cells play a central role in the pathogenesis of diseases such as allergic asthma, rhinoconjunctivitis, urticaria, anaphylaxis, and systemic mastocytosis; they may also be important players in other chronic inflammatory disorders such as inflammatory bowel disease and rheumatoid arthritis (Galli, 1993; Theoharides, 1996). Mast cells may also participate in sterile inflammatory conditions exacerbated by stress, such as atopic dermatitis, interstitial cystitis, irritable bowel syndrome, migraines, and multiple sclerosis (Theoharides, 1996). Basophils, the circulating "equivalent" of the tissue mast cells, are now considered as important cells in the pathogenesis of late phase allergic reactions (Lemanske and Kaliner, 1988; Grant and Li, 1998).

The proliferation of mast cells is regulated importantly by stem cell factor, a ligand for the c-kit receptor (Galli, 1993). Early work by Nagai and coworkers (1975) showed that baicalein and some of its derivatives could inhibit mast cell proliferation. Nagai et al. (1995) later showed that genistein inhibited stem cell factor-induced histamine release from rat peritoneal mast cells.

In early experiments, Moss et al. (1950) described inhibition of anaphylaxis in guinea pigs treated with catechin. Quercetin (by oral administration) could substantially inhibit the development of bronchoconstriction in sensitized guinea pigs challenged with aerosol antigen (Dorsch et al., 1992). Silybin was also found to inhibit anaphylactic shock in rats sensitized to ovalbumin (Lecomte, 1975).

Both mast cells and basophils possess high-affinity receptors for IgE (Fc $\epsilon$ RI) in their plasma membranes. Cross-linking of these receptors is essential to trigger the secretion of histamine and other preformed, granule-associated mediators and to initiate the generation of newly formed phospholipid-derived mediators (Galli, 1993). Various flavonoids have been shown in several systems to inhibit this secretory process (Middleton, 1986). Definitive evidence of flavonoid regulation of secretion was first provided by Fewtrell and Gomperts (1977a,b) in studies of the secretion of histamine from rat mast cells stimulated with antigen, mitogen, or the divalent cation ionophore A23187; similar results were obtained on the release of  $\beta$ -glucuronidase from stimulated rabbit leukocytes (Bennett et al., 1981). Quercetin,

kaempferol, and myricetin were found to inhibit the release of rat mast cell histamine. Phloretin also proved to be an effective inhibitor of histamine release (Grossman, 1988). Middleton et al. (1981, 1982) undertook an examination of the effect of several naturally occurring flavonoids on the secretion of histamine from human basophils. Quercetin inhibited antigen-stimulated human basophil histamine release (Middleton et al., 1981) in a concentration-dependent manner and was instantaneous in onset of action. This effect was not significantly affected by increased extracellular  $\text{Ca}^{2+}$  concentrations or by theophylline, suggesting that inhibition was not a cyclic AMP-dependent process.

Subsequent experiments revealed critical structure-activity relationships governing the flavonoid effect on antigen-induced histamine release (Middleton and Drzewiecki, 1982). Inhibitory activity was associated with the following structural features: a C4 keto group, an unsaturated double bond at position C2-C3 in the  $\gamma$ -pyrone ring, and an appropriate pattern of hydroxylation in the B ring. These characteristics were nearly identical to those identified for other inhibitory activities. The flavonoid glycosides, rutin and naringin, were inactive, as were the flavanones (reduced C2-C3 bond), taxifolin and hesperetin. Morin, catechin, and cyanidin were also inactive. Polymethoxylated compounds such as nobiletin and tangeretin showed less or no inhibitory activity against antigen-induced histamine release (as compared with their activity as inhibitors of lymphocyte activation (Mookerjee et al., 1986)). Figure 1 shows the structures of some flavon-3-ols. It is important to note that while quercetin, kaempferol, and myricetin were potent inhibitors of histamine release from rat peritoneal mast cells, morin was not. Similarly, Alexandrakis et al. (1999) showed that the same flavonols could inhibit secretion and induce maturation of rat basophil leukemia (RBL) cells, an action absent only when morin was used. The addition of a single hydroxyl group at position 2' (shown in a square) appears to be sufficient to prevent it from inhibiting mast cell secretion. This hydroxyl group may be interacting with the oxygen at position 1, forming a cyclic structure that possibly interferes with some key biological event.

Further studies were undertaken to determine the effect of flavonoids on basophil histamine release stimulated by different triggers: 1) anti-IgE or concanavalin A (IgE-dependent histamine-releasing agents); 2) the chemoattractant peptide, f-MetLeuPhe or the tumor promoter phorbol ester, TPA (both f-MetLeuPhe and TPA are receptor-dependent, IgE-independent, histamine-releasing agents); and 3) the divalent cation ionophore A23187 (bypasses receptor-dependent processes and carries  $\text{Ca}^{2+}$  directly into the cytoplasm). The results showed that the histamine-releasing effect of each of these secretagogues could be inhibited by some, but not all, of the 11 flavonoids representing 5 different chemical classes (Middleton and Drzewiecki, 1984). Not

surprisingly, yet another stimulus of basophil histamine release, i.e., histamine releasing factor, can be inhibited by quercetin (Ezeamuzie and Assem, 1984). The nature of the stimulus for histamine release and the structure of specific flavonoids appeared to determine whether a particular compound would exert inhibitory activity. It appears that active flavonoids were generally those compounds with a planar conformation (Cody et al., 1988). The results suggested that each of the secretagogues may use a different pathway of cell activation (signal transduction) and that these pathways may be differentially sensitive to the action of particular flavonoids. The effect of quercetin to uniformly inhibit basophil histamine secretion stimulated by a variety of agonists strongly suggests that there is a final common pathway used by each of these agonists that is sensitive to quercetin and other structurally appropriate flavonoids.

Stimulation of  $\text{Ca}^{2+}$ -dependent protein phosphorylation during secretagogue-induced exocytosis in rat mast cells was described by Sieghart and coworkers (1978) and Theoharides et al. (1981). Purified rat peritoneal mast cells, which had been labeled with  $^{32}\text{P}$  and then stimulated by addition of compound 48/80, resulted in the phosphorylation of four proteins of apparent molecular weights of 78,000, 68,000, 59,000, and 42,000. Phosphorylation of the proteins with apparent molecular weights of 68,000, 59,000, and 42,000 was evident within 10 s after addition of 48/80; phosphorylation of the mol. wt. 78,000 protein, however, was not evident until 30 to 60 s after addition of the secretagogue. These experiments clearly indicated that the exocytosis of the mast cell was associated with phosphorylation of certain proteins, while recovery from secretion was related to phosphorylation of a unique protein. The same group of investigators (Theoharides et al., 1980) then showed that the "mast cell stabilizing", antiallergic drug disodium cromoglycate (cromolyn), which is structurally related to flavonoids (Fig. 2), promoted the incorporation of radioactive phosphate into a single rat mast cell protein with an apparent molecular weight of 78,000. The time course and dose dependence of phosphorylation of this protein closely paralleled inhibition of mast cell secretion (Theoharides et al., 1980). This finding provided an insight into the mechanism of inhibition by cromolyn of mast cell secretion triggered by an immunologic stimulus, anti-rat IgE. In subsequent experiments, these authors briefly noted that quercetin and kaempferol (10  $\mu\text{M}$ ), known inhibitors of rat mast cell histamine secretion, also increased the incorporation of radioactive phosphate into a single protein band with an apparent molecular weight of 78,000 (Sieghart et al., 1981). Recently, the same group of investigators (Correia et al., 1998) showed that the 78-kDa mast cell phosphoprotein had high homology to moesin, a member of the ezrin-radixin-moesin family of proteins (Furthmayr et al., 1992), which have recently been shown to regulate signal-transduction by coupling the cell surface

to the cytoskeleton (Tsukita et al., 1997). Phosphorylation of this protein was shown to take place by a calcium- and phorbol ester-independent PKC isozyme (Wang et al., 1999). More recently, this 78-kDa phosphoprotein was cloned and was shown to be identical to moesin (Theoharides et al., 2000); it was further shown that its phosphorylation by cromolyn induced some conformational change that permitted covalent binding to actin and resulted in preferential clustering around the mast cell secretory granules, thus possibly preventing them from undergoing exocytosis (Theoharides et al., 2000). Because of its apparent involvement in mast cell inhibition, this protein was also called "MAst Cell Degranulation Inhibitory Agent, MACEDONIA (Theoharides, 1996). The possible involvement of the cytoskeleton in the inhibitory action of quercetin was also suggested by the finding that it blocks heavy water-induced immunologic histamine release from basophils. Indeed, the augmenting effect of D<sub>2</sub>O on antigen-induced basophil histamine release (Gillespie and Lichtenstein, 1972), which is presumably due to an effect of D<sub>2</sub>O on microtubule assembly, was blocked by quercetin (Middleton et al., 1981), suggesting an effect of the flavonoid on cytoskeletal elements. Phosphorylation of moesin was also reported to occur only on threonine-558, the actin binding domain of the carboxyl termini, during thrombin activation of human platelets (Nakamura et al., 1995).

A still unresolved question is just what cellular component in activated mast cells or basophils first interacts with cromolyn or active flavonoids to inhibit the secretory process. Fewtrell and Gomperts (1977b) and Middleton et al. (1981) demonstrated that only activated mast cells or activated basophils were affected by quercetin and other inhibitory flavonoids (i.e., the unstimulated cells could be exposed to the flavonoids, washed, and subsequently shown to react normally to a secretagogue with histamine release.) Fewtrell and Gomperts (1977b) also observed that pretreatment of rat mast cells with cromolyn (30 μM) for 30 min completely abolished the inhibition normally observed upon subsequent exposure to quercetin (30 μM), added together with antigen. This finding suggested that cromolyn and quercetin acted at the same or a closely associated molecular site. The possible nature of that site could have been clarified by the experiments of Pecht and coworkers who described in detail a cromolyn-binding protein isolated from cultured RBL cells, but not from nonbasophil cells (Mazurek et al., 1980, 1982, 1983, 1984). However, this work had certain drawbacks: 1) cromolyn does not inhibit RBL secretion, suggesting that the RBL cromolyn binding site may be irrelevant; and 2) this binding protein apparently constituted a calcium channel, while cromolyn can inhibit 48/80-induced mast cell secretion in the absence of extra-cellular calcium ions. Other experiments suggested that another cromolyn-binding protein may be the enzyme nucleoside diphosphate kinase (Martin et al., 1995).

Basophils could be exposed to quercetin (50 μM) for 30 min and washed twice, resuspended, and then found to respond normally to antigen with histamine release. However, if the histamine secretory reaction was initiated and an active flavonoid such as quercetin was added at 2, 5, 10, or 15 min after addition of antigen, there was at each time point an immediate cessation of further release of histamine (Middleton et al., 1981). These observations indicated that antigen activation of basophils resulted in the generation of a flavonoid-sensitive substance(s), interaction of which with the flavonoid strikingly altered the outcome of the activation process. The nature of the flavonoid-reactive substance(s) is unknown.

Other evidence suggested that calmodulin may be involved in the mechanism of secretion of histamine from granules of mast cells and basophils (Marone et al., 1986). It is of interest, therefore, that quercetin appeared to interact with the Ca<sup>2+</sup>-calmodulin complex with resultant inhibition of Ca<sup>2+</sup>-dependent activities, including the effects of tumor promoters (Nishino et al., 1984a,b,c).

Ternatin (5,4'-dihydroxy-3,7,8,3'-tetramethoxy-flavone), isolated in 1989 from the flowers of *Egletes viscosa*, was found by Souza et al. (1992) to be a fairly potent inhibitor of IgE-dependent passive cutaneous anaphylaxis in mice and also to reduce the severity of the rat carrageenin pleurisy test following intraperitoneal administration.

In other experiments, Ogasawara et al. (1986) described inhibition of anti-IgE-induced H<sub>2</sub>O<sub>2</sub> generation and human basophil histamine release by quercetin, apigenin, and taxifolin. All three flavonoids inhibited the generation of H<sub>2</sub>O<sub>2</sub>, but only quercetin and apigenin inhibited anti-IgE-induced histamine release. These results, together with the data described above, suggested that quercetin and apigenin possess the structural features necessary for inhibition of histamine secretion, whereas all three compounds possess structural features required for inhibition of H<sub>2</sub>O<sub>2</sub> generation (Bors et al., 1990).

Several other investigators have also described inhibition of histamine release from mast cells by certain flavonoids (Ennis et al., 1980; Kubo et al., 1984; Amella et al., 1985; Bronner and Landry, 1985; Grossman, 1988), including some structurally unique flavonoid dimers such as amentoflavone (a biapigenin). Mast cells contain a high concentration of ascorbic acid, which undergoes oxidation to free radical species in stimulated cells (Ortner, 1980), suggesting that it may function as a radical scavenger, thus protecting against oxidative membrane damage during exocytosis. Flavonoids may also act in a similar fashion.

Several flavonoids possess LO inhibitory activity (Yoshimoto et al., 1983; Yamamoto et al., 1984; Welton et al., 1988). Marone et al. (1980) found that basophil histamine release was inhibited by eicosatetraenoic acid, a

unique LO inhibitor, and suggested that some LO-derived product of arachidonic acid metabolism may be required for basophil histamine release. Interestingly, many flavonoid inhibitors of histamine release are also good LO inhibitors. Several flavonoids are relatively selective inhibitors of 5-LO, which initiates the biosynthesis of leukotrienes, considered to be of importance in mediator release, inflammation, and immediate-type hypersensitivity reactions (Lewis and Austen, 1984; Lewis et al, 1990). Cirsiliol (3',4',5-trihydroxy-6,7-dimethoxyflavone) was a potent inhibitor of LO and caused 97% inhibition of the enzyme partially purified from RBL cells. At 10  $\mu\text{M}$ , the compound caused 99% suppression of immunologic release of leukotrienes from passively sensitized guinea pig lung ( $\text{IC}_{50}$ , approximately 0.4  $\mu\text{M}$ ) (Yoshimoto et al., 1983). Dermal mast cells store the proinflammatory cytokine  $\text{TNF-}\alpha$  in their granules, which is released upon mast cell activation. Mast cell-derived  $\text{TNF-}\alpha$  can directly induce the expression of endothelial leukocyte adhesion molecule-1, a critical event in the development of the inflammatory process. Cromolyn, the flavonoid related bis-chromone and mast cell degranulation inhibitor, blocked the induction of the endothelial leukocyte adhesion molecule-1, as did anti-serum against  $\text{TNF-}\alpha$  (Klein et al., 1989). The role of adhesion molecules in the recruitment of eosinophils and basophils has been well discussed by Bochner and Schleimer (1994). Also, Gaboury et al. (1995) indicated that 48/80-induced mast cell degranulation induced P-selectin-dependent leukocyte rolling. As reviewed by Hamawy et al. (1994), adhesion molecules act as regulators of mast cell and basophil function; thus, it is important that certain flavonoids could also modulate the expression of adhesion molecules (Anné et al., 1994; Gerritsen et al., 1995).

Involvement of the PTK family of kinase enzymes in mast cell histamine release has been established (Sagi-Eisenberg et al., 1984; Benhamou et al., 1990). Morita et al. (1988) demonstrated the involvement of PKC in RBL cell histamine secretion. Also, tyrosine kinase-dependent PI turnover and functional responses in the Fc $\epsilon$ R1 signaling pathway were studied in RBL-2H3 rat basophilic leukemia cells by Deanin et al. (1991). Antigen-induced PI turnover, secretion of [ $^3\text{H}$ ]serotonin, ruffling, and actin polymerization were inhibited by genistein (100  $\mu\text{M}$ ). These workers also showed that orthovanadate, a tyrosine phosphatase inhibitor, mimicked antigen stimulation, a nice example of the opposing effects of phosphorylation and dephosphorylation on a specific cellular function. Orthovanadate mimicked Fc $\epsilon$ R1 activation of PLC- $\gamma$  1 in permeabilized RBL cells by shifting the state of the cell to increased protein tyrosine phosphorylation (Atkinson et al., 1993). Based on studies of inhibition of serine-threonine and tyrosine kinases in antigen-stimulated exocytosis in RBL cells, it was determined that both tyrosine phosphorylation of cellular proteins and activation of PKC were necessary precon-

ditions for inositol phospholipid hydrolysis and exocytosis (Yamada et al., 1992). Kawakami and coworkers (1992) found that genistein, added to sensitized mouse bone marrow mast cells before antigen, inhibited PTK activation,  $\text{IP}_3$  formation, and histamine release; this data supported the concept that PTK activation precedes activation of PLC.

Lavens and coworkers (1992) also studied the effects of four different inhibitors of PTK on IgE-dependent histamine release from human lung mast cells and basophils. Genistein inhibited the anti-IgE-induced release of histamine from basophils ( $\text{IC}_{50}$ , 8  $\mu\text{M}$ ) but was less effective in the human lung mast cell. The genistein glycoside, genistin, and another isoflavone, daidzein, failed to affect the anti-IgE-induced histamine release in either cell type. The genistein effect did not appear to be through PKC inhibition because it failed to alter histamine release from basophils challenged with PMA. The authors suggested that different inhibitors of PTKs inhibit IgE-dependent histamine release from human lung mast cells and basophils by affecting different signal transduction mechanisms in the two cell types.

Certain flavonoids, notably quercetin, interfered with the activity of membrane transport ATPases, including the  $\text{Ca}^{2+}$ -dependent ATPase, which is one of the intrinsic cellular mechanisms that maintain low cytosolic  $\text{Ca}^{2+}$  concentrations. Fewtrell and Gomperts (1977a) found a very good correlation between the ability of certain flavonoids to inhibit rat mast cell histamine secretion and inhibition of  $\text{Ca}^{2+}$ -dependent ATPase activity. They suggested that the effect of quercetin to inhibit secretion from stimulated cells was due to its inhibitory effect on plasma membrane  $\text{Ca}^{2+}$ -ATPase. Racker (1986) suggested that the transport ATPases of cell membranes are separate structural entities that constitute the ATP-dependent ion pumps. Some flavonoids, including quercetin, inhibited aerobic glycolysis and growth of certain tumor cells by modulating the ATPase transport system (Suolinna et al., 1974). The "cromolyn-binding" protein of RBL cells, the cell surface  $\text{Ca}^{2+}$ -ATPase, and the molecular weight 78,000 mast cell phosphoprotein may somehow be linked together.

Based on recent studies, Kilpatrick et al. (1995) concluded that cromolyn inhibited in stimulated neutrophils the assembly of an active NADPH oxidase, which is required for the generation of the tissue-damaging oxyradical  $\text{O}_2^-$ . This is a significant observation that indicates that cromolyn, which is structurally related to the flavonoids, may have different mechanisms of action in different cell types.

Preliminary experiments (Middleton and Foreman, 1984) showed that rat mast cells stimulated with anti-IgE released less histamine and [ $^3\text{H}$ ]arachidonic acid, and took up less  $^{45}\text{Ca}^{2+}$ , in the presence of quercetin (10–50  $\mu\text{M}$ ). These results suggested inhibition by quercetin of phospholipase  $\text{A}_2$  and processes involved in  $\text{Ca}^{2+}$  uptake. However, O'Rourke et al. (1992) found that

quercetin inhibited arachidonic acid release in antigen-stimulated RBL cells without affecting levels of inositol phosphate production. The latter finding suggested that quercetin had no effect on PLC in these experiments.

The growth of human cord blood-derived basophils was inhibited by baicalein according to Tanno et al. (1989), an observation suggesting that cytokine-dependent cellular growth stimulation is sensitive to selected flavonoids. Similarly, Alexandrakis et al. (1999) reported that quercetin, myricetin, and kaempferol, but not morin, inhibited the growth and basal secretion from RBL cells and induced maturation.

#### F. Neutrophils

The inhibitory effect of flavonoids on secretory processes is not limited to basophils and mast cells. Bennett et al. (1981) and Showell et al. (1981) showed that several flavonoids were capable of inhibiting stimulated rabbit neutrophil lysosomal enzyme release. Also, Schneider et al. (1979) and Berton and coworkers (1980) reported that concanavalin A-induced secretion of lysosomal enzyme from polymorphonuclear leukocytes of albino guinea pigs and healthy human volunteers was inhibited by quercetin; this flavonoid had no effect on the binding of concanavalin A to the cell membrane receptors. Rutin and morin were inactive, in keeping with the findings of the human basophil experiments. Tyrosine phosphorylation induction by TNF- $\alpha$  in mitogen-activated adherent human neutrophils was inhibited by genistein (Rafiee et al., 1995).

Oxygen free radicals and nonradical reactive oxygen intermediates released by neutrophils and other phagocytes have been increasingly implicated in inflammatory/immune disorders (Fantone and Ward, 1982; Ward et al., 1991). Different classes of flavonoids are known to scavenge oxygen free radicals (Bors et al., 1990). Flavonoids could profoundly impair the production of reactive oxygen intermediates by neutrophils and other phagocytic cells. This may be accomplished by interference with NADPH oxidase, a powerful oxidant-producing enzyme localized on the surface membrane of neutrophils (Tauber et al., 1984). Flavonoids could also inhibit neutrophil myeloperoxidase (MPO), a source of reactive chlorinated intermediates (Pincemail et al., 1988). The effect of flavonoids on the production of reactive oxygen intermediates by neutrophils is discussed below. Impairment by flavonoids of the production of active oxygen intermediates by neutrophils and other phagocytes might contribute to the anti-inflammatory activity of these compounds.

Lee et al. (1982) examined the effect of quercetin on the release of  $\beta$ -glucuronidase from human neutrophils stimulated with opsonized zymosan and found that quercetin inhibited the release of  $\beta$ -glucuronidase, although the effect was not strong. However, these authors found that the release of [ $^3$ H]arachidonic acid from prelabeled neutrophils was also inhibited by quercetin,

strongly suggesting an inhibitory effect of the flavonoid on phospholipase A<sub>2</sub> and in keeping with the findings of Lanni and Becker (1985). Of considerable interest is the finding that human synovial fluid phospholipase A<sub>2</sub> activity was also inhibited by quercetin in vitro; retinoids such as retinal, retinol, retinic acid, and retinol acetate produced similar inhibition of human synovial fluid phospholipase A<sub>2</sub>. These investigators also described inhibition of the Ca<sup>2+</sup>-dependent phospholipase A<sub>2</sub> preparation from human plasma. The enzyme activity in *Naja massambica mossambica* venom was similarly inhibited (Fawzy et al., 1988).

Experiments performed by Busse and coworkers (1984) showed that quercetin and chalcone were weak inhibitors of neutrophil  $\beta$ -glucuronidase secretion stimulated by opsonized zymosan. These investigators also described that quercetin and several other flavonoids were quite effective inhibitors of opsonized zymosan-stimulated generation of superoxide anion. Long et al. (1981) found that quercetin had at least three separate effects on human polymorphonuclear leukocytes: 1) it inhibited the Mg<sup>2+</sup>-dependent ecto-ATPase in a noncompetitive fashion; 2) it inhibited O<sub>2</sub> consumption, glucose oxidation, and protein iodination in cells exposed to opsonized zymosan and TPA; and 3) it inhibited transport of the nonmetabolizable glucose analog, [ $^3$ H]2-deoxyglucose. Tordera et al. (1994) assessed the effects of 24 flavonoids, reported to be anti-inflammatory, on lysosomal enzyme secretion and arachidonic acid release in rat neutrophils. Amentoflavone, quercetagenin-7-O-glucoside, apigenin, fisetin, kaempferol, luteolin, and quercetin were the most potent inhibitors of  $\beta$ -glucuronidase and lysozyme release. These flavonoids significantly inhibited arachidonic acid release from membranes, and there was a correlation between degranulation and arachidonic acid release (PLA<sub>2</sub> activation).

Quercetin inhibited the activation of rabbit peritoneal neutrophils stimulated by f-MetLeuPhe, as determined by measurement of degranulation and superoxide formation; quercetin also inhibited tyrosine phosphorylation, mitogen-activated protein kinase, and phospholipase D (Takemura et al., 1997). Neutrophil protein tyrosine phosphorylation stimulated by chemotactic factors was diminished by genistein (Rollet et al., 1994), while pertussis toxin blocked the tyrosine phosphorylation response to f-MetLeuPhe.

Neutrophil cytokinesis is accompanied by changes in membrane fluidity and polarity caused by movement of active microfilaments toward the leading edge of the moving cell. Interestingly, fisetin, kaempferol, chrysin, flavonol, morin, and quercetin (in decreasing order of activity) enhanced both random and f-MetLeuPhe-directed migration in murine neutrophils in vitro, while flavone inhibited both random and directed movement (Kenny et al., 1990). On the other hand, quercetin administered intraperitoneally in rats reduced in a dose-dependent manner leukocyte migration into carrag-

enin-induced pleural exudates (Mascolo et al., 1988). This flavonoid also reduced the synthesis of PGE<sub>2</sub> and LTB<sub>4</sub> by the inflammatory cells, while apigenin and luteolin decreased leukocyte accumulation and PGE<sub>2</sub> synthesis, but not LTB<sub>4</sub> formation. These results suggested that there was some stereoselectivity of flavonoid inhibition of CO and LO pathways of arachidonic acid metabolism. The generation of human polymorphonuclear leukocytes luminol-enhanced chemiluminescence stimulated by opsonized zymosan, PMA, and f-MetLeuPhe was inhibited in each case by silybin (0.5–25 mg/ml). There was no effect on phagocytosis or response to chemotactic stimuli (Minonzio et al., 1988). Baicalein inhibited ionophore-induced human polymorphonuclear leukocytes LTB<sub>4</sub> and LTC<sub>4</sub> synthesis and degranulation with accompanying  $\beta$ -glucuronidase release, all in a noncyclic AMP-dependent manner (Kimura et al., 1987). From these various experiments, it is clear that the action of flavonoids on arachidonic acid release and metabolism is complex and related to cell type and activation stimulus.

### G. Eosinophils

Ionophore A23187-induced eosinophil secretion of Charcot-Leyden crystal protein and eosinophil cationic protein was inhibited by quercetin, but not by taxifolin (dihydroquercetin), in a concentration-dependent manner (Sloan et al., 1991). Thus, the activated eosinophil appears to respond to these flavonoids in the same fashion as basophils and mast cells. Whether eosinophil degranulation stimulated by other immunologic or nonimmunologic stimuli, such as allergen or PAF, would be inhibitable by selected flavonoids remains to be determined. Eosinophil degranulation stimulated by IgA- or IgG-coated beads was inhibited by genistein; at the same time, several phosphorylated proteins were decreased in quantity, and PLC activation was inhibited (Kato et al., 1995).

### H. Platelets

In addition to their role in hemostasis and thrombosis, considerable evidence implicates platelets as inflammatory cellular elements (Weksler, 1983; Metzger and Page, 1998). Several proinflammatory mediators are derived from platelets, including thromboxane A<sub>2</sub> and serotonin, as well as TGF- $\beta$ , PDGF, and LO metabolites, some of which are implicated in the pathogenesis of asthma (Metzger and Page, 1998). Platelets are also key participants in atherogenesis. Platelet factor 4 concentration increases in plasma of allergic asthmatics after bronchial challenge with specific antigen, but not with the nonimmunologic bronchoconstrictor stimulus, methacholine (Knauer et al., 1981). Blood platelet numbers may decrease in patients undergoing allergen challenge (Maestrelli et al., 1990).

Platelet activating factor (PAF) is a well recognized proinflammatory mediator derived from membrane phos-

pholipids by the enzymatic activity of phospholipase A<sub>2</sub> and an acetyl transferase in mast cells, basophils, eosinophils, and endothelial cells. PAF receptor-coupled activation of phosphoinositide-specific phospholipase C and phosphorylation of several cellular proteins has been reported. Dhar and colleagues (1990) used the isoflavonoid genistein to investigate the possible involvement of tyrosine kinase in PAF-stimulated platelets and the relationship between protein phosphorylation and PLC activation. PAF alone stimulated PLC activity, as measured by the production of IP<sub>3</sub>. Genistein (0.5 mM) decreased PAF-stimulated PLC activity to control levels. At this concentration, genistein also blocked PAF-stimulated platelet aggregation. In addition, genistein also reduced PAF-induced phosphorylation of proteins of mol. wt. 20,000 and 50,000. Taken together, these results strongly suggested that genistein inhibited PTK at an early stage of signal transduction, resulting in inhibition (or associated with inhibition) of PLC; this action could, in turn, result in decreased activation of PKC via reduced PLC-catalyzed formation of DAG. The combined effects would, therefore, result in a reduction of protein phosphorylation. Based on these and other experiments, the authors concluded that tyrosine phosphorylation is involved in the PAF receptor-coupled activation of PLC. It is tempting to speculate that there may be other isoflavonoid or flavonoid compounds, both natural and synthetic, which could affect the outcome of PAF-stimulated pathological states.

In light of the above, it is of interest that several flavonoids significantly (1–10  $\mu$ M) inhibited platelet adhesion, aggregation, and secretion. This subject has been reviewed in detail (Beretz and Cazenave, 1988). Flavonoid effects on platelets have been related to the inhibition of arachidonic acid metabolism by CO (Corvazier and Maclouf, 1985). Alternatively, certain flavonoids are potent inhibitors of cyclic AMP phosphodiesterase, and this may in part explain their ability to inhibit platelet function. The effect of selected flavonoids on platelet aggregation/adhesion is akin to their effect on mononuclear cell adhesion, as described earlier, and is another example of their potential capacity to regulate the expression and activity of adhesion molecules (Beretz et al., 1982). Fisetin (at relatively high concentrations) completely inhibited aggregation of washed human platelets induced by two serine proteases, thrombin and cathepsin G, (Puri and Colman, 1993). The experiments of Tzeng et al. (1991) demonstrated that several flavonoids could act as inhibitors of thromboxane formation, as well as thromboxane receptor antagonists.

Even though genistein inhibited platelet aggregation and serotonin secretion, tyrosine phosphorylation stimulated by thrombin was only weakly affected (Nakashima et al., 1990). On the other hand, this isoflavone suppressed platelet aggregation, serotonin secretion, and protein tyrosine phosphorylation triggered by collagen and stable thromboxane A<sub>2</sub> analogs. These results indicate that the flavonoid effects could depend on the

type of the stimulus, as well as the cell type. Interestingly, genistein competitively inhibited the binding of the stable thromboxane A<sub>2</sub> analog U46619 to washed platelets. Daidzein, an isoflavone lacking a 5-position hydroxyl group, was also capable of inhibiting binding of U46619, even though it was inactive as a PTK inhibitor (Nakashima et al., 1990). Platelet aggregation induced by U46619 was also antagonized by fisetin, kaempferol, morin, and quercetin. The suggestion was made that the antiplatelet effect of flavonoids may be explained by both inhibition of thromboxane synthesis and thromboxane receptor antagonism (Tzeng et al., 1991). A role for tyrosine kinases in control of Ca<sup>2+</sup> entry in stimulated human platelets was provided by Sargeant et al. (1993), who reported that ADP-induced protein phosphorylation and [Ca<sup>2+</sup>] increase were blocked by genistein. Daidzein had no effect on either process, yet another example of striking differences in structure-activity relationships. Through effects on polyphosphoinositide turnover, genistein attenuated thrombin-induced Ca<sup>2+</sup> mobilization in human platelets (Ozaki et al., 1993). Protein phosphorylation induced by thrombin was not affected by genistein, suggesting that its inhibitory activity against polyphosphoinositides was not related to tyrosine kinase inhibition. Murphy et al. (1993) found that Ca<sup>2+</sup> mobilization and influx, IP<sub>3</sub> generation, and phosphorylation of several rabbit platelet proteins stimulated by PAF were inhibited by genistein. On the other hand, while stimulation with  $\alpha$ -thrombin, ionomycin, or TPA showed a profile of genistein-inhibitable protein phosphorylation similar to that induced by PAF, the functional responses were not inhibited by genistein. Human platelets treated with genistein and exposed to thrombin were only slightly inhibited with respect to aggregation and serotonin release. However, the increase in intracellular Ca<sup>2+</sup> concentration was substantially reduced (Ozaki et al., 1993). Genistein also inhibited the CO pathway and the accumulation of IP<sub>3</sub> in a concentration-dependent manner.

Robbins (1988) reported that citrus flavones and *Vaccinium myrtillus* (Bilberry) anthocyanosides inhibited platelet aggregation in an ex vivo study. In studies of human platelet aggregation, epigallocatechin moderately inhibited aggregation and thromboxane synthesis, while gallic acid and epicatechin-3-O-gallate were quite active as inhibitors of H<sub>2</sub>O<sub>2</sub>-induced endothelial cell injury (Chang and Hsu, 1991). At high concentrations, quercetin inhibited porcine platelet aggregation (Tomasiak, 1992). Finally, note that genistein significantly inhibited phosphoinositide phosphorylation in human platelets stimulated with an endoperoxide analog, while flavone and biochanin A were without effect (Gaudette and Holub, 1990).

Several flavonoids from *Eupatorium odoratum* have been isolated and structurally characterized by Trirantana et al. (1991). This plant has long been used as a hemostatic in traditional Thai medicine. One compound,

4',5,6,7-tetramethoxyflavanone, was found to significantly reduce the activated partial thromboplastin time, while having no effect on prothrombin time or thrombin time. This result suggested that this compound could act to enhance blood coagulation by possibly affecting factors XII, XI, IX, and VIII. Several flavonoids (e.g., baicalin and oroxylin A) were found to be potent inhibitors of NAD(P)H:quinone acceptor oxidoreductase (Chen et al., 1993). Most oral anticoagulants are inhibitors of this enzyme and antagonize vitamin K. Consequently, selected flavonoids may be potentially useful anticoagulant drugs.

Hispidulin (4',5,7-trihydroxy-6-methoxyflavone), a naturally occurring flavonoid derived from the flowering parts of *Arnica montana*, inhibited human platelet aggregation stimulated by adenosine monophosphate, arachidonic acid, PAF, and collagen (Bourdillat et al., 1988). The potential of this and related flavonoids as useful antiplatelet agents remains to be tested.

### I. Adhesion Molecule Expression

The development of an inflammatory process requires that local endothelial cells become activated and express adhesion molecules on their surface; these interact with related molecules on the surface of activated circulating leukocytes, which then stick firmly to the endothelium and transmigrate into the inflammatory site (Aplin et al., 1998). Exposure of endothelial cells to cytokines such as IL-1, TNF $\alpha$ , interferon- $\gamma$ , or LPS stimulates the expression of certain adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1). Gerritsen et al. (1995) showed that apigenin (and several other flavonoids) blocked cytokine-induced expression of ICAM-1, vascular cell adhesion molecule-1, and E-selectin on human endothelial cells. Apigenin also proved to be an active anti-inflammatory agent in the rat paw carrageenin model and in a contact sensitivity test in mice. Similar findings were obtained by Anné et al. (1994) where quercetin inhibited the generation of ICAM-1 in umbilical vein endothelial cells (HUVECs) stimulated with LPS, with accompanying reduction of lymphocyte adhesion to the endothelial cells. Panés et al. (1996) characterized the effect of apigenin on TNF-stimulated ICAM-1 expression in different rat tissues in vivo. Apigenin blocked ICAM-1 up-regulation in all tissues, but to a variable degree. Naringenin, structurally related to apigenin, had no effect, indicating significant structure-activity relationships.

As noted with other cellular processes, different classes of flavonoids behave differently with respect to adhesion molecule expression. For example, Tiisala and coworkers (1994) found that genistein enhanced ICAM-mediated adhesion. It actually induced the expression of ICAM-1 and its counter-receptors in several different cell lines by potentiating the up-regulating action of TNF and interferon (IFN)- $\gamma$ . McGregor and coworkers (1994) found that genistein inhibited up-regulation of

neutrophil and monocyte adherence, but had no effect on lymphocyte adherence on HUVECs stimulated with the cytokines IL-1 and TNF. In contrast, apigenin and quercetin did inhibit lymphocyte adherence. Possible loci of action for the effect of active flavonoids are as follows: 1) TNF interaction with its cellular receptor, 2) G protein-coupled activation of phospholipases, 3) generation of free radicals, and 4) damage to nuclear DNA by endonucleases (Larrick and Wright, 1990). Tanetin (6-hydroxykaempferol 3,7,4'-trimethyl ether), a new lipophilic flavonol found in the ancient traditional medicinal plant, feverfew, was shown to contribute to the anti-inflammatory properties of the plant inhibiting the generation of proinflammatory arachidonic acid derivatives (Williams et al., 1995). Synthetic flavonoids were also investigated for effects on adhesion molecule gene expression and synthesis (Wölle et al., 1996).

In investigations of skin inflammation in rats, apigenin-7-glucoside proved to be an effective anti-inflammatory agent in these animals treated with different generators of reactive oxygen species and free radicals (Fuchs and Milbradt, 1993). Gabor and Razga (1991) found several flavonoids to be active inhibitors of croton oil-induced ear edema and carrageenin-induced paw edema. Myricetin and delphinidin also exhibited marked anti-inflammatory effects. Another biflavonoid, called procyanidin (actually a bicatechin), was a moderately effective inhibitor of rat paw edema induced by serotonin, carrageenin, or PGE (Blazsó and Gábor, 1980). An immunologically-stimulated chronic ileitis of guinea pigs (resembling Crohn's disease) was modified favorably by genistein, with reduction of granulocyte infiltration, reduction in NO production, and improved mucosal architecture (Sadowska-Krowicka et al., 1998). These observations showing an inhibitory effect of low molecular weight flavonoids on inflammation are important because they suggest that consumption of dietary flavonoids may have inflammatory-disease-preventing properties. These results also point to the possible development of new therapeutic agents.

#### IV. Effects of Flavonoids on Other Cells

##### A. Smooth Muscle and Cardiac Muscle Cells

Early studies (Gabor, 1979) suggested that some flavonoids could affect smooth muscle contractility in response to various agonists. For example, Foucard and Strandberg (1975) observed that phloretin derivatives antagonized the contractile activity of human bronchial smooth muscle stimulated with prostaglandin  $F_{2\alpha}$  at concentrations that had no effect on the response of the same smooth muscle to histamine. In addition, polyphloretin phosphate inhibited antigen-induced histamine release from human lung tissue that had been passively sensitized with IgE antibodies from serum of individuals allergic to birch pollen or horse dander.

Several flavonoids were shown to possess moderately potent activity (10–50  $\mu\text{M}$ ) against agonist-induced contractile responses of guinea pig ileal longitudinal smooth muscle stimulated by histamine, acetylcholine, and PGE<sub>2</sub> (Macander, 1986). Quercetin inhibited both the initial phase and the sustained tonic components of an antigen-induced anaphylactic contraction of longitudinal smooth muscle from ileum of guinea pigs sensitized with ovalbumin (Fanning et al., 1983). Inhibition of the anaphylactic contraction was concentration-dependent with an IC<sub>50</sub> of approximately 10  $\mu\text{M}$ . The initial portion of the contractile response is related to the availability of membrane-bound  $\text{Ca}^{2+}$ , while the tonic (sustained) phase is related to the availability of extracellular  $\text{Ca}^{2+}$  (Chang and Triggle, 1973). The results of these experiments suggested that quercetin could affect the ultimate availability of  $\text{Ca}^{2+}$  to the contractile machinery of the smooth muscle, but effects on crucial enzyme systems, such as myosin light chain kinase, for example, were not ruled out.

Quercetin potently stimulated secretion in a human colonic tumor cell line (T<sub>84</sub>) (Nguyen et al., 1991). Using the same in vitro model of colonic secretion, Nguyen and Canada (1993) studied the effect of several citrus flavonoids on colonic T<sub>84</sub> cell secretion. Tangeretin and nobiletin stimulated sustained electrogenic chloride secretion. The glycosylated compounds naringin and hesperidin were essentially inactive. The secretion stimulated by the polymethoxylated flavonoids was synergistic with carbachol, but not with vasoactive intestinal peptide. These flavonoids did not stimulate cAMP formation. Quercitrin increased colonic fluid absorption in mice and rats (antidiarrheal effect), but only in the presence of secretagogues such as PGE<sub>2</sub>. (Galvez et al., 1993).

Stern and coworkers (1989) demonstrated that baicalin, a potent LO inhibitor, strikingly reduced the in vitro contractile response of artery rings to angiotensin II, in contrast to norepinephrine, which had no effect. It appeared, therefore, that LO blockade led to a direct and selective inhibition of angiotensin II-induced vasoconstriction and that products of the LO pathway could play a significant role in mediating the pressor effect of angiotensin II.

In studies using isolated rat vascular smooth muscle, Duarte et al. (1993) found that the contractile responses induced by high KCl,  $\text{Ca}^{2+}$ , and PMA were inhibited by quercetin in a concentration-dependent manner. The authors considered that the vasodilator action was mainly related to inhibition of PKC.

The spasmolytic effect of methanolic extracts of *Psidium guajava L* has been attributed to quercetin, a flavonoid contained in this plant (Lozoya et al., 1994). Quercetin produced smooth muscle relaxation on isolated guinea pig ileum previously contracted by a depolarizing KCl solution (Morales et al., 1994). Quercetin

inhibited intestinal contraction induced by different concentrations of calcium.

Apigenin inhibited the contractile response of rat thoracic aorta to several agonists. It caused relaxation in precontracted muscle, which was endothelium-cyclic nucleotide-independent. Apigenin apparently caused relaxation in this preparation by decreasing  $\text{Ca}^{2+}$  influx through both voltage- and receptor-operated  $\text{Ca}^{2+}$  channels (Ko et al., 1991). The spasmolytic action of quercetin may be explained by its inhibition of  $\text{Ca}^{2+}$  entry into smooth muscle cells (Morales and Lozoya, 1994). A recently described flavanone, 7-*O*-methylepideriodytyol, isolated from *Artemesia monosperma*, also possessed smooth muscle relaxing activity in several rat preparations (Abu-Niaaj et al., 1993). Cirsiliol also proved to inhibit rat isolated ileum stimulated with acetylcholine through an effect on calcium movements (Mustafa et al., 1992).

Sodium vanadate, a potent inhibitor of protein tyrosine phosphatases, caused smooth muscle contraction and enhanced phosphorylation, events that appear to be coupled; both processes were inhibited by genistein (Di Salvo et al., 1993). Huckle and Earp (1994) found that ionophore-induced tyrosine phosphorylation in rat liver epithelial cells was strikingly increased by a combination of vanadate plus flavonoids containing catechol nuclei. Working along similar lines, Lutterodt (1989) found quercetin to cause a morphine-like inhibition of acetylcholine release from stimulated guinea pig ileum. Interestingly, quercetin is a major component of several plants used for centuries as antidiarrheal remedies.

In rat and rabbit pulmonary artery cells, the voltage-gated  $\text{K}^+$  current was blocked in a concentration-dependent manner (20–100  $\mu\text{M}$ ) by genistein, but not by its close chemical relative, daidzein (Smirnov and Aaronson, 1995). The flavonoid hispidulin (5,7,4'-trihydroxy-6-methoxyflavone) was shown to have variable effects on guinea pig tracheal, ileal, and pulmonary vascular smooth muscle. The authors considered that this compound may act by interfering with agonist- $\text{Ca}^{2+}$  receptor protein coupling (Abdalla et al., 1988). The exocytotic, isoproterenol-stimulated release of amylase from parotid acinar cells was inhibited by genistein, but not by daidzein, the closely related isoflavone. Genistein also inhibited the exocytotic action of two cAMP derivatives (Takuma et al., 1996).

The biflavonoid amentoflavone (biapigenin) appeared to have antiulcerogenic properties in rats and guinea pigs; such properties appeared to be of interest with respect to the adverse effect of gastric ulceration, which develops commonly in subjects taking anti-inflammatory drugs (Gambhir et al., 1987). Oral quercetin was also shown to have antiulcer and gastroprotective activity; additionally, quercetin also caused a marked increase in gastric mucus (Alarcon de la Lastra et al., 1994).

Exposure of rabbit pericardial cells to EGF and insulin-like growth factor-I cooperatively increased hyaluronic acid synthase activity and hyaluronic acid synthesis. Pretreatment with genistein affected the growth factor activity but had no direct effect on hyaluronic acid synthase activity (Honda et al., 1991).

Mulberry is the source of two complex flavonoids, kwanon G and H, which can antagonize the binding of gastrin-releasing peptide to gastrin-releasing peptide-preferring bombesin receptors in murine Swiss 3T3 fibroblasts (Mihara et al., 1995). A cytoprotective, antiulcer (gastroprotective) effect of the citrus flavonoid naringin has been described (Martin et al., 1994).

The effects of flavone on myocardial postischemic reperfusion recovery was studied by Ning and coworkers (1993). Rabbit hearts were made modestly hypothermic (34°C) and left ventricular functional recovery was evaluated. Flavone treatment caused significantly better recovery of left ventricular developed pressure; end-diastolic pressures were significantly lower in the flavone-treated group compared with control. In addition, myocardial oxygen consumption was higher in the flavone-treated group. The salutary effects of flavone infusion were abolished by SKF 525-A, a P450 inhibitor, thus indicating a relationship between the flavone effect and P450 metabolism. The hypertrophic response of cultured rat ventricular myocytes to phenylephrine was prevented by genistein (Thorburn and Thorburn, 1994). Genistein also inhibited the phenylephrine-induced activation of three promoters: fos, atrial natriuretic factor, and MLC-2, all of which are activated in the hypertrophic response. Phenylephrine also induced activation of MAP kinases Erk 1 and Erk 2 and also inhibits GTP loading of the Ras proteins (Thorburn and Thorburn, 1994). Taken together, these results suggested that a genistein-sensitive step may be critical for activation of the Ras-MAP kinase pathway by phenylephrine.

The protective effect of silybin on spontaneously hypertensive rats subjected to acute coronary artery occlusion was studied by Chen et al. (1993). Silybinin reduced mortality and blood pressure, as well as the severity of ventricular hypertrophy. Baicalein is a component of the traditional Japanese herbal medicine (Kampo, TJ-960) used for treatment of epilepsy (Hamada et al., 1993).

### B. Effects on Nerve Cells

Electrical stimulation of the guinea pig myenteric plexus preparation causes acetylcholine release and smooth muscle contraction; it is of interest that quercetin effectively inhibited the release of (preloaded) [ $^3\text{H}$ ]choline as well as the contractile response (Kaplita and Triggle, 1983). It is intriguing that electrically driven acetylcholine release, a secretory process roughly analogous to basophil histamine release, was also inhibited by quercetin.

According to Nielsen et al. (1988), the brain possesses benzodiazepine receptors, which bind the biflavonoid

amentoflavone with an  $IC_{50}$  of 6  $\mu M$  in vitro, an affinity comparable with diazepam. Amentoflavone, however, did not inhibit [ $^3H$ ]flunitrazepam binding to brain benzodiazepine receptors. Another flavonoid with central benzodiazepine receptor-binding activity was chrysin (5,7-dihydroxyflavone). In a murine test system, chrysin proved to have anxiolytic activity, without inducing sedation and muscle relaxation (Wolfman et al., 1994). Another observation of real interest along these lines is the fact that 7-bromoflavone was a high-affinity ligand for central benzodiazepine receptor and had anxiolytic activity equivalent to diazepam (Marder et al., 1996). Neuronal protein synthesis initiation was depressed by genistein, but at quite high concentrations. Nevertheless, this finding led the investigators to consider that a protein tyrosine kinase in neurones was involved by affecting the activity of eukaryotic initiation factor-2 (Hu et al., 1993).

Nerve growth factor stimulates the extension of PC12 pheochromocytoma nerve fibers with an accompanying increase in arachidonic acid metabolism. The LO inhibitor baicalein (but not CO inhibitors) proved to be a potent blocker of nerve fiber growth (DeGeorge et al., 1988). Apigenin inhibited proliferation (at G2/M) of rat B104 neuronal cells and induced morphological differentiation of these cells (Sato et al., 1994). Quercetin protected sensory ganglion cells from GSH depletion-induced death (Skaper et al., 1997).

Amine uptake into human neuronal and neuroendocrine cell lines has been investigated by Sher et al. (1992). Diosmetin, but not the glycoside diosmin, concentration dependently inhibited the uptake of [ $^3H$ ]dopamine ( $IC_{50}$ , 4  $\mu M$ ) thus indicating an effect of certain flavonoids on plasma membrane amine transporters. On the other hand, Morita et al. (1988) discovered that flavone markedly increased tyrosine uptake into cultured bovine adrenal chromaffin cells, while apigenin caused a moderate effect. Myricetin, phloretin, luteolin, and several other flavonoids proved to be relatively weak inhibitors (100  $\mu M$ ) of ATP-dependent  $Ca^{2+}$  uptake by rat liver plasma membrane vesicles (Thiyagarajah et al., 1991).

### C. Calcium Homeostasis

Flavonoid effects extend to osteoclasts. Ipriflavone, a synthetic isoflavone, inhibited bone resorption in bone organ cultures; osteoclastogenesis appeared to be inhibited, but with no effect on mature osteoclasts (Notoya et al., 1993). However, Albanese and coworkers (1994) claimed that ipriflavone inhibited osteoclastic activity in isolated osteoclasts via an effect on intracellular free  $Ca^{2+}$ . This compound has been shown to be active in clinical settings of osteopenic and osteoporotic women (Brandi, 1993). Valente et al. (1994) reported increases in bone mineral density of postmenopausal women treated with ipriflavone for one year. Genistein was found to inhibit the proliferation of osteoblast cell line

G292 stimulated by EGF (Stephan and Dziak, 1994). Genistein inhibited  $Ca^{2+}$  influx mediated by thapsigargin (Yule et al., 1994).

Geniste and coworkers (1984) reported that 5,7,3',4'-tetrahydroxyflavan was an effective compound in the treatment of experimental periodontitis in the golden hamster. Whether the effect of this compound was caused by the preservation of an efficient microcirculation of the bone and gingiva was not clear. The flavonoid did diminish alveolar bone loss, as demonstrated histologically, and thus appeared to slow down the process of bone resorption.

Ipriflavone inhibited the differentiation and activity of osteoclasts but also promoted differentiation of osteoblast-lineage cells, a double-barreled approach to staving off osteoporosis (Ozawa et al., 1992). The experiments of Yamazaki and Kinoshita (1986) showed that ipriflavone increased the sensitivity of the thyroid gland to estrogen to secrete calcitonin in response to calcium. Mousavi and Adlercreutz (1993) demonstrated that genistein was an effective stimulator of sex hormone-binding globulin formation by human hepatocarcinoma cells, indicating the capacity of this isoflavonoid to up-regulate the gene responsible for sex hormone-binding globulin production. Genistein also inhibited the proliferation of these cells in tissue culture.

## V. Endocrine and Metabolic Effects

The effects of flavonoids on estrogen receptors are discussed in the section dealing with their effects on estrogen-dependent tumor cells.

An infertility syndrome of sheep, first described in western Australia, is recognized to be caused by ingestion of certain species of clover containing the phytoestrogen isoflavonoid formononetin, which is transformed by gut microflora to equol (Bennetts et al., 1946). Equol has estrogenic properties and is absorbed into the circulation. Also, equol competitively antagonized estradiol-17- $\beta$  binding to cytoplasmic estrogen receptors. Perhaps of clinical significance for human infertility is the finding of urinary excretion of equol in human urine by gas chromatography-mass spectrometry and NMR (Axelson et al., 1982).

In related studies, Adlercreutz et al. (1993) measured the concentrations of several isoflavonoids (genistein, daidzein, equol, and *O*-desmethylangolensin) in plasma of Japanese and Finnish men. The geometric mean levels were 7 to 110 times higher in the Japanese than in the Finnish men, which correlates with the high intake of dietary sources of isoflavonoids, particularly soybeans, soy meal, and tofu, by the Japanese. Taken together with the antiproliferative and other activities of genistein, this diet may account for the low mortality from prostatic cancer in Japanese men. Genistein concentrations in urine of subjects consuming a traditional soy-rich Japanese diet were in the micromolar range,

while these concentrations were 1/30th or less of those in urine of omnivores (Adlercreutz et al., 1991).

Bannwart et al. (1984) described the presence of the phytoestrogen daidzein in human urine by GC-MS. The isoflavonic phytoestrogens have been shown to bind with relatively high affinities to the estrogen receptors of human mammary tumor cells (Martin et al., 1978). They may, therefore, be implicated in the inhibition of breast carcinoma cell growth mediated by estrogen. Plasma concentrations of the isoflavonoid phytoestrogens genistein, daidzein, and equol have been measured in postmenopausal Australian women and were found to increase when the diet was supplemented with soya (Morton et al., 1994).

Acacetin and luteolin by oral administration showed a dose-dependent capacity to inhibit implantation of fertilized eggs in Wistar albino rats (Hiremath and Rao, 1990). The antifertility properties of flavonoids require further study.

Isoflavones, in the form of a diet rich in soy protein, were studied for their effect on the menstrual cycle of premenopausal women (Cassidy et al., 1994). Mid-cycle increases of luteinizing hormone and follicle-stimulating hormone were significantly reduced during the dietary intervention. Isoflavones such as genistein could, because of their antiestrogen effects, be useful especially in the management of women at high risk for breast cancer and may also help explain the relatively low incidence in Japanese and Chinese women with a high soy intake.

Extracts of some plants contain antihormonal components, explaining some long-standing uses in traditional medicine. Miksicek (1995) surveyed the structural features of polycyclic phenols associated with estrogenic activity. Natural estrogens belong to several chemically related classes: chalcones, flavanones, flavones, flavonols, and isoflavones. Aufmkolk et al. (1986) noted the action of aurones from plant extracts to inhibit rat liver iodothyronine deiodinase, the regulator of extrathyroidal thyroxine metabolism. Some aurones produced potent, concentration-dependent inhibition of three different metabolic monodeiodination pathways catalyzed by rat liver microsomal type I iodothyronine deiodinase. The most potent plant-derived inhibitors of the deiodinase system ( $IC_{50}$ ,  $0.50 \mu\text{M}$ ) were the 3',4',4,6-(tetra)trihydroxyaurones. Computer graphic modeling studies were used to confirm aurone conformations with the conformation of the thyroid hormones and suggested the possibility of using this procedure to design other deiodinase inhibitors (Koehrlle et al., 1986).

Genistein strongly inhibited the effect of an  $A_1$ -adenosine receptor agonist on thyroid-stimulating hormone-induced PLC activation in FRTL-5 thyroid cells. Genistein also competitively inhibited adenosine-induced cAMP accumulation in pertussis toxin-treated cells (Okajima et al., 1994).

Quercetin proved to be an effective inhibitor of insulin receptor tyrosine kinase-catalyzed phosphorylation of a

glutamic acid-tyrosine random copolymer, while insulin stimulated autophosphorylation of the receptor itself. In rat adipocytes, quercetin inhibited glucose transport, oxidation, and incorporation into lipids (Shisheva and Shechter, 1992). With respect to alteration of transmembrane transport systems, it is worth noting that hexose transport in a human diploid fibroblast cell line was inhibited by quercetin (Salter et al., 1978). Vera et al. (1996) also showed that genistein was an inhibitor of hexose and dehydroascorbic acid transport through the glucose transporter GLUT.

Davis et al. (1983) reported that quercetin suppressed thyroxine stimulation of human red blood cell  $Ca^{2+}$ -ATPase activity in vitro and interfered with the binding of the hormone to red blood cell membranes in the concentration range of 1 to  $50 \mu\text{M}$ . In contrast, however, quercetin stimulated  $Ca^{2+}$ -ATPase activity at low concentrations and inhibited the ATPase at  $50 \mu\text{M}$  in the absence of any thyroid hormone. Interestingly, the effects of quercetin at the low concentrations (stimulation of  $Ca^{2+}$ -ATPase and inhibition of membrane binding of thyroid hormone) mimicked those of thyroxine. The results were considered consistent with the thyroxine-like structure of quercetin. Several other flavonoids, including fisetin, hesperetin, tangeretin, and chalcone, were also shown to reduce the sensitivity of membrane  $Ca^{2+}$ -ATPase to hormonal stimulation. In preliminary reports, Richardson and Twente (1987) showed that quercetin was capable of inhibiting in vitro and in vivo the stimulated secretion of rat pituitary growth hormone.

Silibinin, an antioxidant flavonoid from the European milk thistle, had a biphasic effect on secretion of steroids from adenomatous, hyperplastic, and atrophied adrenals. High concentrations of silybinin were inhibitory, while low concentrations significantly increased secretion of several corticosteroids in adrenocorticotropin-stimulated hyperplastic and adenomatous cells (Rácz et al., 1990).

In studies of the role of LO pathway in angiotensin II stimulation of aldosterone secretion from adrenal glomerulosa tissue, Natarajan et al. (1988) showed that baicalein, a 12-LO inhibitor, inhibited angiotensin II-mediated aldosterone secretion.

Ikeda et al. (1992) studied the flavonoid constituents of tea, namely, the tea catechins: (-)-epicatechin, (-)-epigallocatechin, (-)-epicatechin gallate, and (-)-epigallocatechin gallate (EGCG). Diverse pharmacological activities have been attributed to these compounds, including antioxidant, antimutagenic, and antihypertensive effects (Ikeda et al., 1992). These investigators found that partially purified catechin mixtures reduced cholesterol absorption from rat intestine (as measured by thoracic duct content) due to reduction of cholesterol solubility in mixed bile salt micelles.

Bourdeau and coworkers (1992) found that the 12-LO inhibitor baicalein ( $0.1 \mu\text{M}$ ) blunted the high  $Ca^{2+}$ -induced inhibition of parathyroid secretion while the 5-LO

pathway, 12-LO antagonist nordihydroguaiaretic acid did not restore hormone secretion, which was inhibited by high  $\text{Ca}^{2+}$ . Thus, 12-LO products could act as second messengers in parathyroid cells. Ong and Khoo (1996) studied the insulinomimetic properties of myricetin and found that this polyhydroxylated flavonol stimulated lipogenesis and glucose transport in rat adipocytes. The compound was without effect on insulin receptor autophosphorylation or glucose uptake. The authors speculated that myricetin might play a role in the management of non-insulin-dependent diabetes mellitus. In studies of insulin release from MIN6 cells, a glucose-sensitive insulinoma cell line, Ohno and coworkers (1993) found genistein to increase glucose-stimulated insulin release in a  $\text{Ca}^{2+}$ -dependent fashion. This effect was accompanied by cAMP accumulation, which was considered possibly related to phosphodiesterase inhibition.

The relationship of the flavonoids to the human endocrine system has been reviewed by Michael Baker (1997). It is now well recognized that flavonoids can interact with some hormone-transporting proteins and inactivating enzymes, all of which can alter the tissue concentrations of hormones such as steroids, prostaglandins, thyroid, and retinoids. Sequence analysis has revealed that dihydroflavonol 4-reductases (required for flavonoid pigment formation) share a common ancestor with human 3- $\beta$ -hydroxysteroid dehydrogenase. Other similar relationships have also been discovered (Baker, 1990, 1992, 1995). For instance, genistein ( $\text{IC}_{50}$ , 10  $\mu\text{M}$ ) inhibited lactogen-mediated stimulation of protein and DNA synthesis in Nb2 cells (a pre-T rat cell line) (Carey and Liberti, 1993).

## VI. Antiviral Effects

Naturally occurring flavonoids with antiviral activity have been recognized since the 1940s (Selway, 1986), but only recently have attempts been made to make synthetic modifications of natural compounds to improve antiviral activity. Quercetin, morin, rutin, dihydroquercetin (taxifolin), dihydrofisetin, leucocyanidin, pelargonidin chloride, apigenin, catechin, hesperidin, and naringin have been reported to possess antiviral activity against some of 11 types of viruses (Selway, 1986). The antiviral activity appears to be associated with nonglycosidic compounds, and hydroxylation at the 3-position is apparently a prerequisite for antiviral activity. Ishitsuka and coworkers (1982) isolated 4',5-dihydroxy-3,3' 7-trimethoxyflavone from the Chinese medicinal herb *Agastache folium* and detected antiviral activity against representatives of the picornavirus group ( $\text{IC}_{50}$  values in the range of 0.09–1.45  $\mu\text{M}$ ). Among other synthesized derivatives, only 4',6-dichloroflavan was observed to have high in vitro activity ( $\text{IC}_{50}$  values in the range of 0.007–10  $\mu\text{M}$ ) against rhinovirus

serotypes (Bauer et al., 1981). Unfortunately, this compound proved unsuccessful in clinical trials.

Although there was an early suggestion that (+)-cyanidanol-3 [(+)-catechin] may be of benefit in viral hepatitis (Blum et al., 1977), the true value of this compound in treatment of hepatitis remains to be thoroughly evaluated along with other hepatoprotective flavonoids such as silymarin.

In Belgium, pronounced antiviral activity noted in extracts of *Euphorbia grantii* was isolated in four related 3-methoxyflavones that exhibited significant activities against picornaviruses and vesicular stomatitis virus (Van Hoof et al., 1984). All of the active antiviral compounds were derivatives of 3-*O*-methylquercetin. In tissue culture, 90% inhibition of polio type 1 and coxsackie B viruses was achieved at concentrations of approximately 0.01 mg/ml, as compared with a 50% cytotoxic concentration of 40 mg/ml. Mice were protected from viremia and lethal infection from coxsackie B<sub>4</sub> virus by 3-*O*-methylquercetin administered at a daily dose of 20 mg/kg for a period of 9 days (Van Hoof et al., 1984). The mechanism of action of 3-*O*-methylquercetin and 3,3'-dimethylquercetin, another active derivative, suggested these substances prevent a virally induced shut-down of host protein synthesis (Van Hoof et al., 1984; Vrijssen et al., 1987).

Further studies of the mechanism of action of 3-*O*-methylquercetin by Rombaut et al. (1985) led to a comparison of effects of the flavonoid and the antiviral agent arildone (4-[6-(2-chloro-4-methoxyphenoxy)-hexyl]-3,5-heptanedione). At an early stage of replication, polio viruses were inhibited by these compounds. Although arildone is known to inhibit uncoating of polio virus, other experiments revealed that 3-*O*-methylquercetin and arildone interacted directly with the virus capsid. Thermal denaturation of polio virions and the alkaline disruption of procapsids to smaller subunits were affected. In polio virus-infected cells, viral protein and RNA synthesis were markedly reduced provided that 3-*O*-methylquercetin was added between 1 and 2 h after infection with the virus (Vrijssen et al., 1987).

Naturally occurring 4'-hydroxy-3-methoxyflavones possessed antiviral activity against rhino- and poliomyelitis viruses. Comparison with synthetic derivatives indicated that high antiviral activity was associated with the 4'-hydroxyl and 3-methoxyl groups, a substituent in the 5-position and a poly-substituted A ring (De Meyer et al., 1991).

Mucsi and Pragai (1985) demonstrated the inhibitory effect of four flavonoid compounds in human herpes simplex virus type I and Suid (a) herpes virus type I (Pseudorabies virus); there was a relationship between viral inhibition and the ability of flavonoids to increase cyclic AMP in the HEp-2 cells and chicken embryo fibroblasts. A direct relationship between the antiviral activity of quercetin, quercitrin, rutin, and hesperidin and the ability to stimulate cyclic AMP synthesis in the cells

seemed to exist. Quercetin and quercitrin were the most active compounds, although high concentrations were required.

The effect of quercetin, naringin, hesperetin, and catechin on the infectivity and replication of HSV-1, polio virus type 1, parainfluenza virus type 3, and respiratory syncytial virus has been studied in cell culture monolayers using the technique of viral plaque reduction. Kaul et al. (1985) observed that quercetin caused a concentration-dependent reduction in the infectivity of each virus, and in addition, intracellular replication of viruses was reduced when monolayers were infected and subsequently cultured in medium containing quercetin. Hesperetin had no effect on infectivity, but did reduce intracellular replication of each virus. The infectivity, but not the replication of respiratory syncytial virus and HSV-1, was noted with catechin, a compound that had negligible effects on the other viruses. Naringin had no effect on either infectivity or replication of any of the viruses studied. The structural basis for the antiviral activity of naturally occurring flavonoids was further studied by Wleklik et al. (1988). Inhibition of HSV-1 replication in RK-13 cells was examined. Hydroxylation at positions 3', 4', 3, 5, and 7 was associated with highest antiviral activity. Genistein (>25  $\mu\text{M}$ ) inhibited the replication of HSV-1 accompanied by phosphorylation of tyrosine residues in particular viral peptides (Yura et al., 1993). Daidzein was inactive, while prunetin, also a PTK inhibitor, showed activity similar to genistein.

The possibility of synergistic antiviral effects when flavonoids are combined with other antiviral agents was suggested by the work of Mucsi (1984) and Veckenstedt et al. (1987). Quercetin in combination with 5-ethyl-2'-deoxyuridine had antiviral activity on HSV-1 or pseudorabies infection in vitro; quercetin together with murine  $\alpha/\beta$ -interferon was also effective for the treatment of mice infected with Mengo virus. Enhanced antiviral activity against herpes viruses in cell culture could be achieved by combining acyclovir with flavonoids such as quercetin, quercitrin, and apigenin (Mucsi et al., 1992).

An interesting interaction between ascorbate and quercetin was observed by Vrijssen et al. (1988). Quercetin exhibited antiviral activity only when oxidative degradation was inhibited by ascorbate. Luteolin was as active as ascorbate-stabilized quercetin.

Among a large number of flavonoids isolated from *Scutellaria baicalensis*, two were found to have a remarkable ability to inhibit EBV-EA activation using the EBV genome-bearing lymphoblastoid Raji cell line. EBV-EA activation was induced by TPA, and thus the flavonoids could be acting as inhibitors of PKC, which is directly activated by TPA. The most active inhibitory flavones were 5,7,2'-trihydroxy- and 5,7,2',3'-tetrahydroxyflavone (Konoshima et al., 1992). The biflavone ginkgetin from the leaves of *Cephalotaxus drupacea* possessed antiherpes virus activity as well as activity against human cytomegalovirus (Hayashi et al., 1992).

Ginkgetin decreased viral protein synthesis and strongly suppressed transcription of immediate-early genes without evidence of cytotoxicity at low concentrations. Further studies from this group (Li et al., 1993) established that baicalin inhibited 1) syncytium formation on CEM-ss monolayer cells, 2) HIV-1-specific p24 core antigen expression, and 3) HIV-1 RT from infected 119 cells. Clearly, baicalin and related flavonoids require further clinical investigation.

The antiviral activity of TNF was greatly augmented by quercetin with vesicular stomatitis virus, encephalomyocarditis virus, and HSV-1 in WISH cells (Ohnishi and Bannai, 1993). Luteolin, genistein, kaempferol, and rutin were without effect. Antibodies to IFN- $\beta$  totally blocked the TNF- or TNF/quercetin-induced antiviral activity. This finding indicated that the TNF- or TNF/quercetin-induced antiviral state was mediated by induction of IFN- $\beta$ . Also, 2',5'-oligo-adenylate synthetase was markedly enhanced in those cells which were exposed to both TNF and quercetin. Notably, this activity was abrogated in the presence of antibodies to IFN- $\beta$ . Thus, the induction of the synthetase by TNF or TNF/quercetin appeared to be mediated via TNF-induced IFN- $\beta$ .

Hu and coworkers (1994) found that an acacetin glycoside isolated from chrysanthemum inhibited HIV replication in H9 cells. Another flavonoid, chrysin, was also a potent inhibitor. Overall, the antiviral studies suggest that selected dietary flavonoids may have prophylactic activity against certain viral infections. Epidemiological studies are warranted.

## VII. Antitoxic, Hepatoprotective, and Cytoprotective Effects

The liver is subject to acute and potentially lethal injury by several substances, including phalloidin (the toxic constituent of the mushroom *Amanita phalloides*),  $\text{CCl}_4$ , galactosamine, ethanol, and other compounds. Silymarin has been shown to have hepatoprotective effects in vivo. Both silymarin and silybin dihemisuccinate have been shown to be effective protective agents against the hepatotoxicity of  $\text{CCl}_4$ , phalloidin, and  $\alpha$ -amanitin (Hahn et al., 1968). It was considered possible that the flavonoid exerts a membrane-stabilizing action, thus inhibiting lipid peroxidation (Greimel and Koch, 1977). Silymarin has been widely used in Europe in the treatment of alcoholic liver disease and diseases associated with increased vascular permeability and capillary fragility (Perrissoud, 1986). The protective effect of (+)-catechin against acute liver injury extended also to protection against galactosamine as described by Perrissoud and Weibel (1980). A placebo-controlled, double blind pilot study of the silybinphosphatidyl complex (IdB1016) in chronic active hepatitis was conducted by Buzzelli et al. (1993). The silybin-lipid complex (a 1:1 M ratio of silybin to phosphatidylcholine) was given p.o.,

and after seven days there was a significant reduction of the plasma concentration of three liver enzymes and bilirubin, but not in malondialdehyde (MDA), a measure of lipid peroxidation.

It was reported that in vivo treatment with silymarin protected against lipid peroxidation and hemolysis induced in rat erythrocytes when incubated with phenylhydrazine (Valenzuela et al., 1985a). In addition, in vivo treatment with silybin dihemisuccinate was shown to inhibit the release of MDA induced by phenylhydrazine in the perfused rat liver (Valenzuela and Guerra, 1985). Silymarin also prevented liver glutathione depletion and lipid peroxidation induced by an acute intoxication with ethanol in the rat (Valenzuela et al., 1985b). These effects attest to the suggested action of the flavonoid as a cytoprotective agent. Intraperitoneal administration (50 mg/kg) of silybin dihemisuccinate to rats inhibited lipid peroxidation, methemoglobin formation, and osmotic fragility induced in vitro by phenylhydrazine in erythrocytes (Valenzuela et al., 1987). Effects on osmotic fragility were thought to be a consequence of the membrane-stabilizing properties of the flavonoid. These effects were also ascribed to the antioxidant properties of the flavonoid, since spontaneous or induced oxidative stress could labilize cell membranes. The observed novel pharmacological action of silybin dihemisuccinate, primarily used in the treatment of hepatic diseases, could have other therapeutic implications. Several drugs are metabolized to hydrazine derivatives producing not only liver damage, against which silybin has been shown to have a protective effect (Valenzuela and Guerra, 1985), but also hematological disorders. Prophylactic or therapeutic treatment with the above flavonoids has been suggested to confer protection against these deleterious effects (Valenzuela et al., 1987).

Rat 3Y1 fibroblasts can be transformed by the E1A gene of adenovirus type 12 (E1A 3Y1 cells) and are highly sensitive to the cytotoxic/cytolytic effect of 1,3-dilinoleoylglycerol. The LO inhibitor baicalein reduced the 1,3-dilinoleoylglycerol-dependent selective cytotoxicity; CO inhibitors had no effect. The authors concluded that lipid peroxidation could play a critical role in cytotoxicity against E1A-transformed cells and that the multiple pore-type destruction of the cell membrane with round defects may account for cell death (Matsuzaki and coworkers, 1989).

X-Irradiation is known to increase capillary permeability. Parmar and Ghosh (1977) studied the effect of two flavonoid compounds and one "citrus bioflavonoid compound" mixture on X-irradiation-induced increase in the capillary permeability of the rat intestine. All three substances decreased the leakage of Evans blue dye into the irradiated intestine, and some had quite high degrees of protective activity against X-irradiation. Among twelve flavonoids studied by Shimoi et al. (1994), luteolin proved to be the most active inhibitor. The possible

usefulness of flavonoids as antagonists of radiation-induced injury requires further investigation.

Tuchweber et al. (1979) studied the effect of silybin, an active flavonoid derived from the European milk thistle, on phalloidin-induced, acute hepatotoxicity in Swiss mice. Silybin pretreatment prevented phalloidin-induced acute hemorrhagic necrosis of the liver. As determined by electron microscopy, the initial changes induced by phalloidin are observed in the hepatocyte plasma membrane, followed by the subsequent development of cytoplasmic vacuoles. These morphologic alterations in tissue correlate with increased plasma levels of liver enzymes. Pretreatment with a single dose of silybin abolished the morphologic changes induced by phalloidin and significantly reduced the leakage of liver enzymes into the blood stream. Iwu (1985) observed that biflavones isolated from the seeds of *Garcinia kola* were the active principles preventing phalloidin-induced liver injury in mice. Studies by Desplaces and coworkers (1975) disclosed that silymarin, another one of the active principles of the European milk thistle, was capable of dramatically inhibiting liver damage associated with phalloidin poisoning in a dose-dependent fashion. The authors also claimed that there was considerable normalization of metabolic abnormalities that accompany phalloidin toxicity.

The effect of flavonoids on CCl<sub>4</sub>-induced toxicity in isolated rat hepatocytes was studied by Perrissoud and Testa (1986). The ability to interfere with CCl<sub>4</sub>-induced release of aspartate aminotransferase was tested with 55 flavonoid compounds. The more hydrophilic compounds were observed to inhibit the CCl<sub>4</sub>-induced toxicity, whereas the more lipophilic derivatives actually potentiated the toxicity. In several countries, although not in the United States, silybin and other flavonoids are widely used in the treatment of liver diseases and diseases associated with increased vascular permeability and capillary fragility (Perrissoud, 1986). Silymarin (50 mg/kg) given p.o. completely prevented all CCl<sub>4</sub>-induced changes in the metabolism and disposition of acetylsalicylic acid in CCl<sub>4</sub>-induced cirrhosis in rats (Mourelle and Favari, 1988). In addition, it corrected the elevated hepatic and serum esterase activity. Silymarin also reduced the amount of collagen found in CCl<sub>4</sub>-induced cirrhosis (Lapis et al., 1986). Ternatin, a tetramethoxyflavone from *Egletes viscosa* Less., caused marked inhibition of CCl<sub>4</sub>-induced elevation of serum enzymes and morbid histologic changes in rats, indicating that it possesses liver-protective activity (Rao et al., 1994).

A report by Harada et al. (1984) indicated that quercetin supplied at a 1% dietary concentration to male Syrian golden hamsters exposed to cigarette smoke for 13 weeks resulted in improved body weight gain and significant inhibition of thickening of the laryngeal mucosa. The investigators suggested that quercetin could have some ameliorative effects on tissue damage provoked by cigarette smoke.

Elucidation of the mechanism for the protective effect of silymarin against the hepatotoxicity of  $\text{CCl}_4$  has provoked considerable interest. A short report showed decreased amounts of diene conjugates in rats pretreated with silymarin before the administration of  $\text{CCl}_4$  (Rauen et al., 1973). The possible mechanisms for the protective effect of silymarin against the hepatotoxicity of  $\text{CCl}_4$  was further elucidated by Letteron et al. (1990). Intraperitoneal administration (800 mg/kg) of silymarin to mice protected the liver from  $\text{CCl}_4$ -induced lipid peroxidation and hepatotoxicity. Silymarin inhibited the metabolic activation of  $\text{CCl}_4$  in vivo, as suggested by a decreased covalent binding of  $\text{CCl}_4$  metabolites to hepatic lipids in vivo. Decreased metabolic activation of  $\text{CCl}_4$  by cytochrome P450 would depress the initial formation of the trichloromethyl free radical and therefore diminish the initiation of lipid peroxidation. Silymarin (800  $\mu\text{g}/\text{ml}$ ) impaired the irreversible binding of  $\text{CCl}_4$  metabolites to hepatic microsomal protein by only 2%, although it decreased by 72% the in vivo lipid peroxidation mediated by  $\text{CCl}_4$  metabolites. Silymarin treatment in vivo diminished the irreversible binding of  $\text{CCl}_4$  metabolites to hepatic lipids by 39% and depressed by 60% the exhalation of ethane during the first hour after the administration of  $\text{CCl}_4$ . Silymarin (800  $\mu\text{g}/\text{ml}$ ) decreased by 70% in vitro lipid peroxidation mediated by  $\text{CCl}_4$  metabolites and decreased by 90% lipid peroxidation mediated by NADPH alone. In this system, lipid peroxidation is thought to be mediated by the reduction of iron to the ferrous state (Labbe et al., 1987). It was earlier reported that silymarin could prevent lipid peroxidation mediated by the addition of  $\text{Fe}^{2+}$ -ascorbate, cumene hydroperoxide, or *tert*-butylhydroperoxide, suggesting that flavonoids can act as chain-breaking antioxidants (Bindoli et al., 1977; Koch and Loffler, 1985; Valenzuela and Guerra, 1986; Valenzuela et al., 1986; Kandaswami and Middleton, 1994). Letteron et al. (1990) concluded that silymarin prevented  $\text{CCl}_4$ -induced lipid peroxidation and hepatotoxicity in mice by a dual mechanism: by decreasing the metabolic activation of  $\text{CCl}_4$  into free radicals as well as by scavenging free radicals.

Feher et al. (1988) showed that silymarin treatment corrected the decreased SOD activity of erythrocytes and lymphocytes in patients with alcoholic cirrhosis, thus exemplifying the potential therapeutic utility of the flavonoid. Lang and coworkers (1993) demonstrated that lymphocytes and erythrocytes of patients with chronic alcoholic liver disease responded to silymarin with an increase in SOD expression. They speculated that the hepatoprotective properties may in part be due to this antioxidant activity.

Another protective effect of silymarin was described against rat liver injury induced by ischemia (Wu et al., 1993). The induction of hepatic ischemia was accompanied by elevation of hepatocellular enzymes, which were significantly reduced by silymarin pretreatment. Moreover, silymarin decreased the fall in glycogen phosphorylase ac-

tivity during 60 min of in vitro ischemia. Acetaminophen hepatotoxicity is characterized by glutathione depletion, cell death, and occasionally by the induction of lipid peroxidation. Interestingly, silybin protected rats against glutathione depletion in the liver and lipid peroxidation induced by acute acetaminophen toxicity (Campos et al., 1989).

Trichothecene mycotoxins are a chemically related group of secondary metabolites derived from *Fusarium* and some other fungi and are known to be toxic to both humans and animals. Indeed, these compounds have been implicated as the cause of inadvertent food intoxication after fungal contamination of certain foodstuffs. Anecdotal reports from southeast Asia indicate that extracts of plants rich in flavonoids may be successful in treating mycotoxicosis. Markham et al. (1987) observed that quercetin was able to reduce the cytotoxic effect of T-2 mycotoxin on cultured murine thymocytes. Mice simultaneously treated with T-2 mycotoxin and quercetin had a reduced mortality compared with mice not receiving quercetin.

Gastric lesion formation caused by the oral administration of ethanol to rats could be prevented by parenteral pretreatment with quercetin (Mizui et al., 1987). Scavengers of  $\text{O}_2^-$  and  $\text{OH}^-$ , such as sodium benzoate and dimethyl sulfoxide, were ineffective. The authors suggested that an active species, probably derived from iron mobilized by the xanthine oxidase system, contributed to lesion formation in the gastric mucosa after ethanol administration.

The effect of the flavonoid hispidulin (6-methoxy-5,7,4'-trihydroxyflavone) on bromobenzene-induced hepatotoxicity in mice was assessed (Ferrandiz et al., 1994). The compound inhibited liver injury and lipid peroxidation. It also counteracted glutathione depletion induced by bromobenzene in starved mice. The hepatoprotective effects could be related to the antioxidant properties of the flavonoid.

Morin was found to be an effective hepatoprotector in vitro and in vivo. This compound prolonged the survival of rat hepatocytes against oxidative damage (Wu et al., 1993). In a rat model of ischemia reperfusion in the liver, morin was found to be hepatoprotective. For centuries in China, extracts from the edible vine *Pueraria labata* have been widely used as a nonintoxicating inebriation deterrent. Significantly, Xie et al. (1994) found that one of the main constituents, the isoflavone daidzin, when given orally to rats, caused a delay in reaching (as well as reducing) the peak blood alcohol concentrations. The effects were caused by delayed gastric emptying and not on alcohol dehydrogenase. The potential clinical implications of these observations are obvious. Of note also is the finding that two other antioxidants (vitamin E and thiotic acid) were tested and showed effects similar to daidzin. Thus, daidzin's activity may be attributed to its antioxidant activity (Xie et al., 1994).

Sanz et al. (1994) examined the influence of a series of natural flavonoids isolated from Indian medicinal plants for their effect on free radical generating systems and their oxidative effect (bromobenzene-induced hepatotoxicity). All flavonoids inhibited lipid peroxidation in vitro, and some compounds behaved as hydroxyl radical scavengers (deoxyribose degradation assay). Scutellarein and nepetin inhibited xanthine oxidase, while morelloflavone (a biflavonoid) scavenged superoxide anions generated by the xanthine oxidase/hypoxanthine system. Several compounds protected mice against bromobenzene intoxication as detected by decreased serum liver enzyme levels. Only kaempferol-3-*O*-galactoside significantly reduced hepatic lipid peroxidation products and increased the reduced glutathione levels in the liver. Note that morelloflavone increased bromobenzene toxicity, indicating that not all naturally occurring flavonoids are nontoxic.

Thallium-induced hepatotoxicity was reduced substantially by silymarin and, therefore, could ameliorate the toxicity of this substance in other organs as well. In part, its activity could be ascribed to its antioxidant/radical-scavenging properties (Mourelle et al., 1988). The effects of other hepatotoxic drugs, such as erythromycin estolate, amitriptyline, nortriptyline, and *tert*-butylhydroperoxide were also decreased by catechin and silybin (Davila et al., 1989). Silybin appeared to be less effective than selected xanthines and xanthonolignoids in protecting against *tert*-butylhydroperoxide-induced toxicity in isolated rat hepatocytes (Fernandes et al., 1995).

The activity of intravenous administration of a purified fraction (S5682) containing 90% diosmin (a flavone derivative) and 10% hesperidin (a flavanone derivative) was evaluated (25 mg/kg and 50 mg/kg) in the rat by measuring the degree of hyperglycemia provoked by an intravenous injection of alloxan, the metabolism of which produces reactive oxygen species toxic to  $\beta$ -cells of the pancreas. This preparation caused a decrease in hyperglycemia in a dose-dependent manner (Lonchampt et al., 1989). The authors suggested that the radical-scavenging properties of S5682 might explain its diverse pharmacological effects, such as 1) the reduction in capillary permeability induced in the sensitized rat and rabbit by injection of antigen, application of chloroform swabs, or by irradiation and 2) the antiedematous effects seen in inflammatory granulomas in the rat (Lonchampt et al., 1989).

The flavonoids quercetin, kaempferol, catechin, and taxifolin suppressed the cytotoxicity of  $O_2^-$  and  $H_2O_2$  on Chinese hamster V79 cells, as assessed with a colony formation assay (Nakayama et al., 1993). Quercetin and kaempferol showed protective effects at 5 to 10  $\mu$ M concentrations, whereas much higher concentrations of catechin and taxifolin were necessary for the prevention of cytotoxicity. The protective activity was ascribed to the *O*-dihydroxy structure in the B ring, or 3- and 5-OH

groups and the C2-C3 double bond. The authors earlier suggested that the *O*-dihydroxy structure of polyphenols was essential for protection against  $H_2O_2$ -induced cytotoxicity in V79 cells, because antioxidants bearing only one phenolic OH, such as ferulic acid methyl ester and  $\alpha$ -tocopherol, exhibited no protective effects (Nakayama et al., 1992). The observation that kaempferol, lacking the above structure, showed a protective effect seems to be an exception. The conversion of kaempferol to quercetin by hydroxylation under the experimental conditions used might explain this effect.

The mutagenic effect of chrysotile asbestos fibers, zeolite, and latex particles on human lymphocytes in whole blood was inhibited by the antioxidant enzymes SOD and catalase, as well as by radical scavengers such as rutin, ascorbic acid, and bemitil. These results suggested that the mutagenic effects of the particles was mediated by oxygen radicals (Korkina et al., 1992). Of the radical scavengers studied, rutin was the most effective inhibitor of the mutagenic effect of mineral fibers and dusts. The study of lucigenin- and luminol-amplified chemiluminescence of peritoneal macrophages stimulated by the above particles showed that their mutagenic action was probably mediated by different oxygen species. Rutin was more potent than ascorbate in inhibiting luminol-dependent chemiluminescence of peritoneal macrophages activated by chrysotile fibers or zeolite particles (Korkina et al., 1992).

Kantengwa and Polla (1991) reported that erythrophagocytosis induced in human monocytes-macrophages was accompanied by the synthesis of stress proteins, including the classical heat shock protein and heme oxygenase. Quercetin and kaempferol inhibited this induction. The results suggested that 1) erythrophagocytosis-related oxygen radicals were involved in the induction of the stress response in phagocytic cells, 2) the induction of classical heat shock proteins appeared, at least in part, to be dependent on PKC, and 3) the effects of the flavonoids on heme oxygenase were linked to their scavenging activity rather than to protein kinase C modulation.

Cytotoxicity and inhibition of intercellular communication represent two possible mechanisms by which tumor promoters produce their promoting effects (Trosko and Chang, 1984). The prevention of these effects by tea flavans may suggest a mechanism by which these catechins inhibit tumor promotion in vivo.

The cytoprotective effect of three flavonoids, catechin, quercetin, and diosmetin, was investigated on iron-loaded rat hepatocyte cultures, considering two parameters, namely, the prevention of iron-induced increase in lipid peroxidation and the inhibition of intracellular lactate dehydrogenase release (Morel et al., 1993). The cytoprotective potency of these flavonoids was rated as follows: catechin > quercetin > diosmetin. The investigation of the capacity of the above flavonoids to remove iron from iron-loaded hepatocytes revealed that the iron-

chelating capacity of the three compounds followed the same order as did their cytoprotective effect. The authors suggested that this relationship must be taken into consideration in further development of these protective flavonoids, which could have important applications in human diseases. Some flavonoids have been reported to be able to mobilize iron from ferritin (Boyer et al., 1988) and to be capable of reducing  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  (Aruoma, 1991). These considerations were thought to be of importance, although some authors apparently ruled out the possibility that the antiperoxidative action was related to an interaction of the flavonoids with iron ions (Bindoli et al., 1977; Kapus and Lukacs, 1986).

Fuchs and Milbradt (1993) examined the effect of apigenin-7-glucoside on skin inflammation induced by different generators of reactive oxygen species (ROS). Skin inflammation in rats was induced by intradermal application of xanthine oxidase/hypoxanthine ( $\text{O}_2^-$  radical generator) and cumene hydroperoxide (peroxyl radical generator). Subsequent intradermal application of apigenin-7-glucoside inhibited in a dose-dependent manner skin inflammation caused by xanthine oxidase and cumene hydroperoxide. The results were in good agreement with in vitro  $\text{O}_2^-$  radical- and peroxyl radical-scavenging properties and indicated that the antioxidant properties of the compound could have accounted for its anti-inflammatory effect in this system. The relationship of flavonoid structure to superoxide anion-scavenging activity was studied by Hu et al. (1995). The greatest activity was found among nonglycosidic flavonols and the flavanols.

Naringenin was shown to have cytoprotective properties on mucosal injury induced in rats by ethanol (Motilva et al., 1994). Oral pretreatment with the highest dose of naringin (200 mg/kg) was found to be the most effective in ulcer prevention. Histomorphometric evaluation confirmed a significant increase of mucous production accompanied by a parallel reduction of gastric lesions.

Pretreatment of rats subcutaneously with hesperidin (50 and 100 mg/kg), a citrus flavonoid, significantly reduced the paw edema induced by carrageenin in rats and mice (Emim et al., 1994). The effect was equivalent to that produced by indomethacin (10 mg/kg, p.o.).

Topical application of quercetagenin, kaempferol-7-*O*-glucoside, scutellarein, and hispidulin inhibited TPA-induced ear edema in mice with a potency comparable to that of indomethacin (Gil et al., 1994). These flavonoids were also able to inhibit carrageenin-induced mouse paw edema. The blockade of the free hydroxyl at C-7 reduced the anti-inflammatory activity.

### VIII. Antioxidant Activity

The term "reactive oxygen species" (ROS) collectively denotes oxygen-centered radicals such as superoxide ( $\text{O}_2^-$ ) and hydroxyl ( $\cdot\text{OH}$ ) as well as nonradical species derived

from oxygen, such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), singlet oxygen ( $^1\text{O}_2$ ), and hypochlorous acid ( $\text{HOCl}$ ). ROS play a pivotal role in the action of numerous foreign compounds (xenobiotics). Their increased production seems to accompany most forms of tissue injury (Halliwell and Gutteridge, 1990; Halliwell, 1991a; Halliwell et al., 1992). Whether sustained and increased production of ROS is a primary event in human disease progression or a secondary consequence of tissue injury has been discussed (Halliwell, 1991a,b; Halliwell et al., 1992). Whatever may be the case, the formation of free radicals has been implicated in a multitude of disease states ranging from inflammatory/immune injury to myocardial infarction and cancer. The best known antioxidant molecules are vitamins A, E, and  $\beta$ -carotene (Sies and Krinsky, 1995; Krinsky, 1998). These natural substances have also been reviewed for their possible role in the prevention of cancer and cardiovascular disease (Krinsky et al., 1996; Krinsky, 1998).

Some of the well known detrimental effects of excessive generation of ROS in biological systems include peroxidation of membrane lipids, oxidative damage to nucleic acids and carbohydrates, and the oxidation of sulfhydryl and other susceptible groups in proteins (Sies, 1985, 1991; Halliwell, 1991a,b; Halliwell et al., 1992). Oxygen-derived free radicals appear to possess the propensity to initiate as well as to promote carcinogenesis. There is heightened interest in the role of ROS in atherosclerosis, stroke, myocardial infarction, trauma, arthritis, ischemia/reoxygenation injury, and cancer (Halliwell and Gutteridge, 1990; Halliwell et al., 1992). The involvement of ROS in aging and in many chronic diseases has been considered. The defense provided by antioxidant systems is crucial to the survival of organisms. Detoxification of ROS in the cell is provided by both enzymatic and nonenzymatic systems which constitute the antioxidant defense systems. Enzymatic systems include extensively studied enzymes such as SOD, catalase, glutathione peroxidases, D-T diaphorase, and glutathione-regenerating enzyme systems (Sies, 1985, 1991; Krinsky, 1992). Some enzymatic systems such as SOD and catalase act specifically against ROS, while certain other enzyme systems reduce thiols. Nonenzymatic antioxidants are less specific and can also scavenge other radicals, both organic and inorganic. These antioxidants can be classified as water-soluble or lipid-soluble, depending on whether they act primarily in the aqueous phase or in the lipophilic region of cell membranes. Hydrophilic antioxidants include ascorbic acid and urate. Ubiquinols, retinoids, carotenoids, and tocopherols (vitamin E) are some of the lipid-soluble antioxidants (Sies and Krinsky, 1995). Plasma proteins, GSH, urate, and others are some of the endogenous antioxidants, while ascorbic acid, carotenoids, retinoids, flavonoids, and tocopherols constitute some of the dietary antioxidants. These compounds possess the potential to scavenge and quench various radicals (oxygen-centered; carbon-centered; alkoxy, peroxyl, or phenoxy)

TABLE 2  
Reactive oxygen species that can be scavenged or whose formation can be inhibited by flavonoids

$O_2^-$ (Superoxide anion)	One-electron reduction product of $O_2$ . Produced by phagocytes, formed in autoxidation reactions (flavoproteins, redox cycling), and generated by oxidases (heme proteins).
$HO_2^-$ (Perhydroxy radical)	Protonated form of $O_2^-$
$H_2O_2$ (Hydrogen peroxide)	Two-electron reduction product of $O_2$ formed from $O_2^-$ ( $HO_2^-$ ) by dismutation or directly from $O_2^-$ . Reactivity of $O_2^-$ and $H_2O_2$ is amplified in the presence of heme proteins.
OH (Hydroxyl radical)	Three-electron reduction product of $O_2$ generated by Fenton reaction, transition metal (iron, copper)-catalyzed Haber-Weiss reaction; also formed by decomposition of peroxyxynitrite produced by the reaction of $O_2^-$ with $NO\cdot$ (nitric oxide radical).
$RO\cdot$ (Alkoxy radical)	Example: Lipid radical ( $LO\cdot$ ).
$ROO\cdot$ (Peroxy radical)	Example: Lipid peroxy radical ( $LOO\cdot$ ) produced from organic hydroperoxide (e.g. lipid hydroperoxide, LOOH), ROOH by hydrogen abstraction.
$^1O_2$	Singlet oxygen.

TABLE 3  
Characteristics of flavonoid structure for most effective radical-scavenging activity

- The catechol (*O*-dihydroxy) group in the B ring confers great scavenging ability, with exceptions such as those described by Ratty and Das (1983), who thought it did not contribute towards lipid peroxidation in rat brain mitochondria.
- A pyrogallol (trihydroxy) group in ring B of a catechol, as in myricetin, produces even higher activity. The C2-C3 double bond of the C ring appears to increase scavenger activity because it confers stability to the phenoxy radicals produced.
- The 4-oxo (keto double bond at position 4 of the C ring), especially in association with the C2-C3 double bond, increases scavenger activity by delocalizing electrons from the B ring.
- The 3-OH group on the C ring generates an extremely active scavenger; in fact, the combination of C2-C3 double bond and 4-oxo group appears to be the best combination on top of the catechol group.
- The 5-OH and 7-OH groups may also add scavenging potential in certain cases.

radicals) and ROS. Certain radical scavengers are not recyclable, while others are recycled through the intervention of a series of enzyme systems or other nonenzymatic antioxidant systems. The free radical-scavenging and antioxidant activity of plant flavonoids has been reviewed by Kandaswami and Middleton (1994, 1995). ROS that can be scavenged or whose formation can be inhibited by flavonoids are shown in Table 2.

Before looking at particular aspects of the effects of flavonoids on free radicals, it is worth summarizing some important aspects of the flavonoid structure up front. As it will become evident below, many different methods have been used to study the antioxidant potential of flavonoids. This work was reviewed recently by Rice-Evans and Miller (1998) who, themselves, have contributed significantly to our understanding of structure-activity relationships of the antioxidant effects of flavonoids. The structural aspects of the antioxidant activity of flavonoids were also discussed by van Acker et al. (1998). Their conclusions appear to converge and are summarized in Table 3 so that it will be easy to refer to during the rest of the review.

All in all, quercetin appears to be an extremely efficient radical scavenger, with myricetin being even more

active by virtue of the third (pyrogallol) hydroxyl group on the B ring. Kaempferol is a very good scavenger even though it has only one hydroxyl group on the B ring (4'-OH) possibly because of the combination of the other characteristics (C2-C3 double bond, 3-OH group, and 4-oxo group on ring C). Catechin, which has the catechol group on ring B and the 3-OH group on ring C is, nevertheless, a weak scavenger because it lacks the C2-C3 double bond and the 4-oxo group on ring C. These observations are similar to what we have observed for inhibition of mast cell secretion and maturation of RBL cells (Alexandrakis et al., 1999).

#### A. Influence of Flavonoids on Reactive Oxygen Species Production by Phagocytic Cells

Phagocytosis is an important physiological process accompanied by production of  $O_2^-$ . Activated phagocytic cells such as monocytes, neutrophils, eosinophils, and macrophages generate  $O_2^-$  (Curnutte and Babior, 1987; Babior and Woodman, 1990). Radical production by phagocytes is extremely important for their bactericidal and tumoricidal functions. Phagocytosis is accompanied by a dramatic increase in oxygen consumption (respiratory burst) with the attendant production of  $O_2^-$ , catalyzed by a membrane-bound NADPH oxidase system (Curnutte and Babior, 1987; Babior and Woodman, 1990).

$O_2^-$  generated by phagocytes is transformed by dismutates to  $H_2O_2$ , a fairly unreactive molecule, which in turn gives rise to  $\cdot OH$  by reaction with transition metal ions (Halliwell, 1991b; Halliwell et al., 1992). This radical is extremely reactive and is one of the strongest oxidizing agents. The enzyme MPO provides another bacterial killing mechanism in neutrophils by catalyzing the oxidation of chloride ions by  $H_2O_2$ ; this reaction results in the formation of HOCl, a powerful bactericidal agent (Weiss, 1989).

Even though  $O_2^-$  is far less reactive than  $\cdot OH$ , it can attack several biological targets. It can react with nitric oxide ( $NO\cdot$ ), a reactive free radical produced by phagocytes and vascular endothelial cells, to yield an even more reactive species, peroxyxynitrite (Michel and Bors, 1991), which can decompose to form  $\cdot OH$  in a reaction

independent of transition metal ions (Beckman et al., 1990). Endothelium-derived relaxing factor, an important mediator of vasodilator responses, has been identified to be NO<sup>•</sup> (Marletta, 1989; Moncada et al., 1989). O<sub>2</sub><sup>-</sup> has been reported to react with NO<sup>•</sup> and inhibit its action (Gryglewski et al., 1986). By impairing the physiological function of NO<sup>•</sup>, O<sub>2</sub><sup>-</sup> can act as a vasoconstrictor, which could have deleterious consequences in some clinical situations (Laurindo et al., 1991).

While ROS generated by phagocytes play an important physiological function, they can also cause cellular damage. The highly reactive oxygen metabolites, along with other mediators elaborated by neutrophils and macrophages, can promote inflammation and cause tissue damage (Fantone and Ward, 1982). Busse et al. (1984) showed that flavonoids inhibited ROS release (as assayed by the production of luminol-dependent chemiluminescence) by human neutrophils. Quercetin and several other flavonoids were quite effective inhibitors of O<sub>2</sub><sup>-</sup> production by the cells. 'T Hart et al. (1990) recently reported a similar inhibitory effect of different flavonoids on ROS production by activated human neutrophils using the chemiluminescence method. Four selected flavonoids inhibited MPO release, while two of these also strongly inhibited MPO activity. Considering luminol-dependent chemiluminescence production by neutrophils to be an MPO-dependent process, these authors suggested that these effects might mask the effects of flavonoids on ROS production. Using the luminescent probe lucigenin for the exclusive detection of O<sub>2</sub><sup>-</sup> release, 'T Hart et al. (1990) showed that the release of this species by human neutrophils was inhibited by flavonoids. Essential determinants for inhibition of O<sub>2</sub><sup>-</sup> release appeared to be the OH groups located in the B ring of the flavonoid molecule. The formation of O<sub>2</sub><sup>-</sup> is dependent on the activation of NADPH oxidase localized in the plasma membrane, which is also subject to flavonoid inhibition (Tauber et al., 1984). The inhibition of PKC by flavonoids (Ferriola et al., 1989) could also be implicated in the impairment of the NADPH oxidase activation.

Antioxidant catechins (flavans) isolated from Chinese green tea showed scavenging activity against H<sub>2</sub>O<sub>2</sub> and O<sub>2</sub><sup>-</sup> generated by the xanthine-xanthine oxidase system (Ruch et al., 1989) (Table 3). The flavans also prevented oxygen radical-induced cytotoxicity and inhibition of intercellular communication in cultured B6C3F1 mouse hepatocytes and keratinocytes (NHEK cells).

A novel antioxidant flavonoid, flavone-3-hydroxyfarerol, inhibited the respiratory burst in human neutrophils activated by f-MetLeuPhe with an IC<sub>50</sub> of 20 μM (Ursini et al., 1994). This effect might also be linked to the observed inhibition of PKC (IC<sub>50</sub>, 50 μM); PTK and caseinkinase-2 were not inhibited. Tumor promoter (TPA)-induced formation of H<sub>2</sub>O<sub>2</sub> was inhibited by genistein in a concentration-dependent manner (1–150 μM) in human polymorphonuclear leukocytes and HL-60 cells (Wei et al., 1995).

In addition to inhibiting the activity of purified human neutrophil MPO, quercetin was also found to depress this activity in a system using intact human neutrophils (Pincemail et al., 1988). In this case, quercetin was significantly more potent than methimazole, a specific inhibitor of MPO (Winterbourn, 1985). Flavonoids could inhibit the formation of O<sub>2</sub><sup>-</sup> and the generation of <sup>•</sup>OH radicals. The inhibition of neutrophil MPO activity by flavonoids could result in the impairment of ROS production. Such impairment could diminish the formation of highly toxic HOCl and the hypochlorite ion (OCl<sup>-</sup>). A consequence of this would be a decrease in the inactivation of α-1-antitrypsin, which could in turn result in the enhanced inactivation of neutrophil-derived and other tissue-damaging enzymes (Stolc, 1979). Quercetin was found to be a potent inhibitor of human neutrophil degranulation and O<sub>2</sub><sup>-</sup> production induced by different secretagogues (Pagonis et al., 1986; Blackburn et al., 1987). Quercetin also inhibited the phosphorylation of neutrophil proteins accompanying neutrophil activation by PMA. Phosphorylation of a specific neutrophil protein (mol. wt. 67,000) was reported to be particularly sensitive to quercetin at concentrations that also diminished neutrophil degranulation and O<sub>2</sub><sup>-</sup> production, suggesting that its phosphorylation may be an important intracellular event associated with neutrophil activation (Blackburn et al., 1987).

Fourteen flavonoids were evaluated for their ability to inhibit chemiluminescence of neutrophils exposed to both luminol and PMA or to an enzymatic system with H<sub>2</sub>O<sub>2</sub>, luminol, and horseradish peroxidase (Krol et al., 1994). It was concluded that the 3-hydroxyl group and C2-C3 double bond were vital for the inhibitory effect of the flavonoids. The two hydroxyl groups on the B ring were considered to be optimal for the inhibitory effect.

A series of flavonoid compounds were assessed for their ability to inhibit the release of ROS by human neutrophils, using two chemiluminescence probes, lucigenin or luminol, after stimulation by f-MetLeuPhe, PMA, or opsonized zymosan in the presence or absence of horseradish peroxidase (Limasset et al., 1993). On the basis of structure-activity relationship analysis, the following B ring substituents proved to be particularly potent: 3',4'-dihydroxy (luteolin, rhamnetin), 3'-methoxy-4'-hydroxy (isorhamnetin), and 3'-hydroxy-4'-methoxy (diosmetin). Quercetin was found to have an ability to directly scavenge HOCl, a highly reactive chlorinated species generated by the MPO-H<sub>2</sub>O<sub>2</sub>-Cl system (Winterbourn, 1985). Several flavonoids were also active superoxide scavengers in a nonenzymatic system, inhibition of nitro blue tetrazolium reduction (Huguet et al., 1990).

#### *B. Effect of Flavonoids on Lipid Peroxidation and Oxyradical Production*

Oxidative stress can damage many biological molecules. Proteins and DNA are significant targets of cellu-

lar injury. Another target of free radical attack in biological systems is the lipids of cell membranes (Halliwell et al., 1992; Halliwell and Chirico, 1993).

As discussed later, lipid peroxidation *in vivo* involves a radical chain reaction consisting of a chain initiation and a chain propagation. During the initiation reaction, an alkyl radical is formed by abstracting one of the two hydrogens on a bisallylic carbon atom from the polyunsaturated fatty acid moiety of phospholipid bilayers or LDL. It is not known which is (are) the initial free radical attacking the phospholipid and initiating the reaction. It could be a perhydroxy radical ( $\cdot\text{OOH}$ ), a peroxyxynitrite ( $\text{ONOO}^-$ ) or a hydroxy radical ( $\cdot\text{OH}$ ), about which most of the comments below are made. In any event, the chain reaction leads to lipid hydroperoxides which continue to attack neighboring polyunsaturated fatty acids. Theoretically, this reaction could be controlled by the presence of lipid-soluble antioxidants such as  $\alpha$ -tocopherol, or the absence of catalytically active iron or copper. Unstable lipid hydroperoxides could also interact with DNA and form unstable adducts. Aldehydes and ketones could also be produced, many of which are toxic on their own. Highly reactive radicals such as  $\cdot\text{OH}$  have the propensity to attack biological molecules by abstracting hydrogen. The most widely studied oxidative damage caused by  $\cdot\text{OH}$  is its capacity to initiate the free radical chain reaction, lipid peroxidation. For instance, this damage readily ensues when  $\cdot\text{OH}$  radicals abstract a hydrogen atom from a methylene carbon of a fatty acid or fatty acid side chain of a lipid. The lipids initially attacked by free radicals become oxidized to lipid peroxides. Lipid peroxides are potentially toxic and possess the capacity to damage most cells (Halliwell and Gutteridge, 1990; Halliwell, 1991b; Halliwell et al., 1992; Halliwell and Chirico, 1993). Accumulation of lipid peroxides has been reported in atherosclerotic plaques, in brain tissues damaged by trauma or oxygen deprivation, and in tissues poisoned by toxins. The idea that lipid peroxidation is often a secondary event consequent to primary cell damage induced by oxidative stress has been discussed (Halliwell and Chirico, 1993). Rises in intracellular "free"  $\text{Ca}^{2+}$ , protein and DNA damage, and abnormalities in cellular metabolism produced by oxidative stress have been considered to be more important than the peroxidation of membrane lipids in causing cellular injury (Halliwell and Chirico, 1993).

Whether lipid peroxidation is a primary event produced by oxidative stress or a consequence of tissue damage, it can still be biologically important in exacerbating tissue injury in view of the potential cytotoxicity of the end products of lipid peroxidation (Esterbauer et al., 1988). Lipid peroxidation products originating from dying cells could exert a cancer promotional effect. Recently, great emphasis was placed on the significant contribution of lipid peroxidation to the development of atherosclerosis, stroke, and myocardial infarction, as

well as to the deterioration of the brain or spinal cord that occurs following trauma or ischemia (Halliwell and Gutteridge, 1990). Lipid peroxidation has also been implicated in several pathologic conditions including aging, hepatotoxicity, hemolysis, cancer, tumor promotion, inflammation, and iron toxicity (Plaa and Witschi, 1976; Tappel, 1978; Recknagel and Glende, 1979; Bus and Gibson, 1979)

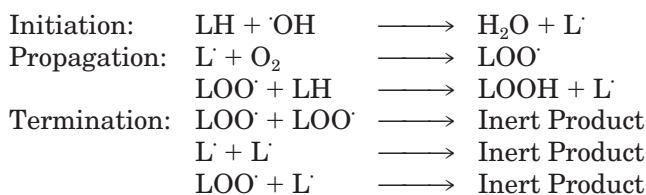
Several flavonoids have been reported to inhibit either enzymatic or nonenzymatic lipid peroxidation. Flavonoids such as quercetin could suppress lipid peroxidation in model systems (Letan, 1966), as well as in several biological systems, such as mitochondria, microsomes (Bindoli et al., 1977; Cavallini et al., 1978), chloroplasts (Takahama, 1983), and erythrocytes (Sorata et al., 1984; Maridonneau-Parini et al., 1986). Several studies have reported the inhibitory effects of (+)-catechin, quercetin, and other flavonoids on *in vitro* lipid peroxidation generally assessed by measuring colorimetrically the formation of thiobarbituric acid-reactive substance (Videla et al., 1981, 1985; Younes and Siegers, 1981; Muller and Sies, 1982; Valenzuela and Guerra, 1986).

Bindoli et al. (1977) demonstrated that silymarin, a 3-OH flavanone present in *S. marianum* (the European milk thistle), protected rat liver mitochondria and microsomes from lipid peroxide formation induced by  $\text{Fe}^{2+}$ -ascorbate and  $\text{NADPH-Fe}^{3+}$ -ADP systems. Its antiperoxidative action was 10-fold higher than that of  $\beta$ -tocopherol at micromolar concentrations. While the impairment of enzymatic lipid peroxidation by this flavonoid might involve its effect on the cytochrome P450 system, inhibition of nonenzymatic lipid peroxidation has been considered to involve interaction of silymarin with free radical species responsible for lipid peroxidation (Bindoli et al., 1977). Cavallini et al. (1978) reported that the inhibitory activity of silybin was superior to that of other flavonoids even with *O*-dihydroxy or trihydroxy substitution patterns. Soybean isoflavones have been examined for their antioxidative potency by measuring the extent of inhibition of soybean LO and by their ability to prevent peroxidative hemolysis of sheep, rat, and rabbit erythrocytes (Naim et al., 1976). The extent of inhibition of the enzyme activity was positively correlated with the number of hydroxyl groups in the isoflavone nucleus. Several isoflavones and their reduced derivatives (isoflavanones and isoflavans) were examined for inhibitory effects on lipid peroxidation in rat liver microsomes (Jha et al., 1985). The parent isoflavones and the isoflavans were by far the most potent inhibitors. Some isoflavans (6,7,4'-trihydroxy- and 6,7-dihydroxy-4-methoxyisoflavans) surpassed  $\alpha$ -tocopherol and butylated hydroxyanisole (a synthetic antioxidant) in terms of inhibitory effect. The 6,7-dihydroxylated isoflavans were 80 times stronger than  $\alpha$ -tocopherol in inhibiting lipid peroxidation. Methylation of the C7-OH of the isoflavones did not reduce the inhibitory effect, while methylation of the C6-OH group or both hydroxyl

groups (C6 and C7) resulted in lower inhibition. The position of the single phenolic group in the chromane ring of  $\alpha$ -tocopherol corresponds to the 6-OH group of the isoflavonoids. A common feature of the active isoflavonoids is an *ortho*-dihydroxybenzene or catechol structure, which is considered to be important for their anti-oxidative effectiveness (Simpson and Uri, 1956; Mehta and Seshadri, 1959; Hudson and Lewis, 1965).

Kimura et al. (1984) reported that flavonoids such as wogonin, oroxylin A, chrysin, skullcapflavone II, baicalein, and baicalin, isolated from the roots of *S. baicalensis* Georgi, inhibited lipid peroxidation induced by ADP-NADP and  $\text{Fe}^{2+}$ -ascorbate in rat liver homogenates. The dried roots of *S. baicalensis* have been used for the treatment of suppurative dermatitis, diarrhea, inflammatory diseases, hyperlipidemia, and atherosclerosis in Chinese and Japanese traditional medicine. Another flavonoid isolated from these roots by Kimura et al. (1984), 2',5,5',7'-tetrahydroxy-6',8-dimethoxyflavone, was found to be a very potent inhibitor of lipid peroxidation (Kimura et al., 1984). It exhibited over 90% inhibition toward lipid peroxidation induced by both ADP plus ascorbate and ADP plus NADPH in rat liver mitochondria and microsomes at a concentration of 100  $\mu\text{M}$ . Wogonin, at the same concentration, inhibited the ADP plus NADPH-induced lipid peroxidation of rat liver microsomes by 90%, whereas it inhibited the ADP plus ascorbate-induced lipid peroxidation of rat liver mitochondria by only 19%. It is worth noting that wogonin does not possess any hydroxyl substitution in its B ring.

It was reported that lipid peroxidation could be inhibited by flavonoids possibly acting as strong  $\text{O}_2^-$  scavengers (Baumann et al., 1980b) and  $^1\text{O}_2$  quenchers (Sorata et al., 1984). Although  $\text{O}_2^-$  itself does not appear to be capable of initiating lipid peroxidation,  $\text{HO}_2^-$  (the protonated form of  $\text{O}_2^-$ ) appears to do so in isolated polyunsaturated fatty acids (Halliwell and Gutteridge, 1990). The role of  $^1\text{O}_2$  in lipid peroxidation appears to be minor. The initiation of lipid peroxidation can be induced by  $\cdot\text{OH}$  and metal ion-free radical (such as perferryl and ferryl) complexes (Halliwell and Gutteridge, 1990). The scavenging of  $\cdot\text{OH}$  by flavonoids can impair lipid peroxidation. The induction of lipid peroxidation is shown below:



Lipid peroxidation may be prevented at the initiation stage by free radical scavengers, while the chain propagation reaction can be intercepted by peroxy-radical scavengers such as phenolic antioxidants. The chain-

breaking antioxidant action of the flavonoids (F) can be represented as shown below:



where FL-OH represents flavonoid.

Termination of lipid radical ( $\text{L}'$ ), lipid peroxy radical ( $\text{LOO}'$ ), and alkoxy radical ( $\text{LO}'$ ) (formed by reinitiation of lipid peroxidation induced by metal ions) by phenolic antioxidants is shown below:



where A-OH represents phenolics (e.g.,  $\alpha$ -tocopherol, flavonoids) and  $\text{AO}'$  represents the phenoxy radical.

It has also been proposed that flavonoids react with lipid peroxy radicals ( $\text{LOO}'$ ) leading to the termination of radical chain reactions. The oxidation of quercetin and rutin by lauroyl peroxide radicals is suggestive of such a mechanism (Takahama, 1983). The autoxidation of linoleic acid and methyl linoleate was inhibited by flavonoids such as fustin, catechin, quercetin, rutin, luteolin, kaempferol, and morin (Torel et al., 1986). Morin and kaempferol were the most inhibitory for the autoxidation of linoleic acid. Yet, morin had minimal inhibitory activity as compared with kaempferol toward mast cell secretion. Such differences indicate that different constituents are important for different biological activities of flavonoids. The inhibition of the formation of *trans-trans* hydroperoxide isomers of linoleic acid by flavonoids suggested that there was inhibition of the autoxidation of fatty acids by radical chain reaction termination (Torel et al., 1986).

Ratty and Das (1988) showed that several flavonoids inhibited both ascorbic acid and ferrous sulfate-induced lipid peroxidation in rat brain mitochondria. The concentrations of the flavonoids tested were (0.1–4.0  $\mu\text{M}$ ). Structural requirements for antiperoxidative activity included a 3-OH substitution, a 4-keto group, a C2-C3 double bond, and OH substitutions on rings A and B. The presence of OH groups in the B ring (3',4'-OH) had no particular effect in increasing the inhibitory potency.

The mechanism of antiradical action of quercetin and its glycoside, rutin, was evaluated by Afanas'ev et al. (1989) using NADPH- and carbon tetrachloride ( $\text{CCl}_4$ )-dependent lipid peroxidation of rat liver microsomes and iron ion-induced peroxidation of lecithin liposomes. Both flavonoids were significantly more effective inhibitors of the iron ion-dependent lipid peroxidation system due to their chelation of iron ions. The chelating mechanism of inhibition was more important for rutin than for quercetin. Neither flavonoid impaired the activity of cytochrome P450 as assessed by their influence on microsomal aminopyrine demethylase. It is surprising that no effect of quercetin was found on this mixed function oxidase activity. The inhibitory action of rutin and quercetin was demonstrated in all the peroxidation (iron ion-dependent and independent) systems studied. This

action was explained by both chelating and antioxidative properties of the flavonoids.

The inhibitory effects of both quercetin and rutin were more pronounced on NADPH-dependent than on  $\text{CCl}_4$ -dependent lipid peroxidation in rat liver microsomes. Microsomal NADPH-dependent lipid peroxidation is known to be catalyzed by NADPH cytochrome P450 reductase and proceeds in the presence of iron ions (Svingen et al., 1979). On the other hand, the activation of  $\text{CCl}_4$  involves cytochrome P450 and does not require iron ions (Albano et al., 1982). A much stronger inhibitory effect of the flavonoids on NADPH-dependent peroxidation was ascribed to their metal-chelating properties. The flavonoids were reported to chelate iron ions and to form inert complexes unable to initiate lipid peroxidation, yet they retained their free radical-scavenging properties. Ascorbate, instead, could exhibit antioxidant activity only in the absence of transitional metal ions (Halliwell, 1991a). The stronger inhibitory effect of quercetin in both peroxidation systems was thought to be attributable to its additional phenolic group (3-OH). Quercetin was also found to be oxidized by radicals generated in the decomposition of linoleic acid hydroperoxide in the presence of cytochrome *c*. The authors surmised that quercetin and rutin were able to suppress free radical processes by inhibiting the formation of  $\text{O}_2^-$ ,  $\cdot\text{OH}$ , and lipid peroxy radicals.

Baicalein was found to be a strong inhibitor of lipid peroxidation in rat forebrain homogenates (Hara et al., 1992). Its  $\text{IC}_{50}$  ( $0.42 \mu\text{M}$ ) was lower than that of quercetin ( $1.2 \mu\text{M}$ ). Flavone was found to be inactive. Baicalein also showed free radical-scavenging action against 1,1-diphenyl-2-picrylhydrazyl (DPPH). This flavone also inhibited phorbol ester-induced ear edema in mice, a process thought to involve lipid peroxidation.

Polymethoxylated flavones and C-glycosyl derivatives of flavones isolated from medicinal plants were studied for their influence on lipid peroxidation induced by  $\text{FeSO}_4$  plus cysteine in rat liver microsomes (Mora et al., 1990). Several hydroxylated flavones, C-glycosyl flavones, methoxyflavones, and flavonols, as well as the flavanol, leucocyanidol, and the biflavone, amentoflavone, showed inhibitory activity at a concentration of  $100 \mu\text{M}$ . Some hydroxyflavones were as effective as hydroxylated flavonols in inhibiting lipid peroxidation. The same was the case with C-glycosylflavonols (e.g., rutin) and C-glycosylflavones (e.g., orientin and isoorientin). Some methoxyflavones were also quite potent in inhibiting lipid peroxidation, although their  $\text{IC}_{50}$  values were much higher than those of hydroxyflavones. The flavanone glycoside, naringin, displayed no inhibition even at high concentrations ( $100 \mu\text{M}$ ). However, the corresponding flavone apigenin (with a C2-C3 double bond) was a potent inhibitor. Galangin, a flavonol possessing no B ring hydroxyl groups, was as effective as quercetin in inhibiting lipid peroxidation.

Cirsiliol and sideritoflavone, potent LO inhibitors (Alcaraz and Ferrandiz, 1987), showed no inhibitory activity, indicating that the inhibition of arachidonic acid metabolism by these compounds is dependent on flavonoid-enzyme interactions and is not related to possible antioxidant properties. A similar conclusion was also made by Laughton et al. (1991), who investigated the ability of various flavonoids to inhibit 5-LO and CO activities of rat peritoneal leukocytes and lipid peroxidation induced by  $\text{FeCl}_3$  plus ascorbate in rat liver microsomes. Several flavonols were potent inhibitors of lipid peroxidation in this system. Rutin was far less potent than quercetin. The lipid peroxidation inhibitory capacity of the flavonoids was not significantly correlated with their ability to inhibit LO or CO activity, suggesting that their mode of inhibition of 5-LO/CO is not simply due to scavenging of peroxy radicals generated at the active site of the enzymes. Robak et al. (1988) examined a series of flavonoids, isolated from plants, for their influence on soybean LO activity, CO activity, and inhibition of ascorbate-stimulated lipid peroxidation in rat liver microsomes. Most of the tested flavonoids stimulated CO when arachidonic acid was used as a substrate at  $100 \mu\text{M}$ . Several flavonoids were inhibitors of soybean LO activity and of lipid peroxidation. The most active inhibitors possessed vicinal hydroxyl groups in the B ring.

An isoflavonoid glycoside containing OH groups at positions 3 and 4 of the B ring, isolated from the roots of *P. labata*, was found to inhibit enzymatic (NADPH-induced) and nonenzymatic (ascorbate or  $\text{H}_2\text{O}_2$  plus  $\text{Fe}^{2+}$ -induced) lipid peroxidation in rat liver microsomes (Sato et al., 1992). On the other hand, wogonin, a flavone with no OH substitution in the B ring, inhibited only the enzymatically induced lipid peroxidation (Sato et al., 1992). Formation of  $\text{Fe}^{2+}$  by NADPH-dependent cytochrome P450 reductase was inhibited by wogonin, but not by the isoflavonoid glycoside. The glycoside had no effect on terminating radical chain reaction during lipid peroxidation in the enzymatic system or in the linoleic acid hydroperoxide-induced peroxidation system, suggesting that its antioxidant activity was probably caused by its ability to scavenge free radicals involved in the initiation of lipid peroxidation.

Laughton et al. (1989) found that both quercetin and myricetin were powerful inhibitors of iron-induced lipid peroxidation in rat liver microsomes. In these studies peroxidation was induced by adding  $\text{Fe}^{2+}$  (as ferrous ammonium sulfate),  $\text{Fe}^{3+}$  (as ferric chloride),  $\text{Fe}^{3+}$ -ascorbic acid,  $\text{Fe}^{3+}$ -EDTA or  $\text{Fe}^{3+}$ -ADP/NADPH. Myricetin possesses *o*-trihydroxy substitution (pyrogallol structure) in its B ring. The inhibitory effect was particularly pronounced when lipid peroxidation was stimulated by adding  $\text{Fe}^{3+}$ /ascorbate. At low concentration, the phenols caused a "lag period" during the course of lipid peroxidation. This effect was attributed to their action as lipid-soluble chain-breaking inhibitors of the

peroxidative process, scavenging intermediate peroxy and alkoxy radicals. At 100  $\mu\text{M}$ , both quercetin and myricetin accelerated the generation of  $\cdot\text{OH}$  radicals from  $\text{H}_2\text{O}_2$  in the presence of  $\text{Fe}^{3+}$ -EDTA.  $\cdot\text{OH}$  production was inhibited by catalase and SOD, which prompted the authors to suggest a mechanism in which the phenols oxidize to produce  $\text{O}_2^-$ , which then induces  $\cdot\text{OH}$  generation from  $\text{H}_2\text{O}_2$  in the presence of  $\text{Fe}^{3+}$ -EDTA. At concentrations up to 75  $\mu\text{M}$ , quercetin and myricetin accelerated bleomycin-dependent DNA damage in the presence of  $\text{Fe}^{3+}$ , which was suggested to be caused by the reduction of the  $\text{Fe}^{3+}$ -bleomycin-DNA complex to the  $\text{Fe}^{2+}$  form. These phenols, however, caused no acceleration of microsomal lipid peroxidation in the presence of  $\text{Fe}^{3+}$  or other iron complexes. The authors contended that the chain-breaking antioxidant activity of the phenolics outweighed any iron-reducing activity. In view of their observed prooxidant effects, the authors remarked that these phenolics could not be classified simplistically as "antioxidants". At this juncture, it may be recalled that both  $\alpha$ -tocopherol and ascorbate have similar prooxidant effects (Girotti et al., 1985; Husain et al., 1987b; Yamamoto and Niki, 1988).

Semisynthetic hydroxyethyl, water-soluble derivatives of flavonols have also been shown to display antioxidant action (Rekka and Kourounakis, 1991). Several hydroxyethyl rutosides and 7,3',4'-trihydroxyethyl quercetin exhibited considerable inhibition of rat liver microsomal lipid peroxidation induced by  $\text{FeSO}_4$  and ascorbate. They were less active than quercetin. They were also shown to be potent  $\cdot\text{OH}$  scavengers and interacted with DPPH stable free radical. Increasing substitution on the phenolic groups resulted in a concomitant diminution in the observed inhibition of lipid peroxidation.

The antioxidant action of the flavonoids silybin and (+)-cyanidanol-3 [(+)-catechin] was assessed in a peroxidation system consisting of linoleate and  $\text{Fe}^{2+}$  (Valenzuela et al., 1986). At the high concentration of 200  $\mu\text{M}$ , silybin (a water-soluble preparation of silybin as dihemisuccinate disodium salt) inhibited  $\text{Fe}^{2+}$ -induced linoleate peroxidation. The antioxidant effect exerted by (+)-catechin was far greater than that of silybin at high concentrations (250  $\mu\text{M}$ –2.0 mM). At a concentration of 200  $\mu\text{M}$ , the inhibitory action of silybin was comparable to that of butylated hydroxyanisole, while the antioxidant effect of (+)-catechin was similar to that obtained with butylated hydroxytoluene, one of the most powerful synthetic antioxidants. (+)-Catechin has been shown to have a powerful free radical-scavenging activity and to inhibit lipid peroxidation in different experimental systems (Videla et al., 1981, 1983; Videla, 1983). These included the inhibition of ethanol-induced enhancement of liver conjugated dienes (Videla et al., 1981) and of the chemiluminescence of rat liver in situ (Videla et al., 1983).

Fraga et al. (1987) reported that (+)-catechin, eriodictyol, and myricetin, at low concentrations ( $\text{IC}_{50}$ , 3–15  $\mu\text{M}$ ), inhibited the *tert*-butyl hydroperoxide-initiated chemiluminescence of mouse liver homogenates; this reaction is associated with lipid peroxidation resulting from the formation of hemoprotein-catalyzed radicals following rupture of the hydroperoxide (Boveris et al., 1985). Administration of eriodictyol and (+)-catechin to mice also depressed the enhancement of in situ liver chemiluminescence produced by  $\text{CCl}_4$ , which reacts with cytochrome P450 to initiate in vivo lipid peroxidation (Slater, 1984). Both carbon- and oxygen-centered radicals (McCay et al., 1984) and excited species (Chance et al., 1979) are formed during this process. The observed inhibition of chemiluminescence was proposed to involve free radical scavenging as well as excited species quenching.

When a light mitochondrial fraction of rat liver was incubated in the presence of xanthine oxidase and xanthine, the free activity of *N*-acetylglucosamine increased as a result of the deterioration of the lysosomal membrane (Decharneux et al., 1992). Certain flavonoids were able to prevent this phenomenon. Comparative activity studies suggested the importance of the presence of two OH groups in ortho substitution in the B ring and of an OH group in the C-3 position. It was suggested that the protective effect of flavonoids on lysosomes exposed to ROS did not only originate from their scavenging and antilipoperoxidative properties, but also from a direct action on lysosomal membranes making them more resistant to oxidative attack. Flavonoids could account for the protective effect of *G. biloba*, observed previously by the authors, on lysosomes exposed in vitro to ROS and osmotic stress.

Sorata and coworkers (Sorata et al., 1984) demonstrated that quercetin and rutin inhibited human erythrocyte lipid peroxidation accompanying photohemolysis. Several flavonoids were observed to inhibit *N*-ethyl maleimide-induced lipid peroxidation in human platelets (Koch and Loffler, 1985). Very low  $\text{IC}_{50}$  values were observed, and silymarin appeared to be particularly active. Kappus et al. (1979) showed the inhibition of lipid peroxidation in isolated rat hepatocytes by (+)-catechin. Using phenazine methosulfate as an intracellular generator of oxygen free radicals, Maridonneau-Parini et al. (1986) reported a heterogeneous effect of flavonoids on  $\text{K}^+$  loss and lipid peroxidation induced by oxygen radicals in human erythrocytes.

Cholbi et al. (1991) described the activity of apigenin, luteolin, gardenin D, galangin, datiscetin, and morin, as well as catechin, as inhibitors of  $\text{CCl}_4$ -induced rat liver NADPH-dependent microsomal lipid peroxidation. The polymethoxylated flavone, gardenin D, possesses OH groups at 5- and 3'-positions, and  $\text{OCH}_3$  groups at 6-, 7-, 8-, and 4'-positions. Its potency was reported to be comparable to that of (+)-catechin, showing its strong inhibitory effect on cytochrome P450.

The flavonols quercetin, rutin, and morin, as well as the flavanones naringin and hesperidin, were studied as chain-breaking antioxidants for the autoxidation of linoleic acid in cetyl trimethylammonium bromide micelles (Wang and Zheng, 1992). All three flavonols exhibited antioxidant activities, while the two flavanones, naringin and hesperidin, did not suppress the oxidation appreciably. The 7-hydroxy group of the flavonoids is considered to be the first to dissociate and is thus the most likely site of attack by peroxy radical (Mabry et al., 1970; Bors et al., 1990). The 7-hydroxy group is unsubstituted in quercetin, rutin, and morin, while it is blocked with a glycoside in naringin and hesperidin. Thus, the former compounds exhibited active antioxidant activity, whereas the latter were inactive.

Terao et al. (1994) reported that (-)-epicatechin, (-)-epicatechin gallate, and quercetin retarded the accumulation of phosphatidylcholine hydroperoxides when the suspension was exposed to a water-soluble radical indicator, 2,2'-azobis (2-amidinopropane) hydrochloride. Their inhibitory effects lasted longer than that of  $\alpha$ -tocopherol. The catechin derivatives, when mixed in the liposomes, disappeared in favor of  $\alpha$ -tocopherol. It was suggested that the localization of the flavonoids near the surface of phospholipid bilayers suitable for scavenging aqueous oxygen radicals prevents the consumption of lipophilic  $\alpha$ -tocopherol.

Middleton, Drzewiecki, and Kandaswami (unpublished results) examined the scavenging action of a wide range of flavonoids against DPPH radical. Several flavonols, flavones, and flavan-3-ols were active, although flavone, apigenin, naringin, naringenin, and chrysin showed no activity. The C2-C3 double bond and the 3-OH group appeared to increase the radical-scavenging potency at lower concentrations.

Bors and Saran (1987) studied the radical-scavenging efficiencies of different classes of flavonoids by using the method of pulse radiolysis. Aroxy radicals were generated by univalent oxidation of several flavonoids by azide ( $N_3$ ) radicals at pH 11.5. Compounds with a saturated ring were predominantly attacked at the *O*-dihydroxy site in the B ring and the semiquinones formed were quite stable. For a substance to act as an antioxidant, the stability of the radicals formed from it is of prime importance. Radicals derived from flavonoids with a C2-C3 double bond and both 3- and 5-OH substituents (flavonols) apparently did not seem to possess a higher stability. The very high rate constant of formation and the relative stability of some of the aroxy radicals led to the supposition that the biological function of flavonoids might be the scavenging of radicals. In a study dealing with the reaction of fatty acid peroxy radicals, both kaempferol and quercetin turned out to be exceptionally good scavengers of linoleic acid peroxy radicals (Erben-Russ et al., 1987).

In further studies, using the method of pulse radiolysis, Bors et al. (1990) examined the radical-scavenging

and antioxidant potential of different classes of flavonoids. They demonstrated the effective radical-scavenging capabilities of most flavonoids and indicated the existence of multiple mesomeric structures for aroxy radical species of flavonoids. Three structural groups were important determinants for radical-scavenging and for antioxidant potential: 1) the *O*-dihydroxy (catechol) structure in the B ring, the obvious radical target site for all flavonoids with a saturated C2-C3 double bond (flavan-3-ols, flavanones, cyanidin chloride); 2) the C2-C3 double bond in conjunction with a 4-oxo function; and 3) the additional presence of both 3- and 5-OH groups for maximal radical-scavenging potential. The capacity of flavonoids to scavenge  $O_2^-$ , OH, and lipid radicals has been frequently reported (Ueno et al., 1984; Takahama, 1985, 1987; Torel et al., 1986; Husain et al., 1987a; Robak and Gryglewski, 1988; Huguet et al., 1990). Flavonoids do react rapidly with  $\cdot$ OH because of the generally high reactivity of this radical with aromatic compounds. In contrast, even for the very efficient flavonol radical scavengers kaempferol and quercetin (Takahama, 1987; Robak and Gryglewski, 1988), only very low rate constants were found for  $O_2^-$  (Bors et al., 1990). Bors et al. (1990) have questioned reports on the specific scavenging of different radicals by flavonoids. Sichel et al. (1991) have reported the scavenger activity of some flavonoids against  $O_2^-$  using electron spin resonance spectrometry. These authors suggested that the presence of hydroxyl groups in the B ring of flavonoids is essential for this scavenging activity. Cotelle et al. (1992) showed the formation of stable radicals from synthetic flavonoids by electron spin resonance spectroscopy.

Certain flavonoids have been shown to inhibit mitochondrial succinoxidase and NADH oxidase and other oxidase activities. In a structure-activity investigation of 14 different flavonoids, four flavonoids, quercetin, kaempferol, myricetin, and delphinidin chloride, were shown to generate a cyanide-insensitive respiratory burst in the presence of isolated beef heart mitochondria and to autoxidize in buffer alone. Subsequently, the same flavonoids were shown to autoxidize with the concomitant production of semiquinone radicals,  $O_2^-$ ,  $\cdot$ OH, and  $H_2O_2$ . The inhibition of the above mitochondrial enzymes by flavonoid compounds was suggested to contribute to their antineoplastic activities. The inhibition of enzymes that catalyze oxidation-reduction reactions by flavonoids may involve flavonoid-generated ROS (Hodnick et al., 1986, 1987, 1988a,b, 1989; Elliott et al., 1992).

Quercetin effectively inhibited lipid peroxidation with microsomes from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats. The pathologic effects induced by TCDD (hepatic necrosis, bone marrow depression, immunotoxicity, carcinogenesis, etc.) are mediated by an intracellular protein called Ah (aromatic hydrocarbon) which binds TCDD. The action of quercetin may be

related to inhibition of PLA<sub>2</sub> shown to be involved in hepatic microsomal lipid peroxidation induced by TCDD in rats (Al-Bayati and Stohs, 1991). Interaction of flavonoids with the free radical 1,1-diphenyl-2-picrylhydrazyl was studied by Ratty et al. (1988); antiperoxidative flavonoids included quercetin, quercitrin, rutin, myricetin, phloretin, phloridzin, catechin, morin, and taxifolin.

The autoxidation of flavonoids such as quercetin and myricetin (having catechol and pyrogallol configuration in the B ring, respectively) in aqueous media at pH 7.5 has been described (Canada et al., 1990). This autoxidation resulted in the generation of O<sub>2</sub><sup>-</sup>, H<sub>2</sub>O<sub>2</sub>, and ·OH. The autoxidation was, however, quite slow at pH 7.5 for quercetin. Such prooxidant effects are of interest in the context of tumor cell cytotoxicity, while not considered to have toxicological consequences.

A large number of studies have emphasized the potential health-promoting and disease-preventing effects of fruits and vegetables in the diet. The beneficial effects of fruits and vegetables have frequently been attributed to ascorbic acid and the carotenoids present in these foods. However, as stated elsewhere, fruits and vegetables contain a multitude of flavonoids and related phenolic compounds that also act as natural antioxidants. Flavonoids can function as 1) metal chelators and reducing agents, 2) scavengers of ROS, 3) chain-breaking antioxidants, 4) quenchers of the formation of singlet oxygen, and 5) protectors of ascorbic acid; conversely, ascorbic acid can protect flavonoids against oxidative degradation. In many of the studies reported, it is not certain whether flavonoids inhibit the formation of ROS or scavenge them. Nevertheless, it is obvious that flavonoids react with OH and, therefore, can be very important chain-breaking antioxidants. They could also play an important role in conserving tocopherols in biological membranes.

### IX. Actions in Relation to Coronary Artery Disease and Vascular Disorders

Increased LDL and especially oxidized LDL are recognized as risk factors in coronary artery disease (CAD). De Whalley et al. (1990) showed that certain flavonoids were potent inhibitors of the modification of LDL by mouse macrophages with IC<sub>50</sub> values in the micromolar range (e.g., 1–2 μM for fisetin, morin, and quercetin). Flavonoids also inhibited the cell-free oxidation of LDL mediated by CuSO<sub>4</sub>. The flavonoids appeared to act by protecting LDL against oxidation caused by the macrophages, as they inhibited the generation of lipid hydroperoxides and protected α-tocopherol, a major lipophilic antioxidant carried in lipoproteins, from being consumed by oxidation in the LDL. Thus the flavonoids protected α-tocopherol (and possibly other endogenous antioxidants) in LDL from oxidation, maintained their levels for longer periods of time, and delayed the onset of lipid peroxidation. While the mechanisms by which fla-

vonoids inhibit LDL oxidation are not certain, the following possibilities have been advanced. First, they may reduce the generation or release of free radicals in the macrophages or may protect the α-tocopherol in LDL from oxidation by being oxidized by free radicals themselves. Second, flavonoids could regenerate active α-tocopherol by donating a hydrogen atom to the α-tocopheryl radical; the latter is formed when it transfers its own OH hydrogen atom to a lipid peroxy radical to terminate the chain reaction of lipid peroxidation. Third, flavonoids may sequester metal ions, such as iron and copper, thereby diminishing the engendered free radicals in the medium. Preliminary evidence indicated that the isoflavone genistein inhibits Cu-mediated LDL oxidation in a time- and concentration-dependent fashion (Tsai and Chait, 1995). Nevertheless, since some flavonoids at a concentration of only 10 μM completely inhibited the modification of LDL by 100 μM Cu<sup>2+</sup>, it was felt that metal complexation by flavonoids alone could not explain all their effects. In any event, polyhydroxylated aglycone flavonoids were potent inhibitors, pointing once more to the importance of OH groups in the flavone nucleus.

The oxidation products of LDL induced by UV radiation attack mainly the lipid core of the LDL, in contrast to the cell- or copper-mediated oxidation, which primarily attacks the LDL surface components (Negre-Salvayre et al., 1990). Negre-Salvayre et al. (1991b) reported the protection of lymphoid cell lines against peroxidative stress induced by oxidized LDL using a combination of α-tocopherol, ascorbic acid, and the quercetin glycoside, rutin. These investigators also showed that the cytotoxicity of oxidized LDL could be prevented by flavonoids in two ways: either by inhibiting the lipid peroxidation of LDL (induced by UV irradiation) or by blocking at the cellular level the cytotoxicity of previously oxidized LDL (Negre-Salvayre et al., 1991a). Their studies showed that 1) probucol (25 μM), a synthetic antioxidant, was very effective in preventing UV-induced lipid peroxidation of LDL and their subsequent cytotoxic effects on lymphoid cell lines (EBV-transformed cell lines), but it could not protect cells against the cytotoxicity of previously oxidized LDL; 2) vitamin E (100 μM) weakly prevented the lipid peroxidation of LDL, but it was able to abrogate the cellular oxidative stress and cytotoxicity induced by previously oxidized LDL; and 3) catechin (10 μM) inhibited the peroxidation of LDL and protected the cells against the toxicity of previously oxidized LDL. In subsequent studies, these investigators showed that both quercetin and rutin exhibited effects similar to catechin, i.e., inhibiting the lipid peroxidation of LDL and blocking at the cellular level the cytotoxicity of previously oxidized LDL (Negre-Salvayre and Salvayre, 1992). Flavone was completely inefficient in exerting any of these effects.

The inhibition of LDL lipid peroxidation by the flavonoids correlated well with the prevention of the cyto-

toxicity of oxidized LDL. In the protection of the cells by polyphenolic flavonoids, two lines of defense were inferred: 1) from studies using quercetin or rutin at moderately high concentrations ( $IC_{50}$ , 10–20  $\mu M$ ), there was inhibition of lipoprotein oxidation and subsequent cytotoxicity; and 2) at relatively low concentrations ( $IC_{50}$ , 0.1 and 3  $\mu M$ ), there was direct protection of cells against the cytotoxic effect of oxidized LDL. The cellular mechanisms for this direct prevention of the cytotoxic effect of oxidized LDL are unknown, but could involve the following: a) prevention of oxidative attack of membrane lipids by sparing vitamin E or regenerating it, as does ascorbic acid in the maintenance of  $\alpha$ -tocopherol levels; b) inhibition of lipoxygenases, which are known to be stimulated by lipid peroxides and which can be involved in oxidative stress, as suggested by their role in LDL oxidation in cells; and c) inhibition of cellular enzymes involved in signal transduction. The above results suggest that dietary flavonoids or related compounds could be involved in the prevention of atherosclerosis not only by inhibiting LDL oxidation, but also by increasing the cellular resistance to the deleterious effects of oxidized LDL. Recruitment of different flavonoids effective in directly protecting cells represents a novel approach in the prevention of atherosclerosis by nutritional intervention.

Negre-Salvayre et al. (1995) demonstrated that LDL mildly oxidized by copper ions or UV radiation exhibited a cytotoxic effect on cultured endothelial cells, which could be inhibited by rutin, ascorbic acid, and  $\alpha$ -tocopherol. The compounds acted to inhibit LDL oxidation and to increase the resistance of the cells to the cytotoxic effect of oxidized LDL. A mixture of the three compounds had a "supra-additive" effect.

Mangiapane et al. (1992) reported that (+)-catechin (50  $\mu g/ml$ ) inhibited oxidation of LDL induced by the mouse transformed macrophage cell line, 1774, human monocyte-derived macrophages, and vascular endothelial cells isolated from umbilical cords. LDL reisolated from cell incubations in the presence of (+)-catechin was endocytosed and degraded at rates similar to native LDL. The compound appeared to inhibit the uptake and degradation by macrophages of cell-modified LDL. Several epidemiological studies have examined the relationship between flavonoid and coronary heart disease. These studies were reviewed recently (Samman et al., 1998). One study from The Netherlands showed an inverse correlation between dietary flavonoid intake and the incidence of CAD in elderly men (Hertog et al., 1993a). In this Zutphen elderly study, the relative risk from CAD was reduced significantly, while the risk from myocardial infarction was borderline. The individuals with the lowest dietary intake of flavonoids had the highest incidence of heart disease. Interestingly, the relative incidence of heart disease among men who had the highest intake of flavonoids was only one third of those who had the lowest intake of flavonoids. The result

was the same even after adjustment for age, body fat, smoking, cholesterol, blood pressure, physical activity, coffee consumption, and the intake of calories, vitamin C, vitamin E, betacarotene, and dietary fiber. The main sources of dietary flavonoids for the above individuals were apples, onions, and tea.

In the same Zutphen study conducted in The Netherlands (Keli et al., 1996), dietary flavonoids, mainly quercetin, were inversely associated with stroke incidence (after adjustment for potential confounders including antioxidant vitamins). One implication of this interesting observation is the possibility that certain flavonoids may be stored in blood vessels and there exert anti-atherogenic effects. In another publication (the seven countries study), The Netherlands group reported that the mortality from coronary heart disease was inversely associated with average intake of flavonoids (Hertog et al., 1995). At least one other study, however, showed no significant correlation between flavonoid consumption and CAD mortality, either in males or females, in spite of large sample size (Knekt et al., 1996)

Cholesterol is considered to be a major risk factor for coronary artery disease. Consumption of diets high in saturated fat and cholesterol is associated with increased risk of coronary artery disease. According to Setchell (1985), the hypocholesterolemic effect of soy may be related to its content of phytoestrogen isoflavones, since soy from which the phytoestrogens had been extracted had a minimal effect in monkeys (Anderson et al., 1995; Erdman, 1995).

Epidemiological evidence indicates that heart disease is less frequent in the French than expected, based on saturated fat intake and cholesterol levels. This unusual effect, known as the "French paradox", has been attributed to drinking red wine. The biochemical/pharmacological basis of the wine question was addressed in an editorial by David Goldberg (1995), who reminded us that red wine contains quercetin, rutin, catechin, and epicatechin (among other flavonoids). Red wine also contains a unique, although rather obscure, trihydroxystilbene known as resveratrol; this compound is recognized as an herbal component in Japanese folk medicine and has been used in the treatment of heart, lipid, and inflammatory disorders. Resveratrol was recently shown to have anti-inflammatory activity (Bertelli et al., 1999). Quercetin and phenolic compounds isolated from red wine effectively impaired copper ion-catalyzed oxidation of LDL, while  $\alpha$ -tocopherol exhibited only 60% of the potency of wine phenolics or quercetin (Frankel et al., 1993).

Several flavanoid glycosides in orange were reported to have vasodilatory activity (Kumamoto et al., 1986). Ning et al. (1993) reported that flavone administration markedly improved functional recovery in the reperfused rabbit heart after a bout of global ischemia. The effects of the compound on postischemic recovery were proposed to be caused by its stimulation of the cyto-

chrome P450 system. Cytochrome P450 reductase, which transfers electrons from NADPH to cytochrome P450 during P450-dependent catalysis, is capable of reducing oxygen to yield  $O_2^-$ ; the oxygenated intermediates of P450 themselves then decompose in a side reaction to release  $O_2^-$  (White and Coon, 1980; Halliwell and Gutteridge, 1985). It was advanced that flavone might be acting as an allosteric effector that improves catalytic efficiency, thereby diminishing detrimental ROS production. Ning et al. (1993) have highlighted the potential utility of flavonoids as a means of enhancing myocardial ischemic tolerance or resistance to reperfusion injury, or both. They also drew attention to the recent identification of an interesting isoflavonoid compound, puerarin (8-C-C-glycopyranosyl-1-4'-7-dihydroxyisoflavone), as an active ingredient in *R. pueriae*, a traditional Chinese medicinal herb that has been used for many decades for the treatment of hypertension and angina pectoris in China (Fan et al., 1985).

Two flavonoids, quercetin and silybin, were reported to exert a protective effect by preventing the decrease in the xanthine dehydrogenase/oxidase ratio observed during ischemia-reperfusion in the rat (Sanhueza et al., 1992). The results indicated the conversion of xanthine dehydrogenase to xanthine oxidase during the early stages of kidney ischemia. The enzyme xanthine oxidase, implicated in tissue oxidative injury after ischemia-reperfusion, is a source of ROS and is formed from a dehydrogenase during ischemia (McCord, 1985). The protective effect of quercetin and silybin on the xanthine dehydrogenase/oxidase ratio, observed in the above study, was postulated to be caused by the inhibition of the dehydrogenase-to-oxidase transformation by the flavonoids. The inhibition of xanthine oxidase activity by flavonoids had also been described (Iio et al., 1986).

Myricetin and quercetin, flavonoid constituents of *G. biloba*, impaired the oxidation of 2,7'-dichlorofluorescein (DCFH) by cellular  $H_2O_2$  within the neurons dissociated from rat brain, at concentrations ranging from 3–10 nM (Oyama et al., 1994). Incubation with each flavonoid also decreased the oxidative metabolism of DCFH without affecting the cellular content of DCFH or of the intracellular concentrations of  $Ca^{2+}$ . Such an antioxidant effect of myricetin or quercetin might partly account for the beneficial effects of *G. biloba* on brain neurons subject to ischemia.

The vascular endothelium is extremely sensitive to oxidative damage mediated by ROS released from inflammatory cells (Sacks et al., 1978; Weiss et al., 1981). Of these metabolites,  $H_2O_2$  appears to be an important mediator of acute cellular injury in a variety of settings (Weiss et al., 1981). Such oxidative damage may play a role in the pathogenesis of atherosclerosis (Mazzone et al., 1983). The flavan-3-ol compounds, epigallocatechin-3-O-gallate and epicatechin-3-O-gallate, isolated from tea, were effective in preventing  $H_2O_2$ -induced injury to bovine endothelial cells in culture (Chang and Hsu,

1991). These observations suggest a possible role for these catechins in maintaining vascular homeostasis.

Beretz et al. (1982) reviewed the inhibitory effect of flavonoids on platelet aggregation. Dhar and colleagues (1990) showed that genistein blocked platelet aggregation stimulated by PAF. Moreover, Tzeng et al. (1991) showed that several flavonoids inhibited thromboxane formation. Inhibition of platelet aggregation was also reported by Robbins (1988) and Tomasiak (1992). Gryglewski and coworkers studied the mechanism of the antithrombotic action of flavonoids (1987). Four flavonoids (quercetin, rutin, cyanidanol, and meciadonol) each inhibited platelet LO activity and ascorbate-induced rat liver microsomal lipid peroxidation, whereas only quercetin and rutin stimulated CO and bound to platelet membranes. Quercetin and rutin were capable of dispersing platelet thrombi adhering to rabbit aortic endothelium in vitro and prevented platelets from aggregating over a blood-superfused collagen strip (adhesion-related phenomena). The in vivo counterpart of these experiments involved the infusion of quercetin and rutin into an extracorporeal stream of blood. Quercetin and rutin inhibited the deposition of platelet thrombi on the blood-superfused collagen strip at calculated plasma concentrations of 0.05 and 0.03  $\mu$ M. Analogously, in the model for studying platelet-endothelium interactions, quercetin and rutin, when infused into the stream of blood that superfused a rabbit aortic endothelial surface, caused the disaggregation of preformed platelet thrombi, again at low concentrations. Clearly, the expression and/or activity of platelet/endothelium adhesion molecules were affected by the flavonoids. The authors concluded that flavonols were antithrombotic because they are bound selectively to mural platelet thrombi and, because of their free radical-scavenging properties, modify damaged endothelial cells and permit normal prostacyclin and NO synthesis (Gryglewski et al., 1987). More detailed discussion appeared under *Platelets*.

The isoflavone orobol (and quercetin) was an effective inhibitor of 15-LO and the formation of 15-hydroxyeicosatetraenoic acid in mouse peritoneal macrophages (Kohyama et al., 1994). 15-LO is also implicated in LDL oxidation and atherogenesis and is found in substantial quantities in atherosclerotic lesions. This flavonoid requires further study as an antiatherogenic agent. Testifying to the potential health-promoting, disease-preventing activity of flavonoids are the remarkable experiments of Demrow et al. (1995), who examined the effects of red wine and grape juice in the Folts model of mechanically stenosed coronary arteries and intimal damage in dogs; intravenously or intragastrically administered grape juice or red wine could reduce or abolish coronary artery cyclic flow reduction used as the outcome measure in this model.

Importantly, olive oil, the beneficial effects of which (along with fruits and seeds in what is known as the

Mediterranean diet) are well known (Trichopoulou et al., 1995, 2000), contains several flavonoids (Boskou, 2000). Another possible mechanism for inhibition of atherogenesis is the smooth muscle antiproliferative effect of certain flavonoids such as baicalein (Huang et al., 1994b). In rat dietary experiments, Monforte et al. (1995) determined that hesperidin, an important citrus flavanone, increased HDL while it lowered cholesterol LDL, plasma triglycerides, and total lipids. These changes occurred in normolipidemic rats, as well as in rats with hyperlipidemia. The potential clinical significance of these observations is obvious.

The protective role of flavonoids in cardiac ischemia may also be related to their ability to inhibit mast cell secretion (discussed above). Mast cells have been increasingly implicated in cardiovascular inflammation (Frangogiannis et al., 1998), especially that induced by acute stress (Pang et al., 1998). In fact, mast cell-derived mediators may be involved in cardiovascular inflammation, which is now considered a key factor in coronary artery disease (Ridker et al., 1998). Mast cell chymase (Schwartz, 1987) has been identified as the enzyme responsible for the conversion of angiotensin I to angiotensin II in the heart (Urata and Ganten, 1993; Takai et al., 1999). Moreover, IL-6 was recently shown to be a key factor in CAD (Yudkin et al., 2000). IL-6 is known to be released from mast cells (Kruger-Krasagakes et al., 1996). We recently showed that IL-6 is released from the heart in acute CAD (Deliargyris et al., 2000). Moreover, acute stress in mice induces release of IL-6 from cardiac mast cells, an effect entirely absent in  $W/W^v$  mast cell-deficient mice; release of IL-6 under acute stress was manyfold higher in Apo-E knockout mice that develop atherosclerosis (Huang et al., 2000).

Flavonoids could be important in protecting LDL from oxidation, thus reducing their atherogenicity. In general, flavonoids could potentially influence disease states in which lipid peroxidation products are intricately involved, especially vascular disorders and coronary artery disease. The anti-inflammatory and mast cell inhibitory actions of flavonoids provide new evidence of their possible ability to modulate inflammation, which is increasingly implicated in CAD. Moreover, genistein inhibited TNF-stimulated induction of endothelial cell adhesion molecules (Weber et al., 1995) in keeping with the effects of several other flavonoids as described by Anné et al. (1994) and Gerritsen et al. (1995). Very likely, the selective induction of VCAM-1 expression by IL-13 in HUVECs (Bochner et al., 1995) would be similarly affected by particular flavonoids.

In summary, flavonoids may be protective against CAD by influencing several processes such as 1) decrease in LDL oxidation, 2) increase in HDL levels, 3) reduction of cardiac mast cell mediator release, and 4) decrease in cardiovascular inflammation.

## X. Flavonoid-Vitamin C Interactions

There is growing interest in the multiple aspects of ascorbic acid biochemistry and the role of this vitamin in human nutrition and physiology (Block et al., 1991). Ascorbic acid is a universal component of plant cells. Ascorbic acid and flavonoids coexist in many plants, and thus the two may be consumed together in the diet (McClure, 1975; Hughes and Wilson, 1977). A large body of literature has accumulated concerning the interactions of flavonoids with ascorbic acid in biological systems (Clemetson and Anderson, 1966; Hughes and Wilson, 1977; Clemetson, 1989). Several flavonoids serve as antioxidants for ascorbic acid (Harper et al., 1969). In vitro studies indicated that flavonoids had considerable capacity to retard the conversion of ascorbate to dehydroascorbate. One mechanism for this protection might involve the chelation of copper and other trace metals by flavonoids, resulting in the retardation of metal-catalyzed oxidation of ascorbic acid. Another protective mechanism is based on the ability of flavonoids to act as free radical acceptors since free radical formation is considered to be an all-important phase of ascorbate oxidation. Several physiological interactions of ascorbic acid with plant flavonoids have been considered (Hughes and Wilson, 1977), such as 1) an increase in ascorbic acid absorption, 2) stabilization of ascorbic acid, 3) reduction of dehydroascorbate to ascorbate, and 4) metabolic sparing of ascorbic acid by flavonoids. The sparing effect of flavonoids on ascorbate oxidation may explain many of the interactions of flavonoids with ascorbic acid described in the voluminous literature on these compounds.

The role of vitamin C on immune function has been reviewed by Meydani and Blumberg (1989). Vitamin C supplementation augmented [ $^3$ H]thymidine incorporation in mitogen-stimulated lymphocytes. A possible explanation of the immunostimulatory effect of vitamin C may be through its antioxidant effect to reduce lipid peroxidation. In early work, Clemetson (1980) found that low levels of plasma ascorbic acid were accompanied by markedly elevated whole blood histamine concentrations and that oral administration of ascorbic acid (1 g for 3 days) led to a reduction of blood histamine levels. Such observations need further study for their potential relevance to atopy and allergic diseases. Human studies showed increased tissue concentration of ascorbic acid as well as increased urinary output of the vitamin (Hughes and Wilson, 1977; Jones and Hughes, 1984). Considerable evidence indicates that flavonoids may influence the metabolism of ascorbic acid, although the basis of this is not understood (Hughes and Wilson, 1977; Clemetson, 1989).

Clemetson and Anderson related ascorbate-protective capacity to the structure of the flavonoids (Clemetson and Anderson, 1966; Clemetson, 1989). They examined the effect of 34 different flavonoids on the oxidation of

ascorbic acid at physiological pH and concluded that significant antioxidant activity was confined to compounds possessing 3',4'-OH groups of the B ring and the 3-hydroxy-4-carbonyl grouping of the  $\gamma$ -pyrone ring. In conformity with this, quercetin and rutin were found to have a greater ascorbic acid-protective capacity than the other flavonoids examined (Hughes and Wilson, 1977). An apparent exception to the above generalization is hesperidin, which did not conform to the prescribed pattern and yet had in vitro protective capacity and in vivo increased tissue ascorbic acid concentrations (Bhagvat, 1946; Wilson et al., 1976). However, it was known that commercial samples of hesperidin contained other flavonoids as impurities (Clemetson and Anderson, 1966).

Leung et al. (1981) demonstrated a synergistic interaction between vitamin E and vitamin C with respect to peroxidation of membrane phospholipids. An analogous situation could exist with flavonoid-flavonoid or flavonoid-vitamin interactions.

Thiol compounds such as glutathione are potential hydrogen donors for the reduction of dehydroascorbic acid to ascorbic acid (Parrot and Gazave, 1951; Hughes and Wilson, 1977). Flavonoids such as quercetin and hesperidin were shown to enhance the reduction of dehydroascorbic acid by glutathione. Parrot and Gazave (1951) reported that (+)-catechin potentiated the reduction of dehydroascorbic acid by glutathione. The possibility that flavonoids might stimulate the tissue reduction of dehydroascorbic acid was examined by Zloch (1973). Guinea pigs were given a standard diet of dehydroascorbic acid with and without flavonoids (rutin, epicatechin), and it was shown that the tissue ascorbic acid content was 30 to 100% greater in the flavonoid-treated group.

Flavonoids have been considered to function as antioxidants and UV light filters in higher plants (McClure, 1975, 1986). This antioxidant activity was related to their protection against ascorbic acid oxidation. The protection of ascorbic acid by flavonoids could have important biological implications, as emphasized by Hughes and Wilson (1977). Ascorbic acid metabolites can be mutagenic for mammalian cells (Stich et al., 1976). An increased production of these metabolites could be a key factor in aging, according to the intrinsic mutagenesis theory of aging (Burnet, 1974). Flavonoids and other factors that suppress the breakdown of ascorbic acid (Davidek, 1960) could, therefore, function as antiaging factors. Conversely, ascorbate may also protect flavonoids from oxidation. Purified cyanidin 3-gentiobioside, cyanidin 3-rhamnoside, and pelargonidin 3-glucoside were decolorized by low levels of  $H_2O_2$  and horseradish peroxidase. Ascorbate added to this system inhibited the decolorization of the anthocyanins to one-tenth the rate of the control, apparently by reducing an early oxidation product of anthocyanin breakdown (McClure, 1975). The physiological relevance of these findings remains to be established because it may be limited

to the concentrations of ascorbate and the in vitro test system used.

Sorata et al. (1988) studied the promoting effect of ascorbate on quercetin-induced suppression of photohemolysis in human erythrocytes. The authors suggested that the cooperation of quercetin with ascorbate in photohemolysis was attributable to the reduction of oxidized quercetin by ascorbate, resulting in the regeneration of the flavonol. Takahama's (1985) studies also suggested the reduction of oxidized quercetin to quercetin by ascorbate. Jan et al. (1991) reported that the antioxidative function of quercetin in inhibiting the photooxidation of  $\alpha$ -tocopherol was enhanced by ascorbate, which reduced oxidized quercetin. Takahama (1986) showed that the intermediates formed during the oxidation of flavonoids by the horseradish peroxidase- $H_2O_2$  system might be reduced by ascorbate; the oxidized product that could be reduced by ascorbate appeared to be an *ortho*-quinone derivative.

In a pulse radiolysis study, Bors et al. (1995) examined the interaction of flavonoids with ascorbate with determination of their redox potentials. All compounds with the catecholic hydroxyl groups in the B ring and the C2-C3 double bond had a higher redox potential than ascorbate and as a result were able to oxidize it to the ascorbyl radical.

An example with potential clinical relevance is the preservation of antiviral activity of quercetin in the presence of ascorbate, which inhibits the oxidative degradation of the quercetin (Vrijssen et al., 1988). Maintenance of biological activity of other flavonoids by ascorbate was also suggested by the experiments of Kandaswami et al. (1993), who found that ascorbic acid augmented by about 2-fold the antiproliferative effect of fisetin and quercetin on proliferation of HTB 43 squamous cell carcinoma in tissue culture. Flavone had no effect, indicating the requirement for hydroxylation. In other experiments (Middleton, Drzewiecki, and Kandaswami, unpublished observations), it was demonstrated that low concentrations of ascorbic acid completely blocked the oxidation of quercetin in aqueous medium at pH 7.5 as determined spectrophotometrically over a 24-h period. Our preliminary experiments clearly indicated that autoxidation of quercetin could be prevented by low concentrations of ascorbic acid in vitro, suggesting that one possible function of ascorbic acid in the diet is to prevent flavonoid oxidation, thus possibly retaining the biologically active flavonoid structure in vivo (Middleton and Drzewiecki, 1993). Considering the redox potentials for the reduction of ascorbic acid and metal ions, ascorbic acid can itself reduce cupric and ferric ions. Metal ions like  $Cu^{2+}$  are known to oxidize flavonols such as quercetin in aqueous media (Kochi, 1978). Chelation of the vicinal hydroxyl groups of quercetin by  $Cu^{2+}$  would result in its conversion to a quinone. The reduction by ascorbic acid of the quinone to the flavonol could enhance its biological activity.

Roy and Liehr (1989) studied the effect of ascorbic acid on metabolic oxidation of diethylstilbestrol to diethylstilbestrol-4',4-quinone in Syrian hamsters. Hamsters pretreated with ascorbic acid or  $\alpha$ -naphthoflavone had approximately 50% reduction in quinone metabolite levels, which correlated nicely with the 50% reduction in diethylstilbestrol-induced renal tumors. The data summarized above strongly suggest that there could be important flavonoid-ascorbate interactions in vivo that require clinical investigation. For example, ascorbate could protect the active antiviral, antiallergic, or even anticancer conformation of certain flavonoids in vivo.

## XI. Cancer-Related Properties

Before discussing the beneficial effects of flavonoids in cancer, it would be prudent to review any possible detrimental effects. Since flavonoids are regular edible constituents of our ordinary diet (Bate-Smith, 1954; Herrmann, 1976; Brown, 1980; Singleton, 1981; Pierpoint, 1986), examination of their genotoxic effects has received increasing attention in recent years. Following early reports on the bacterial mutagenicity of plant flavonoids (Bjeldanes and Chang, 1977; Sugimura et al., 1977; Hardigree and Epler, 1978), further work has developed in the following directions: 1) screening of numerous flavonoids in different strains of *Salmonella typhimurium* and other microorganisms to clarify the structural requirements for any mutagenicity, 2) mutagenicity testing of flavonoid-containing foods, 3) testing for genetic effects in nonmicrobial systems in vitro and in vivo, and 4) testing for carcinogenicity using experimental animals. These are described below.

### A. Microbial Mutagenicity Studies

More than 70 flavonoids have been tested for mutagenicity in different strains of *S. typhimurium* by the Ames test (Hardigree and Epler, 1978; MacGregor and Jurd, 1978; Brown and Dietrich, 1979; Nagao et al., 1981). Only aglycone flavonoids exhibited appreciable mutagenic activity (Brown and Dietrich, 1979). MacGregor and Jurd (1978) reported that 10 flavonoids, including quercetin, myricetin, kaempferol, tamarixetin, and morin, were active as mutagens. Among the 16 flavonol derivatives tested by Nagao et al. (1981), all except the 3-alkoxy derivatives were mutagenic. Among these, quercetin, rhamnetin, and kaempferol were the most mutagenic to *S. typhimurium* strains TA 98 and TA 100. Among the 22 flavone derivatives tested in another study, only one compound, wogonin, was active (Nagao et al., 1981). Cross and coworkers (1996) studied the genotoxic potential of quercetin and cisplatin alone and together in the *Salmonella* tester strain and by assessment of unscheduled DNA synthesis in rat hepatocytes. The investigators concluded that the mutagenic potential of the combination of cisplatin plus quercetin did not exceed that associated with the individual compounds.

In hepatocytes, however, quercetin did inhibit to some extent the repair of cisplatin-induced DNA damage.

At least two distinct classes of mutagenic flavones seem to emerge based upon structural and metabolic activation requirements for mutagenic activity in *Salmonella* and on relative strains (MacGregor, 1986; MacGregor and Wilson, 1988). Examples of the first class are quercetin and structurally related flavonols (3-hydroxyflavones), which are active in both TA 98 and TA 100 strains, the activity being higher in the former. They appear to be metabolically activated to DNA-reactive intermediates, probably invoking initial oxidation of *ortho*- or *para*-hydroxyl groups in ring B to quinonoid intermediates. A free hydroxyl group at position 3 appears to be essential for this activity. Quercetin, with its vicinal hydroxyl groups in the B ring, was mutagenic without metabolic activation. Kaempferol, which has only one hydroxyl group in the B ring, seems to require both an NADPH-generating system and microsomes for activity. The substituted flavones without the 3-hydroxy group constitute the second class of mutagenic flavonoids. Norwogonin and related flavones with hydroxy/methoxy substitutions at positions 5, 7, and 8 of the A ring were most active in strain TA 100 and showed only a minor or very weak activity in strain TA 98. They required metabolic activation by the cytosolic fraction, which was enhanced by the addition of NADP or NADPH, suggesting thereby the possible involvement of a redox reaction in their activation.

Information available on the mutagenicity of flavonoids in other test systems is limited. Quercetin displayed mutagenic activity in tester strains of *E. Coli* and *Saccharomyces cerevisiae* (Brown, 1980; Llagostera et al., 1987). Quercetin and kaempferol were reported to increase the frequency of sex-linked recessive mutations in *Drosophila melanogaster* (Watson, 1982). The flavonols quercetin, kaempferol, and myricetin, extracted from green tea and black tea, were suggested to account for the mutagenic activity of tea in *S. typhimurium* (Uyeta et al., 1981). The fraction containing astragalol extracted from bracken fern was found to be mutagenic using the Ames test (Fukuyoka et al., 1978). Quercetin, kaempferol, isorhamnetin-3-sulfate, and quercetin-3-sulfate were suggested to be the constituents contributing to bacterial mutagenicity in spices and dill seed (Seino et al., 1978; Fukuyoka et al., 1980). Several authors have proposed that the mutagenic activity of red wine and other complex mixtures such as tea in the Ames mutagenicity test is due to flavonols (Tamura et al., 1980; Rueff et al., 1986; Yu et al., 1986). However, studies using the forward mutation assay, Ara test (L-arabinose-resistance test) of *S. typhimurium*, considered to be more sensitive than the Ames test (Dorado and Pueyo, 1988), reported that flavonols were not the major putative mutagens in complex mixtures such as wine (Jurado et al., 1991).

Mutagens derived by cooking proteinaceous foodstuffs have been shown to be bacterial mutagens and to be carcinogenic in experimental animals. Alldrick et al. (1986) studied the effects of plant-derived flavonoids and several polyphenolic acids on the activity of mutagens from cooked food. While the polyphenolic acids failed to exhibit an effect, the flavonoids generally *inhibited* the mutagenic activity of IQ (2-amino-3-methylimidazo-[4,5-f] quinoline), MeIQx (2-amino-3,8-dimethylimidazo-[4,5-f] quinoxaline), Trp-P-1 (3-amino-1,4-dimethyl-5-*H*-pyrido[4,3-*b*] indole), and Trp-p-2 (3-amino-*l*-methyl-5-*H*-pyrido[4,3-*b*] indole) using *S. typhimurium* T98 as indicator and a metabolic activating system.

On the other hand, some flavonoids acted as enhancers of 2-acetylaminofluorene in the *S. typhimurium* T98 test system (Ogawa et al., 1987). Greatest activity was associated with a 3-OH, C2-C3 double bond, and hydroxylation in the B ring.

### B. Genetic Effects of Flavonoids in Mammalian Cells

While several reports have appeared on the genetic effects of flavonoids in mammalian cell systems, quercetin appears to be the only flavonoid that has been evaluated in various cell types for different end points (i.e., frequencies of gene mutation, chromosomal aberration, and sister chromatid exchange). Maruta et al. (1979) reported that quercetin and kaempferol were mutagenic to V79 hamster fibroblasts. Other studies reported genetic effects of quercetin in mammalian cells, such as morphological transformation of hamster embryo cells (Umezawa et al., 1977), induction of chromosomal aberrations and sister chromatid exchanges in cultured human and Chinese hamster cells (Yoshida et al., 1980), induction of mutation at the thymidine kinase locus in L5178Y mouse lymphoma cells (Amacher et al., 1979), DNA single-strand breaks in L5178Y mouse cells (Meltz and MacGregor, 1981), induction of mutations in Chinese hamster lung cells (Nakayasu et al., 1986), and weak transformation of BALB/c 3T3 cells (Meltz and MacGregor, 1981).

When single populations of Chinese hamster ovary cells were exposed to quercetin, kaempferol, and galangin, all three flavonoids were found to increase the frequencies of chromosomal aberrations and mutations at the thymidine kinase locus, with little or no effect on the sister chromatid exchange frequency or on gene mutation at the three other loci (*hgp<sub>r</sub>t*, *ap<sub>r</sub>t*, and Na<sup>+</sup>/K<sup>+</sup>-ATPase) (Carver et al., 1983). The absence of pronounced clastogenic effects with shorter exposure periods raised the possibility of indirect effects caused by interference with cell replication, rather than a direct alkylation of DNA by reactive flavonoid intermediates. The marked increase in the frequency of chromosomal aberration with little or no effect on the incidence of specific locus mutation is reminiscent of the characteristics of ionizing radiation (Perry and Evans, 1975),

which is considered to cause free radical-induced DNA damage (Birnboim, 1986).

Flavonoids possessing vicinal hydroxyl groups, such as quercetin, can autoxidize in aqueous media at biologically relevant pH. Autoxidation to a quinone, followed by intracellular reduction in the presence of molecular oxygen (redox-cycling), may generate oxygen free radicals, which could cause strand scission of DNA. This could explain their observed effects on the frequency of chromosomal aberrations in cultured cells as noted above. The significant increase in mutation at the *hgp<sub>r</sub>t* locus reported earlier was seen in an unidentifiable population of hamster 79 cells that survived two days of exposure to very high concentrations of quercetin (Maruta et al., 1979); such pharmacological levels may, therefore, not be representative of the biologically attainable amounts as discussed by MacGregor (1984). Experiments of Suzuki et al. (1991) suggested that quercetin could induce recombinational mutations in BMT-11 mouse fibrosarcoma cells. The authors suggested that this may provide a molecular basis for its effect on the tumorigenic and metastatic properties of these cells (Ishikawa et al., 1987). Popp and Schimmer (1991) studied 19 naturally occurring flavonoids for their ability to induce sister chromatid exchanges, polyploidy, and micronuclei in human lymphocyte cultures. Some of the compounds exhibited the capacity to induce these genotoxic changes in cells that were exposed for a period of 48 h at quite high concentrations.

Quercetin and calf thymus DNA interacted in a fashion that appeared to stabilize the secondary structure of the DNA, possibly by interaction between base pairs (Alvi et al., 1986). Prolonged incubation of DNA with quercetin, however, resulted in disruption of the double helix and extensive hydrolysis by the S1 nuclease. Possibly, the oxidative degradation products of quercetin, which occur in the presence of oxygen and light, were responsible for the DNA damage (Alvi et al., 1986). In subsequent studies, the same group reported that rutin, galangin, apigenin, and fisetin were as effective as quercetin (Rahman et al., 1992). The DNA strand scission reaction was inhibited by superoxide dismutase and catalase, establishing a role for the reactive oxygen species in the reaction. Whether quercetin could cause DNA strand scission in intact cells has not been demonstrated.

### C. Mutagenicity Studies in Vivo

The flavonol glycosides are not mutagenic by themselves (Brown, 1980), even though they remain in the gut fairly unabsorbed; many of them are susceptible to hydrolysis by glycosidases of intestinal microorganisms (Baba et al., 1983; Bokkenheuser et al., 1987). Cultured cell-free microbial preparations of human feces and saliva also possess the glycosidase rutin-hydrolyzing activity (MacDonald et al., 1983). Even though free flavonols released in the intestine might have mutagenic activity,

rapid metabolic disposition (Ueno et al., 1983), methylation of the hydroxyl groups by catechol-*O*-methyltransferase, and ring scission by bacteria could significantly diminish their harmful effects. Interestingly, a human intestinal bacterium (*Clostridium orbiscindens* sp. nov.) cleaving the flavonoid C ring was reported (Winter et al., 1991). Mutagenicity assays with *S. typhimurium* TA 98 showed moderate mutagenic activity in the urine and fecal extracts, but not in plasma samples from rats treated with a single dose of quercetin, ranging from 500 to 2000 mg/kg of body weight (Crebelli et al., 1987).

Flavonoids do not appear to be mutagenic in mammals in vivo. MacGregor et al. (1983) reported no increase in the frequency of sister chromatid exchange in the peripheral lymphocytes of rabbits given doses of up to 250 mg/kg. intraperitoneally of quercetin. There was also no increase in the incidence of nuclear anomalies in the colonic epithelium of mice fed a 4% quercetin-containing diet for 7 days (Wargovich and Newmark, 1983). Some mutagenic effect was reported in the micronucleus test following intraperitoneal administration of quercetin or kaempferol at the high dose of 200 mg/kg of body weight, but no statistical evaluation was possible because of the small number of mice used (Sahu et al., 1981). Aeschbacher et al. (1982) gave oral doses of 1 to 1000 mg of quercetin per kg of body weight to male mice and found no mutagenic effect with either the micronucleus test or the host-mediated assay employing the *Salmonella* tester strain TA 98 as an indicator organism. MacGregor et al. (1983) did not observe any increase in frequencies of micronucleated erythrocytes in mice exposed to quercetin and other flavonoids under a variety of exposure conditions. Cea et al. (1983), however, reported some increase in the induction of micronuclei in mouse bone marrow erythrocytes after intraperitoneal treatment with 0.5 to 2.0 mg of 5,3',4'-trihydroxy-3,6,7,8-tetramethoxyflavone. This report was surprising considering the lack of in vivo toxicity of flavonoids at concentrations manyfold higher. A recent report showed that quercetin is clastogenic in the murine micronucleus test (Heo et al., 1992).

Sahu and Gray (1994) also found that kaempferol induced nuclear DNA damage and lipid peroxidation in rat liver isolated nuclei. The results support the prooxidant properties of polyphenolic flavonoids, such as kaempferol and quercetin, which have been traditionally considered as antioxidants and anticarcinogenic.

#### D. Carcinogenicity of Flavonoids?

The issue of carcinogenicity of quercetin has received considerable attention. However, most results published to date have been negative. In initial studies, quercetin was reported to cause no lesions in rats fed up to 1% for 410 days (Ambrose et al., 1952). No carcinogenicity was evident in F344/DuCrj rats fed 1.25 and 5% quercetin in the diet for 2 years (Ito et al., 1989). Kato et al. (1985) reported that quercetin exhibited no initiating activity

in rats treated with partial hepatectomy and given a liver cancer promoter; also, no genotoxic activity was evident with a hepatocyte primary culture/DNA repair test. Pamukcu et al. (1980) reported induction of urinary tract and bladder tumors by quercetin in male rats. However, other studies could not confirm this carcinogenicity (Hirono et al., 1981; Morino et al., 1982; Stoewsand et al., 1984). A related study by Dunnick and Hailey (1992) was equally unimpressive: 2-year administration of high dose dietary quercetin was associated with the development of benign tumors of the renal tubular epithelium.

The effect of several drugs, food additives, and natural products including quercetin were studied by Ito et al. (1984) for their ability to act as promoters in rat urinary bladder carcinogenesis initiated with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine. Five percent quercetin in the diet did not increase tumor yield. BALB/3T3 cells reacted diversely to quercetin in two-stage chemical transformation experiments (Sakai et al., 1990). Quercetin showed no effect on two-stage urinary bladder carcinogenesis in male rats (Hirose et al., 1983). Pennie and Campo (1992), however, demonstrated synergism between bovine papillomavirus type 4 and quercetin in cell transformation in vitro.

The National Toxicology Program (NTP), which completed a 2-year study on the toxicology and carcinogenicity of quercetin in F344/N rats, concluded that there was some evidence of carcinogenic activity in male rats fed 40,000 ppm (4%) quercetin, based on an increased incidence of renal tubular cell carcinoma (NTP Technical Report, 1991). These neoplasms were mostly adenomas and were induced only in male rats. However, Ito (1992) and Hirono (1992) emphasized that a statistically significant result was obtained only after reevaluation of additional step sections of histological tissues. Hirono (1992) suggested that the high dose of quercetin in the NTP study exerted an enhancing effect, which modified the incidence of spontaneously occurring renal tumors. Ito (1992) suggested evaluating the possible involvement of  $\alpha$ -2u-globulin nephropathy in quercetin renal carcinogenicity, in view of the possible role of this nephropathy in chemically induced renal carcinogenicity observed only in male rats (Swenberg, 1991). Soybean isoflavones (together) may not always be beneficial because a particular dose of the mixture may be cancer-promoting instead of anticarcinogenic (Lee et al., 1995).

COMT-catalyzed rapid 3' methylation of flavonoids has been proposed as a possible explanation for the noncarcinogenicity of otherwise suspected mutagenic quercetin and fisetin. Other catechol-type flavonoid mutagens could be similarly metabolized. The presence of COMT in various tissues could modulate the activity of flavonoids in those tissues (Zhu et al., 1994).

Quercetin inhibited the promotion caused by TPA in transformation initiated by 3-methylcholanthrene, but quercetin exhibited weak initiating activity in cells sub-

sequently treated with TPA. In addition to quercetin's capacity to inhibit the TPA-induced activation of PKC, it is of interest that this flavonoid could also decrease the number of phorbol ester receptors in mouse skin (Horiuchi et al., 1986), suggesting yet another mechanism of action of flavonoid-induced modulation of cell function.

#### E. Anticarcinogenic Effects

The critical relationship of fruit and vegetable intake and cancer prevention has been thoroughly documented in a review of the epidemiological evidence by Block et al. (1992). The author suggested that "major public health benefits could be achieved by substantially increasing consumption of these foods". Among many other dietary chemicals of various sorts, the flavonoids are, of course, major components of fruits and vegetables. Barnes (1995) has extensively reviewed the anticancer effects of genistein on *in vitro* and *in vivo* models, and Carroll et al. (1998) reviewed the anticancer properties primarily of flavonoids contained in citrus fruits.

There is evidence that flavonoids have *antimutagenic* activity. Quercetin was shown to inhibit the mutagenic activity of BP, a representative PAH carcinogen, in bacterial mutagenicity studies (Ogawa et al., 1985). Quercetin was also shown to inhibit BP-induced nuclear damage in colonic epithelial cells of mice (Wargovich et al., 1985). Galangin (3,5,7-trihydroxyflavone) proved to be a potent anticlastogenic agent both *in vitro* and *in vivo* against bleomycin-induced clastogenesis in mouse spleen culture (Heo et al., 1994). These investigators found that most of 13 other flavonoids studied were also anticlastogenic when administered orally before and after benz[a]pyrene was given intraperitoneally. It is also noteworthy that several hydroxylated flavonoids were found to inhibit the mutagenic activity of bay-region diol epoxides (putative ultimate mutagens/carcinogens) of BP (Huang et al., 1983).

Sixty-four flavonoids were assessed for their antimutagenic activity against 2-amino-3-methylimidazo[4,5-f]quinoline and other heterocyclic amine mutagens from cooked food (Edenharder et al., 1993). Several flavonols, flavones, and flavanones, as well as the isoflavone biochanin A, were highly active; a carbonyl function at C-4 of the flavone nucleus was found to be essential for antimutagenic activity. Flavone-8-acetic acid was also shown to have antitumor effects (Thomsen et al., 1991).

Chang et al. (1985) found that ellagic acid, robinetin, quercetin, and myricetin inhibited the tumorigenicity of BP-7,8-diol-9,10-epoxide-2 on mouse skin and in the newborn mouse. Moreover, the compounds did not exhibit any tumor-initiating activity on mouse skin nor did they induce lung tumors when injected into newborn mice.

PTK(s) encoded by oncogenes are attractive targets for anticancer drug design (Cunningham et al., 1992; Levitzki, 1992). Quercetin has been reported to inhibit

many biochemical events associated with tumor promotion, such as alteration in PKC activity (Gschwendt et al., 1983), interactions with calmodulin (Nishino et al., 1984a), incorporation of <sup>32</sup>P in membranes (Nishino et al., 1983), and LO activity (Nakadate et al., 1983). It also counteracted the tumor-promoting activity of the phorbol ester tumor promoter, TPA, on mouse skin after treatment with the initiator, DMBA (Kato et al., 1983). When applied topically to mouse skin in conjunction with TPA, certain flavonoids inhibited skin papilloma formation (Nakadate et al., 1983). Aflatoxin B<sub>1</sub> is a highly toxic and mutagenic compound with hepatic carcinogenic activity for several species. Aflatoxin B<sub>1</sub> requires metabolic activation by microsomal enzymes to produce AFB<sub>1</sub>-8,9-epoxide, the ultimate carcinogen, which reacts with DNA to form a covalent DNA adduct. Both the microsome-dependent activation and the adduct formation could be significantly affected by several naturally occurring flavonoids (Bhattacharya and Firozi, 1988).

Topical application of quercetin has been reported to protect mice against DMBA-, BP-, *N*-methyl-*N*-nitrosourea-, and BP-7,8-dihydrodiol-9 IQ-epoxide-induced skin tumorigenesis (Khan et al., 1988; Mukhtar et al., 1988). In related experiments, Balasubramanian and Govindasamy (1996) found dietary quercetin to inhibit DMBA-induced hamster buccal pouch carcinogenesis. Wattenberg and Leong (1970) showed that quercetin pentamethyl ether (3,3',4',5,7-pentamethoxyflavone) feeding caused significant reduction in pulmonary adenoma formation in mice. More recently, it was reported that rats fed a diet with 5% quercetin had a 48% lower incidence of mammary cancer induced by DMBA (Verma et al., 1988). Remarkably, neonatal administration of genistein had a protective effect against the subsequent development of mammary cancer induced by DMBA in Sprague-Dawley rats (Lamartiniere et al., 1995). The mechanism of inhibition of mammary cancer by quercetin is not known, however. Quercetin also inhibited colon cancer in rats and mice induced by azoxymethanol (Deschner et al., 1991, 1993). Quercetin also produced cell cycle arrest in proliferating lymphoid cells (Reed et al., 1992).

The evolution of rat liver preneoplastic foci into nodules and hepatocellular carcinoma in animals treated with 2-acetylaminofluorene appeared to depend upon certain products of arachidonic acid metabolism, according to the studies of Tang et al. (1993). Quercetin was administered in the diet over a period of weeks. It significantly decreased the number of hepatocellular carcinomas in animals treated with the liver tumor promoter phenobarbital.

Most of the chemical carcinogens, such as PAH, seem to require metabolic activation to DNA-reactive intermediates by P450-mediated MFO to exert their carcinogenic action (Dipple et al., 1984). The covalent binding of these reactive intermediates to cellular DNA leading to

adduct formation is considered to be a critical event in the initiation of carcinogenesis (Miller, 1978). Flavonoids may inhibit carcinogenesis by acting as "blocking agents" (Wattenberg, 1985) by one or more of several possible mechanisms: 1) inhibiting the metabolic activation of the carcinogen to its reactive intermediates, 2) inducing the enzymes involved in the detoxification of the carcinogen, and 3) binding to reactive forms of carcinogens, thereby preventing their interaction with critical cellular targets such as DNA, RNA, and protein. In addition, plant flavonoids could also inhibit tumor promotional events as mentioned above.

Wattenberg et al. (1968) demonstrated the modulation of PAH-metabolizing enzymes *in vivo* by naturally occurring plant flavonoids. They showed that gastric administration of flavone and polymethoxylated flavonoids (nobiletin and tangeretin) to rats resulted in an induction of liver microsomal BP hydroxylase activity. In contrast, quercetin was inactive as an inducer. Induction of BP hydroxylase activity, leading to greater detoxification of the carcinogen BP, was suggested to be a protective mechanism. Flavone administration to rats has been shown to induce conjugating enzymes such as glutathione-*S*-transferase involved in the detoxification of carcinogenic intermediates (Trela and Carlson, 1987). It seems that the presence of the free hydroxyl group on the flavonols does not necessarily prevent these compounds from inducing some MFO activities (Siess and Vernevaux, 1982). Dietary quercetin pentamethyl ether was found to be a potent inducer of small intestinal BP hydroxylase activity in mice (Wattenberg and Leong, 1970). This flavonoid, however, had no inducing effect on hepatic BP hydroxylase activity. Intraperitoneal administration of flavone to rats was reported to significantly induce hepatic epoxide hydrolase (EH) while there was no induction by the synthetic 7,8-benzoflavone (Alworth et al., 1980). Le Bon et al. (1992) studied the inhibition of microsome-mediated binding of BP to calf thymus DNA by flavonoids either *in vitro* or after administration in the diet. Flavone, flavanone, tangeretin, quercetin, and chrysin (100  $\mu$ M) used *in vitro* inhibited BP-DNA adduct formation in mixtures containing hepatic microsomes prepared from Aroclor-pretreated rats. Importantly, microsomes prepared from animals fed 0.3% quercetin and tangeretin also resulted in less effective binding of BP metabolites to DNA. Animals fed certain flavonoids had increased aryl hydrocarbon hydroxylase and epoxide hydrolase activities. Brouard et al. (1988) showed that dietary administration of flavone to rats induced certain conjugating enzyme activities in the liver, but not in the intestine. The induction pattern for quercetin pentamethyl ether and flavone thus appears to vary with the tissue.

The induction of intestinal PAH-metabolizing activity by flavonoids may also vary with route of administration of the inducer. When administered in the diet, the P-448 type inducer,  $\beta$ -naphthoflavone, was much more active

in the intestine than the liver when induction of certain MFO activities in rats were studied (McDanell and McLean, 1984). According to Chae et al. (1991), several flavones were more active than their isoflavone and flavanone analogs in inhibiting microsomal cytochrome P450-mediated metabolism of BP to water-soluble, more readily excreted compounds. Microsomes induced by  $\beta$ -naphthoflavone (P-450IA1 and/or P-450IA2), in contrast to phenobarbital, were the most effective inhibitors of BP metabolism.

Topical application of quercetin and myricetin to SENCAR mice has been reported to inhibit PAH metabolism and PAH-DNA adduct formation in epidermis (Das et al., 1987a,b), thus indicating a possible mechanism of chemoprevention of skin cancer by flavonoids. Shah and Bhattacharya (1986) studied the effect of flavonoids on microsome-catalyzed adduct formation between benzo[a]pyrene and DNA. Robinetin, quercetin, isorhamnetin, and kaempferol significantly inhibited adduct formation at low concentrations. The isoflavonoids were inactive. Structural features associated with inhibitory activity were hydroxyl groups in the 3-position of the C ring, 5,7-positions of the A ring, and 3', 4', and 5'-positions of the B ring. Methylation or glycosylation of hydroxyl groups reduced activity. Flavanones with a saturated C2-C3 double bond were also inactive. This set of structural features seems to repeat itself for many flavonoid activities ranging from inhibition of basophil histamine release to antiviral activity and so on.

Using a mammalian cell culture benzo[a]pyrene metabolism assay for detection of potential anticarcinogens, Cassady et al. (1988) found the isoflavone, biochanin A, to be an active inhibitor at moderately low concentrations.

Suppression of genotoxicity of several carcinogens by EGCG, a major polyphenol of green tea, was studied by Hayatsu and coworkers (1992). They concluded that EGCG may act by indirect interception of carcinogen action rather than by direct action between EGCG and the mutagens. It is possible that the induction of P450 IA1 and IA2 isozymes in the intestine by dietary flavonoids could aid in the rapid metabolism and elimination of dietary procarcinogens such as PAHs. Using a transformation inhibition assay with BP-treated rat tracheal epithelial cells, Steele et al. (1990) tested several compounds including quercetin, rutin, and catechin as potential chemopreventive agents. Of the three flavonoids, catechin and quercetin were very active.

The inhibition of poly(ADP-ribose) polymerase by flavonoids was suggested to be involved in the inhibition of carcinogen-induced cellular transformation of human fibroblasts (Milo et al., 1985). Quercetin, which inhibited the nuclear poly(ADP-ribose) polymerase system *in vitro*, depressed cellular transformation of human fibroblasts induced by carcinogens such as *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (Milo et al., 1985).

Using HL-60 cells and a mouse skin tumorigenesis model, Wei et al. (1995) studied the antioxidant and antipromotional properties of genistein. This flavonoid was a potent inhibitor of TPA-induced  $H_2O_2$  production; daidzein was less active, and apigenin and biochanin A were inactive. However, genistein, apigenin, and prunetin were equally potent in inhibiting xanthine/xanthine oxidase generation of  $O_2^-$ . Dietary genistein slightly reduced the activity (after 30 days) of the measured antioxidant enzymes in intestine and/or skin. Finally, the expression of the protooncogene *c-fos* stimulated by TPA in mouse skin was inhibited by genistein. These findings strengthen the notion that genistein could be a useful anticancer agent. Wang and coworkers (1996) showed that genistein could block effects of estradiol even though genistein itself is estrogenic. Genistein caused 50% inhibition of [ $^3H$ ]estradiol binding to the estrogen receptor. However, this compound had a bimodal effect on the growth of human mammary cancer cells (MCF-7); low concentrations ( $10^{-8}$ – $10^{-6}$  M) stimulated growth, while  $10^{-5}$  M or greater caused inhibition.

Tumor promoters cause a variety of *in vitro* effects, including cell adhesion of HL-60 and aggregation of NL-3 cells, among many other effects (Sugimura and Fujiki, 1983; Fujiki et al., 1986). Edwards et al. (1979) reported that quercetin and another catecholic flavonoid (5,7,3',4'-tetrahydroxy-3-glucosylflavone) possessed antineoplastic activity toward Walker carcinoma 256.

#### F. Apoptosis and Cancer

The possible role of phytoestrogens in cancer protection has been reviewed by Adlercreutz (1995), who discussed isoflavonoids and lignans in epidemiological and experimental laboratory terms. The phenomenon of apoptosis (programmed cell death) has been reviewed repeatedly (Cohen, 1993; Kroemer et al., 1995; Duke et al., 1996). Dysregulation of apoptosis could play a critical role in oncogenesis (Williams, 1991). Some anticancer drugs cause apoptosis in human tumor cells. Hirano et al. (1995), in studies of the citrus flavone tangeretin (5,6,7,8,4'-pentamethoxyflavone), found that this naturally occurring flavonoid induced apoptosis in HL-60 cells. Tangeretin caused apoptosis at concentrations greater than  $2.7 \mu M$ . The apoptotic effect was largely abrogated in the presence of  $Zn^{2+}$ , a known inhibitor of the apoptosis-requiring enzyme, endonuclease. In addition, tangeretin's effect was sensitive to cyclohexamide, indicating a requirement for protein synthesis. Importantly, tangeretin's effect was essentially limited to the HL-60 cells, having little effect on the mitogen-stimulated blastogenic response of human peripheral blood mononuclear cells. The implications for cancer treatment are clear from these observations (Kandaswami et al., 1991). Wei et al. (1994) studied the induction of apoptosis by quercetin in several tumor cell lines. Quercetin caused appropriate morphological changes in the cells, and agarose gel electrophoresis showed the char-

acteristic ladder-type fragmentation of DNA. Also, the synthesis of heat shock protein (HSP) 70 was inhibited by quercetin and was associated with enhancement of the induction of quercetin-induced apoptosis. Several other studies have examined the ability of selected flavonoids to induce apoptosis. Tilly et al. (1992) reported that genistein completely blocked the ability of EGF, TGF- $\alpha$ , and basic fibroblast growth factor (bFGF) to suppress apoptosis in cultured rat ovarian granulosa cells. In human myelogenous leukemia HL-60 cell cultures, a population of cells with decreased DNA content and nuclear fragmentation characteristic of apoptosis was observed within 8 h (Traganos et al., 1992). Bergamaschi et al. (1993) studied the effect of genistein and tyrphostin on apoptosis in the leukemic cell lines M07e and HL-60. Both PTK inhibitors induced apoptosis in the cell lines, as determined by appropriate morphologic changes and flow cytometry of DNA. Based on additional studies with the tyrosine phosphatase inhibitor sodium orthovanadate, the authors concluded that the balance between tyrosine kinases and phosphatases determines the fate of the cell.

#### G. Antiproliferative Activity

In addition to its antineoplastic activity, quercetin exerted growth-inhibitory effects on several malignant tumor cell lines *in vitro*. These included Ehrlich ascites cells, L1210 and P-388 leukemia cells (Suolinna et al., 1975), NK/Ly ascites tumor cells (Molnar et al., 1981), gastric cancer cells (HGC-27, NUGC-2, NKN-7, and MKN-28) (Yoshida et al., 1990), colon cancer cells (COLON 320 DM) (Hosokawa et al., 1990b), human breast cancer cells (Markaverich et al., 1988; Hirano et al., 1989b), human squamous and gliosarcoma cells (Castillo et al., 1989; Kandaswami et al., 1991), and ovarian cancer cells (Scambia et al., 1990a). Tumor cell growth inhibition by quercetin may be due to its interaction with nuclear type II estrogen binding sites (EBS) as proposed by Markaverich et al. (1988). Larocca and coworkers (1990) have detected type II EBS in the cells of acute lymphoid and myeloid leukemias; quercetin was able to compete for [ $^3H$ ]17 $\beta$ -estradiol binding ( $10^{-8}$ – $10^{-5}$  M). The relative binding affinity of quercetin for type II EBS correlated well with cell growth inhibition. Rutin and hesperidin were only weakly inhibitory of cell proliferation. Transitional cell carcinoma of the bladder was also found to possess type II EBS, which behaved like type II EBS from other tissues. Quercetin ( $10 \mu M$ ) effectively inhibited the *in vitro* incorporation of bromodeoxyuridine in transitional cell carcinoma cells (Larocca et al., 1994). Type II EBS were also present in human ovarian cancer (Ferrandina et al., 1993).

The mechanism of action of quercetin as an antiproliferative agent in human breast cancer cells was investigated further. Singhal et al. (1995) found evidence of increased signal transduction in those cells, which was

markedly reduced by quercetin, thus suggesting a novel target for chemotherapy.

Ahmad et al. (1998) illustrated the mechanism of action of the antioxidant flavonoid silymarin. Using the human epidermoid carcinoma A431, the authors found that exposure of cells to silymarin resulted in a significant decrease of ligand-induced activation of epidermal growth factor receptor (EGFR) with associated decrease in EGFR intrinsic kinase activity. This was accompanied by striking inhibition of DNA synthesis and cell growth. Together, the results suggested that the skin cancer chemoprotective effects of silymarin are mediated by impaired EGFR signaling.

The relationship of soy intake and cancer risk has been reviewed by Messina et al. (1994). The dietary phytoestrogen isoflavonoid, formononetin, exerted a stimulatory effect on mammary gland proliferation in BALB/c female mice with associated changes in vaginal cytology when given by subcutaneous injection (Wang et al., 1995). In addition, estrogen receptor expression was 2-fold higher in formononetin-treated mice, and plasma prolactin increased 1.7-fold. These results may be explained if the estrogenic activity of this or other isoflavonoids surpasses their antiproliferative effects. Nevertheless, the higher expression of estrogen receptors could make such cells more vulnerable to antiestrogens such as tamoxifen.

Genistein potently inhibited the growth of human breast carcinoma cell lines MDA-468 (estrogen receptor negative) and MCF-7 and MCF-7-D40 (estrogen receptor positive) with  $IC_{50}$  values of 6.5 to 12  $\mu\text{g/ml}$  (Peterson and Barnes, 1991). Biochanin A and daidzein were less effective, and the glycosides of genistein and daidzein were essentially inactive. The activity of the isoflavones was not dependent on the presence of the estrogen receptor. Of interest also was the observation that the growth-inhibitory activity of genistein and biochanin A was not affected in the cell line MCF-7-D40, which overexpresses gp 170, the gene product responsible for multidrug resistance. The low rate of breast cancer in Oriental women may be related to the high isoflavone-containing soy content of their diet. Catechin, epicatechin, quercetin, and resveratrol, which account for more than 70% of polyphenolic compounds in red wine, were shown to inhibit growth of human breast cancer cells at picomolar concentrations (Damianaki et al., 2000). The same compounds were also shown to potently inhibit human prostate cancer cells (Kampa et al., 2000). Retinoids and carotenoids also have inhibitory activity on breast cancer cell proliferation in vitro (Prakash et al., 2000).

3-Methoxyquercetin, quercetin, and ipriflavone (a synthetic flavanone), but not rutin or hesperidin, induced type II EBS in both ER-positive and ER-negative human breast cancer cell lines (Scambia et al., 1993). The quercetin effect was concentration-related and required synthesis of mRNA and protein. The flavonoid-

stimulated enhancement of type II EBS correlated well with increased sensitivity of the tumor cells to the inhibitory effects of low concentrations of quercetin. This same group of investigators also reported that meningiomas possessed type II EBS to which quercetin bound, but not rutin or hesperidin.

Quercetin (but not rutin or hesperidin) effectively inhibited bromodeoxyuridine incorporation into the nuclei of meningioma cells (Piantelli et al., 1993). The authors suggested that the antiproliferative activity of quercetin may be related to its capacity to interact with type II EBS in tumor cells. A similar conclusion was reached after studying the inhibitory effect of quercetin on the in vitro growth of primary human transitional cell carcinomas (Larocca et al., 1994). Evidence was presented demonstrating that selected polyhydroxylated flavonoids interact directly with the estrogen receptor, based on competitive binding studies with [ $^3\text{H}$ ]17 $\beta$ -estradiol and cell-free extracts containing the estrogen receptor (Miksicek, 1993). The flavonoid estrogen-like compounds were  $10^3$ - to  $10^4$ -fold less potent at inducing a biological response, although in the assay system used they did generate an estrogen response.

Avila et al. (1994) reported that quercetin strongly inhibited, in a time- and dose-dependent fashion, the expression of the mutated p53 (tumor suppressor gene) protein, which is the only form present at high levels in the human breast cancer cell line MDA-MB468. Quercetin prevented the accumulation of newly synthesized p53 protein without affecting the steady-state mRNA levels of p53.

Since flavonoids can suppress tumor growth through interaction with type II EBS, these compounds could be useful anticancer agents alone or in combination with other chemotherapeutic agents. Genistein caused 50% inhibition of [ $^3\text{H}$ ]estradiol binding to the estrogen receptor. Of great interest is the observation of Markaverich and Gregory (1993), who found that luteolin (5,7,3',4'-tetrahydroxyflavone) bound irreversibly to type II nuclear estrogen receptor, whereas 4',7-dihydroxyflavone, a related flavone, bound reversibly. Since luteolin has catecholic hydroxyl groups in the B ring, which can transform to a protein-reactive quinone, the authors considered that luteolin bound covalently to the type II estrogen receptor, an alkylation reaction (or, if you will, a flavonylation).

The inhibitory effect of quercetin on proliferation of primary ovarian and endometrial cancer cells could be strikingly potentiated in the presence of *cis*-diamminedichloroplatinum (II) and was accompanied by reduction of bromodeoxyuridine uptake into the neoplastic cells (Scambia et al., 1992). Quercetin exhibited a synergistic antiproliferative effect with cisplatin against drug-resistant leukemia cells in vitro (Hofmann et al., 1989); such a synergistic activity was also observed in vivo (Hofmann et al., 1990). The antineoplastic effect of cytosine arabinoside was effectively augmented in the presence

of quercetin when the combination was tested against HL-60 cells (Teofili et al., 1992). This combination also synergistically inhibited colony formation by human leukemic cells. Rutin did not synergize with cytosine arabinoside nor did it combine with type II estrogen binding sites.

Green tea polyphenols and one of its principal flavonoid constituents, EGCG, inhibited the growth of and caused the regression of experimentally induced skin papillomas in mice (Wang et al., 1992). Possible mechanisms of action that were considered included antitumor promoter activity, inhibition of ornithine decarboxylase, free radical scavenging, and augmentation of immunosurveillance. (-)Epigallocatechin gallate, the main polyphenolic constituent of green tea, also inhibited tumor promotion and chemical carcinogenesis in other experimental animal systems. Taniguchi et al. (1992) reported that the oral administration of EGCG inhibited metastasis of B16 melanoma cell lines, such as B16-F10 and B16, in both experimental and spontaneous systems. In a search for antitumor promoters, Konoshima et al. (1992) found two compounds from the root of *S. baicalensis* that had remarkable activity to inhibit Epstein-Barr virus early antigen activation; the flavonoids were 5,7,2'-trihydroxy- and 5,7,2',3'-tetrahydroxyflavone. The compounds had potent activity in an in vivo two-stage mouse skin carcinogenesis assay.

According to Okita and coworkers (1993), baicalein and baicalin (the glycoside of baicalein) caused a concentration-dependent inhibition of the proliferation of a human hepatoma cell line (HuH-7) in a cell cycle-independent manner. The generation of  $\alpha$ -fetoprotein decreased in baicalein-treated cells in proportion to the inhibition of tumor cell growth, a finding analogous to the appearance of cell markers and functions in tumor cells exposed to other prodifferentiating flavonoids (vide infra). Hirano et al. (1994) examined the antiproliferative effect of 28 naturally occurring and synthetic flavonoids against the promyelocytic leukemic cell line HL-60. Genistein was the most effective flavonoid; interestingly, daidzein was ineffective. The mechanism of action of genistein was not worked out. Agullo et al. (1994) studied the effect of quercetin on actively dividing colon carcinoma HT29 and Caco-2 cells. As noted by others, quercetin's cytotoxic effect was exerted preferentially on actively dividing cells and was associated with inhibition of lactate release. Simultaneously, the growth-inhibited cells exhibited a marked decrease of total cellular ATP content.

The experiments of Scambia and coworkers (1994a) suggested an intriguing mechanism of action of quercetin as an inhibitor of proliferation of human ovarian cancer cells. Quercetin stimulated the synthesis by the ovarian cancer cells of transforming growth factor  $\beta_1$ , an established antiproliferative agent. The possibility that quercetin (and perhaps other flavonoids with the same effect) consumed in the diet may regulate endogenous

levels of transforming growth factor  $\beta_1$  is worthy of further study.

The involvement of  $K^+$  channels in the quercetin-induced inhibition of mouse neuroblastoma cell growth was studied by Rouzair-Dubois et al. (1993), who showed that 10  $\mu$ M quercetin inhibited replication and 70  $\mu$ M quercetin inhibited the  $K^+$  current. Valinomycin (1 nM), the  $K^+$  ionophore, antagonized the antiproliferative effects of quercetin by 80%. Thus, a significant part of the growth-inhibitory action of quercetin appeared to be mediated by  $K^+$  channel blockade. Interestingly, the chromone moiety of quercetin was an important structural feature of the  $K^+$  channel agonist, chromakalin.

Blomgren and Kling-Andersson (1992) studied the effect of cirsiol (3',4',5-trihydroxy 6,7-dimethoxyflavone), an inhibitor of 5-LO, on tumor cell proliferation. The compound was quite active in inhibiting the proliferation of three glioma cell lines. It was suggested that 5-LO products may, in part, regulate the growth of both neoplastic and normal cells (Blomgren and Kling-Andersson, 1992).

5-LO inhibition (e.g., by piroprost) led to inhibition of proliferation of several tumor cell lines (Snyder et al., 1989), suggesting that antiproliferative flavonoids may also act through inhibition of 5-LO. Larocca and coworkers (1991) studied the antiproliferative effect of quercetin on normal bone marrow and leukemia progenitors. Sensitivity to quercetin was found (at low concentrations) with the majority of acute myeloid leukemias and with all acute lymphoid leukemias. The clonogenic efficiency assay used was a good predictor of quercetin responsiveness. CD34 hematopoietic progenitors were found to be resistant to the antiproliferative activity of quercetin. The authors concluded that quercetin could be an effective antileukemic agent without affecting normal hematopoiesis.

Matsuzaki et al. (1996) found that baicalein caused cell death in human hepatocellular carcinoma cell lines by different mechanisms. One cell line succumbed by apoptosis, while the other two died by necrosis. The topoisomerase activity of each cell line, however, was inhibited by baicalein, which also caused concentration-dependent inhibition of proliferation. When the progenitor cell line FDC-PL was treated with genistein before stimulation with the cytokines IL-3 or granulocyte monocyte-colony stimulating factor, cell proliferation was markedly inhibited (Townsend et al., 1993).

Yoshida et al. (1992) studied the effect of quercetin on CEM human leukemic T cells. Quercetin reversibly blocked the cell cycle at 3 to 6 h before onset of DNA synthesis. Quercetin-treated cells lacked a 60-kDa protein, which was promptly synthesized after removal of quercetin, suggesting that this protein is somehow intimately involved in the initiation of DNA synthesis. Proliferation of the human leukemia cell lines CEM-1 and CEM-7 was potently inhibited by luteolin and its chalcone analog. Concurrently, there was striking inhibition

of glucose uptake and marked depletion of cellular ATP content (Post and Varma, 1992), suggesting possible mechanisms of action of these particular flavonoids.

Quercetin inhibited the growth of squamous cell carcinoma cells in culture at high concentrations (Castillo et al., 1989), whereas the polymethoxylated flavonoids, tangeretin and nobiletin, exerted the same effect at relatively low concentrations (Kandaswami et al., 1991). A similar effect was found in human gliosarcoma cells (Kandaswami et al., 1992); interestingly, these flavonoids did not inhibit the growth of normal human diploid fibroblast-like lung cells (CCL 135) in culture for a corresponding period and at similar concentrations. Since these actively dividing cells are relatively unaffected by nobiletin and tangeretin, it is possible that these flavonoids have preferential growth-inhibitory effects on tumor cells, a possibility that remains to be explored.

The growth-suppressive activity of the polymethoxylated flavonoids may, in part, be ascribed to their chemical stability. Quercetin may undergo autoxidation and can also be oxidatively degraded, while methylation of the phenolic groups, as in the case of tangeretin and nobiletin, would be expected to confer greater stability to these flavonoids. In addition, these investigators showed that addition of ascorbic acid at low concentrations augmented the antiproliferative activity of fisetin and quercetin against the HTB 43 squamous cell carcinoma (Kandaswami et al., 1993). This effect may be related to the capacity of ascorbic acid to inhibit the oxidative degradation of the polyhydroxylated flavonoids as discussed earlier.

Genistein inhibited the *in vitro* growth of human T cell leukemia (Jurkat) and L-929 mouse transformed fibroblast cells (Pagliacci et al., 1993). Cell cycle analysis revealed a G<sub>2</sub>/M cell cycle arrest after genistein treatment. Butein (2',4',3,4-tetrahydroxychalcone), quercetin, luteolin, tannic acid, and naringenin had modest antiproliferative activity against HeLa cells and the lymphoblastoid Raji cell line (Ramanathan et al., 1992). Quercetin inhibited the proliferation of a human colon cancer (COL0320 DM). This inhibitory effect was partially reversible and is related to alterations in the cell cycle. Synthesis of a 17-kDa protein was selectively inhibited by quercetin. After removal of the flavonoid, cells progressed into S phase. The synthetic rate for the 17-kDa protein was low in G<sub>1</sub> and high in S phase.

Likewise, (-)-epigallocatechin gallate potently inhibited papilloma growth and/or caused the regression of established chemically induced skin papillomas (Wang et al., 1992). Two isoflavone derivatives, biochanin A and genistein, inhibited cell growth of three stomach cancer cell lines *in vitro* through activation of a signal transduction pathway for apoptosis. Biochanin A suppressed tumor growth of two (HSC-45M2 and HSC-41E6) of these cell lines in athymic nude mice (Yanagihara et al., 1993). Treatment of several established

cancer cell lines of human gastrointestinal origin with biochanin A and genistein at cytotoxic doses resulted in DNA fragmentation indicative of the apoptotic mode of cell death caused by these compounds (Yanagihara et al., 1993). Chromatin condensation and nuclear fragmentation of each cell line was observed. In addition, Pagliacci et al. (1994) found genistein to be an effective inhibitor of MCF-7 human breast cancer cells. Based on detailed analysis of the mechanism of antiproliferative activity, the authors concluded that the growth-inhibitory activity of genistein was the sum of cytostatic and apoptotic effects. Uckun et al. (1995) took advantage of the antiproliferative effect of genistein in a very unique way. The isoflavonoid was incorporated in an immun-conjugate containing a monoclonal antibody (B43) directed against the B cell-specific receptor, CD19. The antibody targeted the genistein to CD19-associated tyrosine kinases and triggered apoptotic cell death in an extremely efficient manner.

Quercetin was found to increase cyclic AMP levels (Graziani et al., 1977) and to decrease DNA, RNA, and protein synthesis in Ehrlich ascites tumor cells (Graziani and Chayoth, 1979). Quercetin has also been reported to inhibit aerobic glycolysis in tumor cells (Suolinna et al., 1975). The increases in DNA, RNA, and protein synthesis and loss of density-dependent inhibition of growth in NY 68-infected chick embryo fibroblasts were all abolished by quercetin (Jullien et al., 1984). The preliminary studies of Cunningham et al. (1987) indicated that quercetin inhibited the growth of Abelson-transformed NIH 3T3 cells, which express the Abelson tyrosine protein kinase. Quercetin was found to inhibit the activity of a tyrosine-specific protein kinase considered responsible for the transformation of nonmalignant fibroblasts to sarcoma cells (Glossmann et al., 1981). The inhibition of this enzyme activity by flavonoids may account in part for their antiproliferative effects on malignant cells. In the case of human gastric (Yoshida et al., 1990) and colon cancer cells (Hosokawa et al., 1990b), growth inhibition by quercetin appeared to involve interference with cell cycle events.

Flavonoid effects extend to yet another fundamental biologic process, i.e., gap junctional intercellular communication (GJIC) (Chaumontet et al., 1994). Both flavonoids enhanced GJIC in rat liver epithelial cells accompanied by an accumulation of connexin 43. Their ability to enhance GJIC could account for their actions as antitumor-promoting agents. Neither apigenin nor tangeretin was cytotoxic at low concentrations (10–25  $\mu$ M). The tea polyphenols, (-)-epicatechin gallate and epigallocatechin gallate inhibited the adhesion of mouse lung carcinoma 3LL cells to the monolayer of bovine lung endothelial cells (Isemura et al., 1993). The data suggested that a search for the cellular protein(s) that bind to these inhibitory catechins would provide a clue to the mechanism of interaction between tumor cells and endothelial cells. The presence of these binding sites in

many primary tumors (Markaverich et al., 1984; Carbone et al., 1989; Piantelli et al., 1990) suggested that quercetin could also exert antitumor effects *in vivo*.

Ranelletti et al. (1992) studied the effect of quercetin on the proliferation of HT-29, COLO 201, and LS 174T human colon cancer cell lines. Concentration-dependent, reversible inhibition of cell proliferation was noted at quercetin concentrations as low as 10 nM and up to 10  $\mu$ M. The growth-inhibitory effect of quercetin was localized to the G<sub>0</sub>/G<sub>1</sub> phase of the cell cycle. In these colon cancer cell lines, the growth inhibiting effect of quercetin and several other flavonoids correlated well with the affinities of the compounds for type II EBS detectable in whole cell assays using 17 $\beta$ -[<sup>3</sup>H]estradiol as tracer. Moreover, tumor cells incubated with quercetin showed a marked reduction in bromodeoxyuridine uptake; similar findings were noted with human meningiomas (Piantelli et al., 1993) and human ovarian cancer (Ferrandina et al., 1993). Using a whole cell assay, Scambia et al. (1990b) further demonstrated that IM-9 cells, a lymphoblastoid cell line, possessed both estrogen receptors and type II EBS. The flavonoids quercetin and rutin (but not hesperidin) and the estrogen inhibitor tamoxifen bound competitively to the type II EBS and caused a concentration-dependent antiproliferative effect between 10 nM and 10  $\mu$ M. In studies of estrogen-induced kidney tumors in Syrian hamsters, Narayan and Roy (1992) demonstrated increased expression of tyrosine-containing membrane phosphoproteins. The tyrosine phosphorylation was concentration dependently inhibitable by quercetin and was increased by the growth factors EGF and insulin-like growth factor-1.

#### H. Differentiating Effects

In addition to the anticancer properties mentioned above, it is of interest that certain flavonoids cause undifferentiated cancer cell lines to differentiate into cells exhibiting mature phenotypic characteristics. For example, low concentrations of genistein together with mitomycin C induced the differentiation of murine erythroleukemia cells, as determined by the appearance of hemoglobin in the differentiated cells; higher concentrations of genistein alone also caused differentiation that differed from the differentiation induced by dimethyl sulfoxide (Watanabe et al., 1989, 1991). Another example of the differentiating potential of a flavonoid is the effect of quercetin on RBL cells. Trnovsky et al. (1993) found that quercetin caused the accumulation of secretory granules in RBL and induced the synthesis of rat mast cell protease II; quercetin also inhibited RBL cell proliferation without affecting cell viability (Alexandrakis et al., 1999). These experiments again demonstrated the capacity of selected flavonoids to affect gene expression. More recent experiments showed that quercetin could also permit RBL cells to mature toward the connective tissue-like mast cells and acquire responsiveness to peptide secretagogues (Senyshyn et al., 1998). A

similar effect was recently reported for IL-4 (Karimi et al., 2000). Furthermore, quercetin and kaempferol induced differentiation of human leukemic mast cells, as shown by accumulation of secretory granules and inhibition of basal mediator release (Alexandrakis et al., 1999). Erythroid differentiation of the human myelogenous leukemia K562 cell line was also induced by genistein, possibly via inhibition of the structurally altered *c-abl* oncogenic protein with tyrosine kinase activity present in K562 cells. A multidrug-resistant subline (K562R) could also be induced to differentiate, as evidenced by increased hemoglobin synthesis (Honma et al., 1990).

Induction of differentiation of human promyelocytic HL-60 leukemia cells by genistein was accompanied by cell surface expression of a mature myeloid cell marker, staining for nonspecific esterase activity, and nitro blue tetrazolium dye reduction capability. K562 cells were also differentiated by genistein in this study (Constantinou et al., 1990). Moreover, these investigators also noted apparent genistein-induced DNA strand breakage possibly mediated by an effect on topoisomerase II. The differentiation of HL-60 cells was markedly affected by caffeic acid, a potent LO inhibitor (Miller et al., 1990). However, not all investigators found genistein to act as a differentiating agent despite effects on PTK activity (Nishimura et al., 1988). In A431 epidermoid carcinoma cells, basal tyrosine phosphorylation/activation (kinase F<sub>A</sub>/GSK-3 $\alpha$ ) was high but could become dephosphorylated/inactivated in a concentration-dependent fashion by genistein (Yu and Yang, 1994).

Genistein induced accumulation of K562 cells in the G<sub>2</sub>/M phase of the cell cycle (Hunakova et al., 1994). It potentiated the effect of herbimycin A, a PTK inhibitor, on the cell cycle (i.e., decreased the proportion of S-phase cells). Genistein induced a marked increase in cell surface expression of CD15 (Lewis X) antigen and down-regulated CD45 (leukocyte common antigen/phosphotyrosine phosphatase) and monocyte-associated CD14 antigen on K562 cells.

Certain citrus flavonoids were active antiproliferative differentiation inducers in mouse myeloid leukemia cells and HL-60 cells (Sugiyama et al., 1993). Jing et al. (1993) also found that the isoflavone daidzein was capable of inducing differentiation of HL-60 promyelocytic leukemia cells both *in vitro* and *in vivo*. Differentiation of HL-60 cells along granulocytic lines was determined by morphological characteristics, phagocytic capability, and nitro blue tetrazolium reduction. Treated cells were arrested in the G<sub>1</sub> phase. Combinations of daidzein with other inducers (retinoic acid, dihydroxyvitamin D<sub>3</sub>, TNF- $\alpha$ , interferon- $\gamma$ ) augmented the differentiating effect of daidzein. Daidzein also exhibited *in vivo* activity.

Remarkably, quercetin showed decreased toxicity toward the colorectal tumor cell line HT29 *after* induced differentiation (detransformation) as compared with the control transformed state (Musk et al., 1995). The recip-

rocal relationship between kinase-catalyzed phosphorylation and phosphotyrosine phosphatase-catalyzed dephosphorylation of cellular protein substrates with respect to control of proliferation and differentiation is important (Frank and Sartorelli, 1988a,b). For example, induced differentiation of HL-60 leukemic cells is associated with a marked decrease in cellular phosphotyrosine content (increased protein tyrosine phosphatase activity).

### *I. Adhesion/Metastasis/Angiogenesis*

To survive, metastases must undergo neovascularization involving angiogenesis (Griffioen and Molema, 2000). Interestingly, mast cells have been implicated in angiogenesis (Kessler et al., 1976) and release TNF, which induces endothelial adhesion molecule expression (Walsh et al., 1991). The possible existence of dietary inhibitors of angiogenesis was examined by Fotsis and coworkers (1993) by fractionating urine of healthy humans consuming a vegetarian diet. One potent fraction contained several isoflavonoids, of which genistein was the most potent; it inhibited endothelial cell proliferation ( $IC_{50}$ , 5  $\mu$ M) stimulated by bFGF and also inhibited in vivo angiogenesis ( $IC_{50}$ , 150  $\mu$ M). Genistein also inhibited TNF-stimulated induction of endothelial cell adhesion molecules (Weber et al., 1995), in keeping with the effects of several other flavonoids as described by Anné et al. (1994) and Gerritsen et al. (1995). Basic bFGF is a well recognized angiogenic factor, which stimulates the production of urokinase-type plasminogen activator (PA) and its physiological inhibitor, PAI-1, in vascular endothelial cells. Plasmin generated from plasminogen (via PA) causes graded proteolytic degradation of matrix proteins, a necessary step for neovascularization. Thus, it is of great interest that genistein strikingly reduced both basal levels and bFGF-induced levels of both PA and PAI-1 (Fotsis et al., 1993). Fotsis and coworkers (1997) also investigated 3-hydroxyflavone, 3',4'-dihydroxyflavone, 2',3'-dihydroxyflavone, fisetin, apigenin, and luteolin and showed that all inhibited the proliferation of normal and tumor cells, in addition to in vitro angiogenesis. Antiangiogenic properties were recently reported for flavone acetic acid (Lindsay et al., 1996).

Extracellular matrix molecules such as laminin are involved with invasion and metastasis of malignant tumor cells. Cellular contacts with laminin strongly influence the adhesion of numerous invasive and noninvasive cell types. The flavonoid (+)-catechin bound to laminin and pretreatment of the laminin-coated surfaces with a high concentration of (+)-catechin (0.5 mM) abrogated the effect of laminin (Bracke et al., 1987) on the morphology and adhesion of two different cell types, MO4 (Kristen murine sarcoma virus-transformed fetal mouse cells) and M5076 (a mouse reticulum cell sarcoma). Bracke et al. (1989) also reported that tangeretin inhibited the invasion of MO4 cells into embryonic chick heart

fragments in vitro. The flavonoid appeared to be chemically stable in tissue culture medium, and the anti-invasive effect was found to be reversible on omission of the compound from the culture medium. Related investigations by Scholar and Toews (1994) showed that a very invasive BALB/c mammary carcinoma could be inhibited by genistein from invading a basement membrane-like material (Matrigel). Low concentrations of genistein inhibited invasion while having no effect on growth. The invasion of MCF-7/6 human mammary carcinoma cells into embryonic chick heart fragments in organ culture was reversibly inhibited in a nontoxic fashion by 3,7-dimethoxyflavone (Parmar et al., 1994). At a concentration of 100  $\mu$ M, tangeretin appeared to inhibit the growth of MO4 aggregates in suspension culture (Bracke et al., 1989). In the case of HTB 43 cells, however, growth inhibition by tangeretin and nobiletin was observed at far lower (5–20  $\mu$ M) concentrations (Kandaswami et al., 1991).

To determine whether prevention might be associated with dietary-derived angiogenesis inhibitors, Fotsis et al. (1993) fractionated urine of healthy human subjects consuming soy-rich vegetarian diet and examined the fractions for their abilities to inhibit the proliferation of vascular endothelial cells. Using GC-MS, these authors showed that one of the most potent fractions contained several isoflavonoids, which the authors also synthesized. Of all the synthetic compounds, genistein was the most potent and inhibited endothelial cell proliferation and in vitro angiogenesis with  $IC_{50}$  values of 5 and 150  $\mu$ M, respectively. The high excretion of genistein in urine of vegetarians suggested that genistein might contribute to the preventive effect of a plant-based diet on chronic diseases, including solid tumors and inflammatory conditions (Adlercreutz, 1990) by inhibiting neovascularization. Genistein may thus represent a new class of diet-derived antiangiogenic compounds.

Of particular interest was a report that acute stress increased metastatic spread of mammary tumors in rats (Ben-Eliyahu et al., 1991). This finding acquires new significance in view of the recent reports that corticotropin-releasing hormone released under stress stimulated mast cell secretion (Theoharides et al., 1998; Singh et al., 1999). Mast cell secretion of neovascularization/angiogenesis agents (Kessler et al., 1976) and stimulation of mast cell migration by tumor-derived peptides (Poole and Zetter, 1983) suggest that mast cells may be involved in tumor growth and metastasis (Scott, 1963; Theoharides, 1988). The strong inhibitory action of many flavonoids on mast cell activation and proliferation may also explain their anticancer effects.

### *J. Effect on Heat Shock Proteins*

A universal and highly conserved response of cells to heat shock (HS) stress is the formation of HSPs accompanied by the activation of a cytoplasmic HS factor, which can react with nuclear HS elements. HSPs are

generally referred to as stress proteins and are important in various cell functions, including protein assembly/folding and transport. In addition to heat stress, these proteins can also be induced by hypoxia, glucose starvation, and exposure to arsenite, heavy metals, or amino acid analogs (Hosokawa et al., 1990a). In light of this, it is striking that the behavior of this ancient system can be modulated by flavonoids. Quercetin and other flavonoids inhibited the induction of heat shock proteins in HeLa cell and colon cancer cell cultures at the level of mRNA accumulation (Hosokawa et al., 1990a). Quercetin also inhibited the acquisition of thermotolerance in a human colon carcinoma cell line, suggesting that quercetin or related flavonoids might improve the efficacy of clinical hyperthermia in cancer therapy (Koishi et al., 1992). Quercetin was also found to be a hyperthermic sensitizer of HeLa cells (Kim et al., 1984). This flavonoid also inhibited arsenite-induced thermotolerance.

HSPs belonging to the 70-kDa family (HSP 70) are involved in the regulation of cell proliferation and differentiation. Elia et al. (1996) studied the effect of quercetin on HSP activation, HSP 70 synthesis, and thermotolerance in human K562 erythroleukemia cells. Quercetin blocked HSP synthesis (K562 erythroleukemia cells) at different levels depending on the temperature used and on the stressor employed (Elia and Santoro, 1994). Quercetin inhibited HSP 70 synthesis following  $\text{PGA}_1$  exposure. In  $\text{PGA}_1$ -treated cells, quercetin suppressed  $\text{PGA}_1$ -induced thermotolerance in a kinetically complex fashion. The authors concluded that their data supported the hypothesis that HSP 70 is important in thermotolerance development in human cells. Koishi et al. (1992) studied the effects of quercetin on the acquisition of thermotolerance in a human colon carcinoma cell line. Treatment with quercetin virtually abolished, in a concentration-dependent manner, the development of thermotolerance, which appeared directly related to inhibition of heat shock protein synthesis.

#### *K. Effect on Multidrug Resistance*

An important cellular defense mechanism against naturally occurring xenobiotics is the Pgp system, which also inhibits the accumulation of anticancer drugs in malignant cells. Importantly, quercetin was found to be an inhibitor of multidrug-resistant human breast cancer cell proliferation (Scambia et al., 1991).

Kioka et al. (1992) reported that quercetin affected the expression of multidrug resistance gene-1 (MDR1) in the human hepatocarcinoma cell line HepG2. The increase of Pgp synthesis (the gene product) and MDR1 mRNA accumulation in these cells caused by exposure to arsenite were inhibited by quercetin (Kioka et al., 1992). This appears to be the first report to describe the inhibition of MDR1 expression by any chemical. Not only did certain flavonoids inhibit the expression of the multidrug resistance gene but, in addition, could act as potent stimula-

tors of the Pgp-mediated efflux of the carcinogen 7,12-dimethylbenz[a]anthracene, resulting in a decreased intracellular burden of this polycyclic compound. The active flavonoids were kaempferol, quercetin, and galangin (Phang et al., 1993). On the other hand, somewhat paradoxically perhaps, genistein was shown to inhibit enhanced drug efflux in non-Pgp-mediated multidrug-resistant malignant cells (Versantvoort et al., 1993). Acting through P-glycoprotein as a possible target, quercetin was found to potentiate the effect of Adriamycin in a multidrug-resistant MCF-7 human breast cancer cell line (Scambia et al., 1994b). Critchfield et al. (1994) found, on the other hand, that several flavonoids (galangin, kaempferol, and quercetin) markedly reduced the accumulation of [ $^{14}\text{C}$ ]Adriamycin and accelerated its efflux in HCT-15 colon cells. In spite of some controversy, the findings provide further support for the possible therapeutic application of quercetin and other flavonoids as potential anticancer drugs either alone or in combination with other drugs, at least in multidrug-resistant breast cancer cell lines.

## **XII. Effects on Xenobiotic Metabolism**

It is now well established that dietary chemicals can affect or modulate drug-metabolizing enzymes. This property suggests that some food chemicals, including flavonoids, may have important pharmacological and toxicological consequences. A case in point is the work of Siess et al. (1992), who studied the effect of flavone, flavanone, and tangeretin in the diet of rats (20–2000 ppm) on the induction of hepatic ethoxyresorufin and pentoxyresorufin dealkylases, EH, GST, arylhydrocarbon hydroxylase, and UDP-glucuronyltransferases (UDPGT). In a concentration-dependent manner, flavone induced the activity of each enzyme. Flavone induced EH, GST, and UDPGT1, but not UDPGT2; and tangeretin had only a slight stimulating effect on UDPGT1 and UDPGT2 at the highest diet dose. In the study by Siess et al. (1992), the experimental doses of flavonoids in the rat diet that had enzyme-inducing effects were quantities that could be consumed in the daily human diet. It is also possible that subthreshold levels of several flavonoids acting together could collectively cause enzyme induction.

Flavonoids have the ability to activate and induce the synthesis of the primary enzyme system involved in metabolism of various lipophilic xenobiotics, such as carcinogens, drugs, environmental pollutants, and insecticides. Naturally occurring and synthetic flavonoids were reported to have striking effects on the P450-dependent monooxygenase system (Sato and Omura, 1978), including the induced synthesis and activation of specific P450 isozymes (Wood et al., 1986). Induction of the monooxygenase system by flavonoids has been described (Conney, 1967). Wattenberg et al. (1968) reported that nine flavonoids, including several flavanone

and chalcone derivatives, given orally to rats 2 days before sacrifice produced substantial increases in the levels of benzo[a]pyrene hydroxylase activity in the lung and liver. The synthetic flavonoid, 5,6-benzoflavone, the most active compound examined, increased induction of enzyme activity in the liver by a factor of 15. Of special interest in human physiology is the observation that the monooxygenase system in liver could be activated not only by the synthetic 7,8-benzoflavone but also by the naturally occurring compounds flavone, tangeretin, and nobiletin, which may be consumed in the daily diet. Polymethoxylated flavonoids such as tangeretin could be demethoxylated by a cytochrome P450-catalyzed reaction (Canivenc-Lavier et al., 1993). Rats pretreated with selected flavonoids resulted in increased microsomal demethylation, a mechanism that might lead to increased availability of more hydrophilic biologically active flavonoids.

Several studies have shown that plant flavonoids affect the activity of P450-mediated monooxygenases. These *in vitro* studies indicated that flavonoids have specific actions related to chemical structure or to enzyme activity (Buening et al., 1981; Sousa and Marletta, 1985). For instance, a large number of hydroxylated flavone derivatives were shown to inhibit BP hydroxylation in human liver microsomes, an effect suggested to be partly due to P450 reductase inhibition (Buening et al., 1981). However, such inhibition was not observed by Sousa and Marletta (1985). On the other hand, flavone and other nonhydroxylated analogs acted as activators of BP hydroxylation and aflatoxin B1 activation (Buening et al., 1981; Huang et al., 1981a), an effect later shown only to occur with some P450 isozymes, while others were inhibited (Huang et al., 1981b). Although flavone activated zoxazolamine metabolism *in vivo* in neonatal rats, it did not activate the *in vivo* metabolism of BP (Lasker et al., 1984). The *in vitro* addition of quercetin and other hydroxylated flavonoids inhibited rat liver microsomal hydroxylation of zoxazolamine, but studies with quercetin and apigenin indicated that these flavonoids had no effect on the *in vivo* metabolism of zoxazolamine. Dietary administration of flavone to rats was reported to cause significant increases in hepatic P450 monooxygenases such as ethoxyresorufin, pentoxyresorufin, and ethoxycoumarin deethylases (Brouard et al., 1988). The induction observed appeared to be characteristic of both phenobarbital- and 3-methylcholanthrene-inducible-type cytochrome P450s; quercetin administration, however, produced no induction of the above hepatic enzyme activities. On the other hand, dietary quercetin was shown to induce hepatic aminopyrine demethylase activity in rats (Siess and Vernevaux, 1982).

The induction of monooxygenase and transferase activities in rat liver following dietary administration of several different flavonoids was studied by Siess et al. (1989). The compounds evaluated included flavone and

flavanone and also tangeretin, quercetin, and chrysin. The activities of these compounds were compared with the two synthetic flavonoids, 7,8-benzoflavone and 5,6-benzoflavone. The polyhydroxylated compounds such as quercetin failed to cause any change in phase I or phase II enzyme activities. Flavone was a potent inducer with a resulting mixed type of induction. Flavanone had no effect on monooxygenase activities, but the increase in phase II enzyme activities was similar to that caused by flavone. Tangeretin caused a mixed pattern of induction, but was less active than flavone. The synthetic flavonoids caused induction of patterns similar to that of 3-methylcholanthrene. Generally, similar results were obtained by Obermeier et al. (1995) in studies of tangeretin, naringenin, flavone, epicatechin, epicatechin-3-gallate, epigallocatechin, and epigallocatechin-3-gallate (tea flavonoids). Further experiments suggested that induction of P450 IA2 by the nonhydroxylated flavones, flavone and tangeretin, might involve a transcriptional and/or post-transcriptional mechanism, again indicating the capacity of particular flavonoids to affect mammalian gene function (Canivenc-Lavier et al., 1996).

The isoenzyme CYP11A4 (P450 IIIA4) is mainly responsible for the primary metabolism of dihydropyridine calcium channel antagonists, such as nifedipine and felodipine; it also participates in the metabolism of other drugs such as quinidine, cyclosporin, phenytoin, and also endogenous steroids. It is of clinical significance, therefore, that there was an increase in the maximum plasma concentration of felodipine and a delay in its clearance when the drug was taken with grapefruit juice, as compared with orange juice or water (Bailey et al., 1993a). Edgar et al. (1992) studied the acute effects of grapefruit juice consumption on the pharmacokinetics and dynamics of felodipine. Grapefruit juice caused an increase in  $C_{max}$  and in the area under the curve, corresponding to an increase of systemic availability of the drug from 15 to 45%. The investigators considered it possible that grapefruit flavonoids inhibited the oxidation of felodipine to inactive dehydrofelodipine. Bailey and coworkers (1991) also showed that grapefruit juice increased the bioavailability of nifedipine, as well as felodipine. Similar findings were reported with nitrendipine (Soons et al., 1991). Although it has not been established with complete certainty, it is possible that grapefruit flavonoids (and perhaps flavonoids from other dietary sources) could affect drug metabolism by an effect on various cytochrome P450 enzymes. Some data suggest that the grapefruit juice effect may be attributable to the flavanone naringenin (Miniscalco et al., 1992), which has been shown to inhibit the hepatic mixed function oxidase responsible for the metabolism of the dihydropyridine calcium channel antagonists, but not attributable to the glycoside, naringin (Bailey et al., 1993b). The effect of several other naturally occurring grapefruit flavonoids (naringenin, quercetin, and kaempferol) on dihydropyridine metabolism was inves-

tigated by Miniscalco et al. (1992), who found that quercetin and kaempferol (flavonols) were active inhibitors of human liver microsomes, while naringenin was essentially inactive. They speculated that a likely mechanism of action of active compounds is inhibition of cytochrome P450 IIIA4, the isoenzyme that catalyzes the oxidation of the dihydropyridine ring. Flavonoid effects were not limited to the CYP IIIA4 isoforms, as shown by Fuhr et al. (1993), who found that grapefruit juice and naringenin inhibit CYP1A2, the isoform metabolizing caffeine and theophylline. Studies by Rashid et al. (1993) indicated that quercetin, a minor grapefruit flavonoid and an *in vitro* inhibitor of CYP IIIA, did not account for the grapefruit juice effect. In a study comparing water, grapefruit juice, and naringin (naringin is the principal bitter flavonoid compound in grapefruit), Bailey and coworkers (1993a) found that only grapefruit juice possessed the capacity to increase the bioavailability of felodipine. It is possible that other flavonoids ingested in the regular diet could affect health adversely by delaying metabolism and clearance of drugs, thus causing an increase in plasma and tissue concentrations to potentially toxic levels. Perhaps the anticarcinogenic activity of particular flavonoids may be related to their capacity to induce carcinogen-metabolizing enzymes.

### XIII. Concluding Remarks

Flavonoids comprise a vast array of biologically active compounds ubiquitous in plants, many of which have been used in traditional Eastern medicine for thousands of years. Of the many actions of flavonoids, antioxidant and antiproliferative effects stand out. Moreover, the inhibitory action on inflammatory cells, especially mast cells, appears to surpass any other clinically available compound. Given that certain substituents are known to be required or increase their actions, the therapeutic potential of select flavonoids is fairly obvious. The areas that hold most promise are chronic inflammatory and allergic diseases, as well as coronary artery disease and breast cancer. Well designed clinical trials are overdue possibly because there is no intellectual property protection. It is encouraging that a US patent was recently allowed on the combined use of flavonoids with proteoglycans, which were recently shown to also inhibit mast cell secretion (Theoharides et al., 2001) for the treatment of mast cell activation-induced diseases.

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