PHYTOESTROGENS in FUNCTIONAL FOODS

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Preface

The phenolics or polyphenols include a wide range of plant secondary metabolic substances possessing in common an aromatic ring with one or more hydroxyl groups; these compounds, located in the vacuole, tend to be water-soluble, as they occur in combined forms with sugars as heterosides.

Polyphenols are major bioactives found in fruits, vegetables, cereals, legumes, seeds, nuts, herbs, spices, chocolate, and beverages such as tea, coffee, or wine. They possess antioxidant, phytoestrogenic, antiproliferative, and enzyme-modulating activities in the human metabolic system. Scientific studies from different disciplines strongly suggest a role in the prevention of degenerative diseases such as cancers, cardiovascular diseases, osteoporosis, and neurodegenerative diseases.

The pace of the polyphenol research has increased rapidly over the past 6 years. However, clinical and epidemiological evidence on their protective effects is still limited, and optimal levels and forms of intake have not been established. Furthermore, some questions about their safety have also been raised. This is especially important in view of the growing number of new food products specifically designed as sources of polyphenols.

Phytoestrogens include flavonoids (isoflavones, coumestans, prenyl flavonoids) and nonflavonoids (lignans, stilbenes, phytosterols, saponins, resorcyclic acid), which are all polyphenolic compounds.

Phytoestrogens represent one of many important bioactive nonnutrients found in many plants commonly consumed in human diet. Knowledge of the chemical composition of foods is fundamental for the planning of human nutrition. Qualitative and quantitative data are essential when this information is applied to prevent and control diseases for individuals and populations.

Phytoestrogens are naturally occurring chemicals of plant origin that possess weak estrogenic properties due to their structural similarities to the human hormone estradiol. Bearing a structural resemblance to 17β-estradiol, they can interfere *in vivo* with mechanisms controlled by the hormone through competition for its receptors. They bind the estrogen receptor and exert hormonal and antihormonal effects. In addition, phytoestrogens may be important antioxidants. They perturb the action of DNA topoisomerase II and ribosomal S6 kinase, which could explain their observed effects on cell cycle, differentiation, proliferation, and apoptosis. Moreover, genistein is a potent tyrosine kinase inhibitor. It has been proposed that through such mechanisms phytoestrogens protect against a wide range of ailments, including breast, prostate, bowel, and other cancers, cardiovascular diseases, osteoporosis, and menopausal symptoms. Of the flavonoids group of phytoestrogens, coumestan and isoflavone classes possess the greatest estrogenic

activity. Since coumestans are found predominantly in clover and alfalfa plants and so are rarer components of the human diet, genistein, which has been reported to have the most potent anticarcinogenic and estrogenic effect among isoflavones, attracts a great deal of interest in current studies.

Despite a large amount of research, the role of phytoestrogens in human health and diseases has not been defined. Epidemiological evidence and experimental data from animal studies are not conclusive; beneficial effects of phytoestrogens on human health are suggestive, but the clinical data to support such effects are not widely available. There is a need for large-scale clinical studies.

There has been an attempt to bring together key information from many fields developed over the last 20 years. But controversial subjects, single case reports or single research results, and commercial documents have been omitted from the main text of the chapters.

The objective of this book is to introduce, organize, and document the scientific, technical, and practical aspects involved with the production, consumption, and safety of phytoestrogens in foods. This volume is designed to serve as a reference book for students, researchers, government officials, and manufacturers of baby foods, functional foods, and dietary supplements. On the other hand, this book will be a good reference book for food chemistry, nutrition, therapeutic nutrition, pharmacology, pediatrics, obstetric, gynecology, biochemistry, and toxicology graduate and undergraduate courses.

The contributors to this volume have been very carefully selected from all over the world of science for their distinguishing publications in this field.

This book is an attempt to integrate all aspects of phytoestrogen research in one volume in a concise manner, something that is not currently available in any other book.

If I have been able to summarize the current knowledge and stimulate further research, I think we will all feel successful.

I thank all of the contributors to this volume and all the individuals who helped make this timely book a reality. I especially wish to thank Anita Lekhwani, Erika Dery, and Susan Lee B. for their assistance during the preparation of this book.

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I would like to thank the publishing company and their personnel for their contribution to the dissemination of science to the world.

Finally, I feel a deep sense of gratitute to my family, my wife, and my children, who gave me the peace of mind to enable me to really focus on the book.

1

Introduction to Phytoestrogens

E. Gültekin and Fatih Yildiz

CONTENTS

1.1 Phytoestrogens

Phytoestrogens are naturally occurring chemicals of plant origin that have the ability to cause estrogenic and/or antiestrogenic effects due to their structural similarities to the human hormone estradiol (17β-estradiol).[1]

1.1.1 Classification of Phytoestrogens

The majority of phytoestrogens belong to a large group of substituted phenolic compounds known as flavonoids. There are several groups of flavonoids with estrogenic properties. Of these, coumestans and isoflavones possess the greatest estrogenic activity.[2] A class of prenylated flavonoids with estrogenic activities intermediate to those of the coumestans and isoflavones has recently been identified.[3] Lignans, a class of nonflavonoid phytoestrogens, have also been shown to exert estrogenic effects.[4] The relationship between these types of phytoestrogens and the names of the compounds most commonly found in food from these four groups is summarized in Figure 1.1.

1.1.2 Structure–Function Similarities of Phytoestrogens

Similarity of phytoestrogens to estrogens at the molecular level provides them the ability to mildly mimic and in some cases act as an antagonist to estrogen.[5] Common features of phytoestrogens and estradiol are listed in Table 1.1.[6]

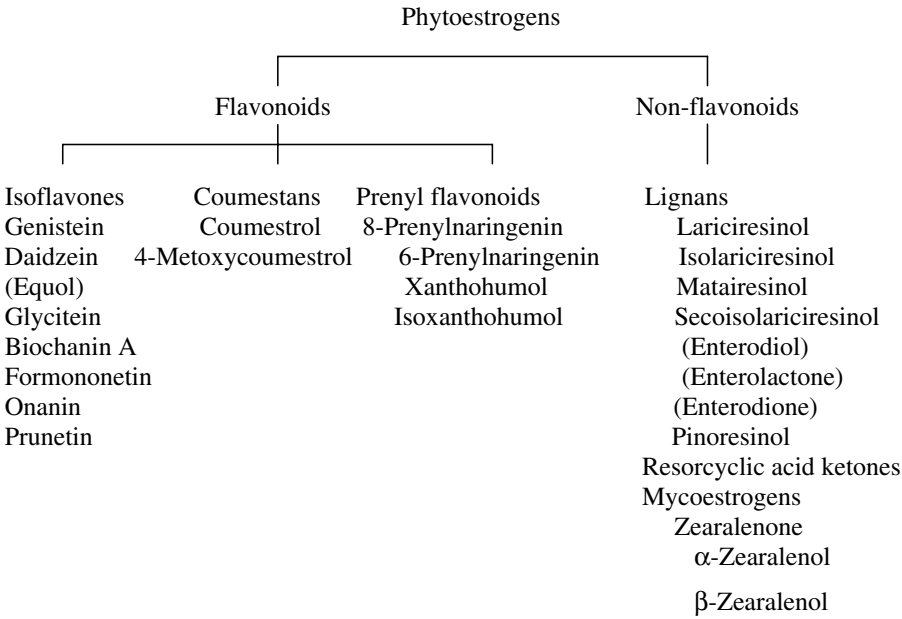

FIGURE 1.1
The relationship between various groups of phytoestrogens (given in bold) and members of each group. (The compounds in parentheses are not inherently present in plants but are oestrogenic products resulting from metabolism of members of that class of phytoestrogens.)

TABLE 1.1

Key Structural Elements Crucial for Estradiol-Like Action

Presence of the phenolic ring indispensable for binding to estrogen receptors (ERs)
Role of the ring of isoflavones mimicking the ring of estrogens at receptor binding
Low molecular weights, similar to that of estradiol ($C_{18}H_{24}O_2$) (MW = 272)
Distance between two aromatic hydroxyl groups in the nucleus of the isoflavones almost identical to the distance between two hydroxyl groups of estradiol
Optimal pattern of hydroxylation, i.e., hydroxyl substituents at 4, 5, and 7 positions (e.g., genistein)

The structural similarities between members of the four main groups of phytoestrogens identified in Figure 1.1 and estradiol are shown in Figure 1.2.[7]

1.1.3 Estrogenic Properties of Phytoestrogens

In the 1940s, it was first realized that some plant-derived compounds could cause estrogenic effects in animals.[8] Sheep grazing on pastures containing red clover had multiple fertility problems. The clover in these pastures had high amounts of the isoflavones formononetin and biochanin A.[9] The phytoestrogens daidzein and genistein were responsible for the infertility of some captive cheetahs fed a soybean-enriched diet subsequently found to contain high quantities of these compounds.[10]

Evidence is beginning to accrue that phytoestrogens may begin to offer protection against a wide range of human conditions, including breast, bowel, prostate, and other cancers; cardiovascular disease; brain function; alcohol abuse; osteoporosis; and menopausal symptoms.[11] The basis for these effects has not been established, but the weak estrogenic activity of isoflavones may be a factor in conferring these properties.[5]

The incidence of a number of cancers, including those of the breast and prostate, has been found to be much higher in Western populations compared with that in countries such as Japan and China. Epidemiological and migrant studies have suggested that racial characteristics and other factors including lifestyle, diet, and fat or fiber intake may play a role in the etiology of these diseases. One notable dietary difference is the relatively high consumption of soy and soy-based foods among Asian populations. Comparison of estimated dietary isoflavone intakes in Western and Eastern (e.g., Japanese and Chinese) populations illustrate that Eastern populations have a significantly higher intake of phytoestrogens. Estimates suggest that the average Japanese consumer is exposed to approximately 25 to 100 mg isoflavones/day, while an average United Kingdom consumer ingests approximately 1 mg isoflavones/day.[7] As such, soy, which has been known to be the richest source of isoflavones, has attracted much attention as a potential chemoprotective factor.[11,12]

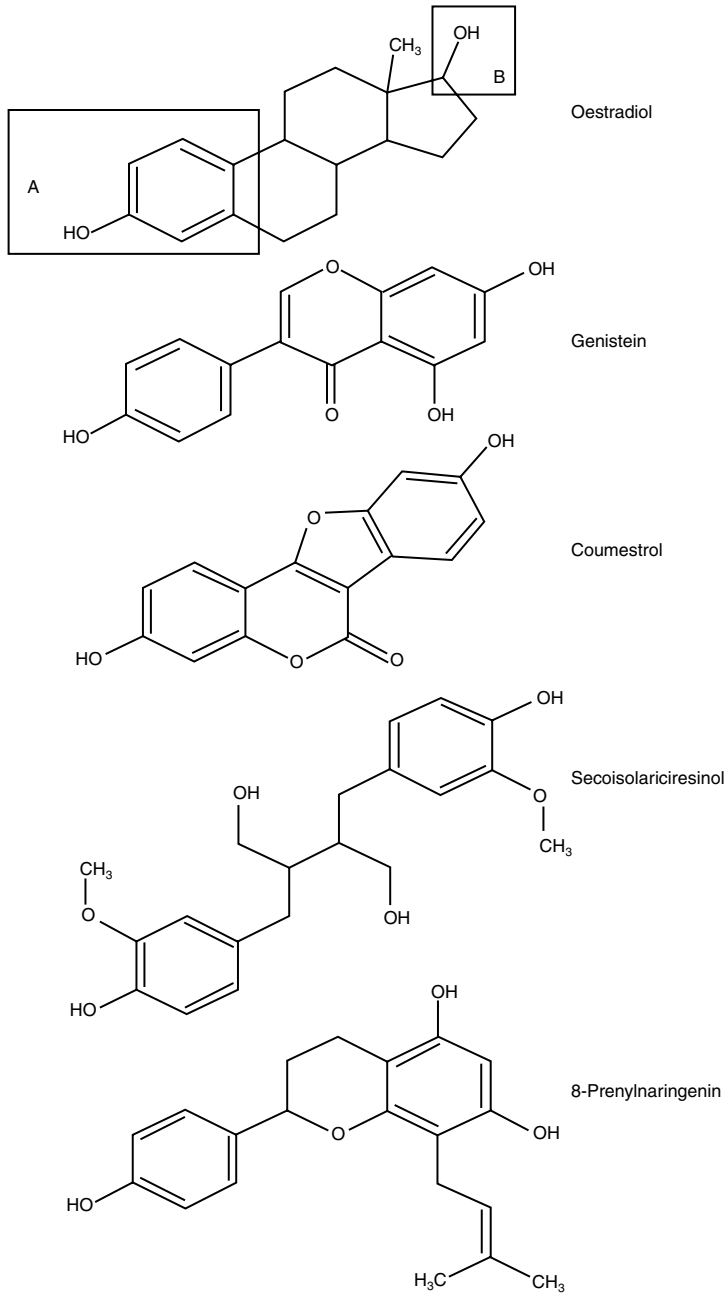

FIGURE 1.2
The structural similarities of phytoestrogens to estradiol. (The similarity of the structure of estradiol and examples from the four classes of phytoestrogens from Figure 1.1. All the structures possess the phenolic [A] and hydroxyl [B] moieties outlined in boxes on the estradiol structure, and the distances between the two groups in each compound are similar.)

1.1.4 Relative Estrogenic Activity of Phytoestrogens

In general, phytoestrogens are relatively weak oestrogens, requiring much higher concentrations than estradiol to produce an equivalent biological response. Since potency values can vary significantly between methods, relative absolute estrogenic potency of phytoestrogens is difficult to determine. However, taking the results of both *in vitro* and *in vivo* studies together, a single rank order of oestrogenic potency of phytoestrogens may be estimated: estradiol coumestrol > genistein, equol > glycitein > 8-prenylnaringenin > daidzein > formononetin, biochanin A, 6-prenylnaringenin, xanthohumol, isoxanthohumol.[7]

Because coumestans, reported to be the most potent phytoestrogen,[13] are found predominantly in clover and alfalfa plants[14] and so are rare components of the human diet,[15] isoflavones attract a great deal of interest in today's studies due to the wider range of foods containing them.

1.2 Isoflavones

Isoflavones are polyphenolic phytoestrogens that occur mainly as glucoconjugates (glucosides) of genistein, daidzein, and glycitein.[16] They enjoy a restricted distribution in the plant kingdom and are predominantly found in leguminous plants.[17] The main dietary sources of isoflavones are soybeans and soy foods.[18]

1.2.1 Classes of Isoflavones

The most prevalent isoflavones present in plant-based foods are as follows[11] (Figure 1.3):

- Genistein
- Daidzein
- Glycitein
- Biochanin A (methylated derivative of genistein)
- Formononetin (methylated derivative of daidzein)

1.2.2 Physical and Chemical Properties of Isoflavones

1.2.2.1 Water Solubility

Isoflavones are low-molecular-weight (MW of daidzein = 254, MW of genistein = 270, MW of biochanin A = 284) hydrophobic compounds. The aqueous solubilities of the isoflavone aglucones are low, and due to the acidic

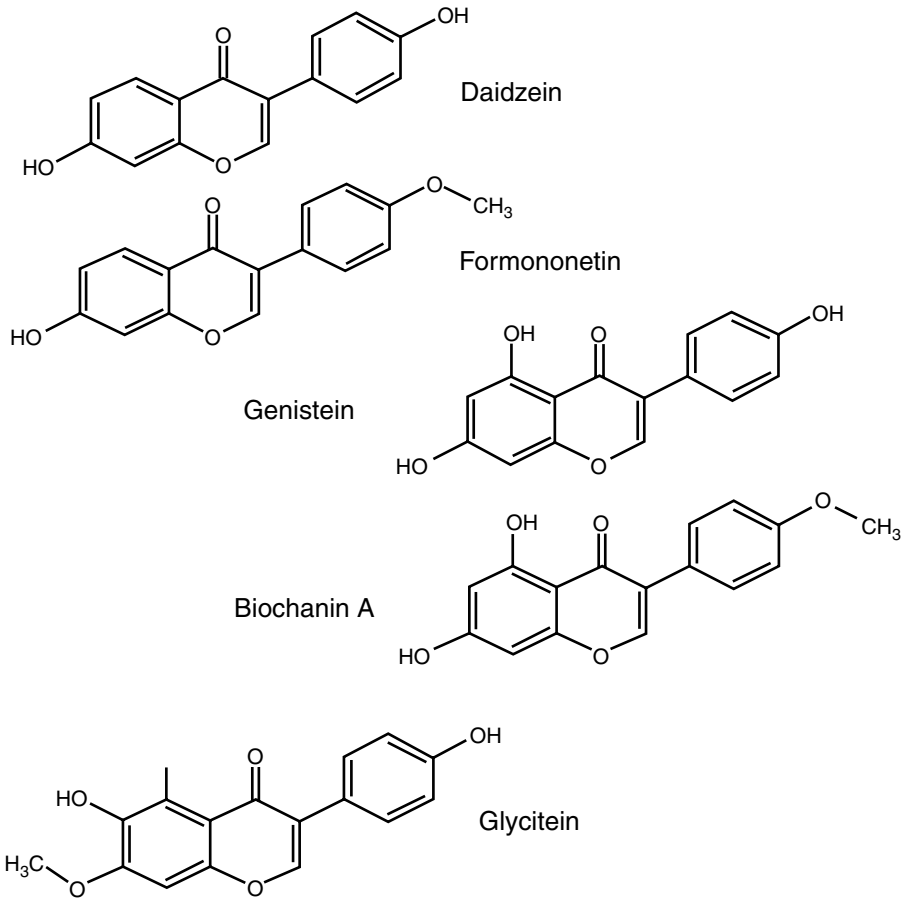

FIGURE 1.3
Isoflavone aglucones: daidzein, formononetin, genistein, biochanin A, and glycitein.

nature of the phenolic groups, they are pH dependent. The methylated derivatives biochanin A and formononetin are less soluble than genistein and daidzein, respectively.[18] Conjugation to glucose, glucuronide, or sulfate groups increases the solubility.[1]

1.2.2.2 Chemical Stability

The isoflavone aglucones are stable under physiological conditions. Under acidic conditions, the glucosides can be deconjugated to give aglucones. In the body, enzymes in the gut and liver can carry out these reactions during metabolism.[7]

1.2.3 Absorption, Distribution, Metabolism, and Excretion of Isoflavones

In plants, isoflavones are present as glucosides. Processing reduces the isoflavone content and can partially convert them to aglucones. Thus, isoflavones are ingested mainly as glucosides and undergo hydrolysis by gut bacterial and mammalian enzymes prior to absorption.[20] Following absorption of the aglucones, these compounds are reconjugated with sulfate and glucuronide and excreted in the bile or urine.[11] Gut microflora can also modify estrogenic isoflavones into more active forms. The methylated isoflavones formononetin and biochanin A are demethylated by gut microflora to daidzein and genistein, respectively.

1.3 Analysis of Phytoestrogens

1.3.1 Isolation of Phytoestrogens

Isoflavones are often present as glucosides in plants. Because acidic conditions cause glucosides to deconjugate into aglucones, most extraction procedures involve acid hydrolysis. Hydrolysis with 1 to 2 *M* hydrochloric acid at 100°C or refluxing with acid in the presence of ethanol has been used to form the aglucones.[21] However, there are some reports indicating that genistein is unstable under acid hydrolysis conditions.[22,23]

For analysis of soy foods, typical extraction conditions that have been used are stirring freeze-dried powdered samples with methanol–water (80:20, v/v) at room temperature or 4°C, or with a mixture of acetonitrile–hydrochloric acid (0.1 *M*)–water.[24–28] Using acidified solvents has been highly recommended by the compilers of the U.S. Department of Agriculture–Iowa State University Database[29]; on the other hand, alcohol extraction has been reported to reduce the isoflavone content of soy products significantly.

In the case of nonsoy foods, since the exact nature and composition of isoflavone glucosides present in foods other than soy is unknown, none of the methods employed for isoflavone detection in nonsoy foods have been used to determine glucoside content. Thus, all the reported sample preparation protocols utilize a hydrolysis step to form aglucones.[29]

Meksem et al.[30] and Lee et al.[31] employed the following sample preparation method: 2 g of ground soybean seeds were mixed with 2 ml of 0.1 *N* HCl and 10 ml of acetonitrile, stirred for 2 h at room temperature, and filtered. The filtrate was dried under vacuum at temperatures below 30°C and then was redissolved in 10 ml of 80% HPLC-grade methyl alcohol in distilled water.

Hutabarat et al.[32] investigated the optimum extraction of daidzein, genistein, formononetin, and biochanin A in soybeans. One gram of ground food was diluted with 40 ml of 96% ethanol and extracted with or without hydrolysis by refluxing and heating on a water bath at 80 or 100°C at varying

pH of phosphoric acid or hydrochloric acid (10 ml) for 0, 1, 2, 3, 4, or 6 h. The optimum extraction was by hydrolysis with 2 *M* hydrochloric acid with refluxing on a water bath at 100ºC.

Pandjaitan et al.[33] evaluated genistin and genistein contents of soy protein concentrates prepared by three basic methods (acid, alcohol, and hot-water leach). The acid leach method gave the highest total genistin+genistein content (0.742 mg/g) compared to soy protein concentrates prepared with the hot-water leach method (0.671 mg/g) and the alcohol leach method (0.070 mg/g). The acid leach, hot-water leach, and alcohol leach methods had 20.3%, 24.2%, and 91.2% losses of total genistin+genistein, respectively.

Yildiz and Gültekin[34] employed the method given in Figure 1.4, which worked well for both soy and nonsoy food phytoestrogen isolation.

1.3.2 Analytical Methods

The most widely used techniques for measurement of phytoestrogens are the following:

- Reversed-phase high-performance liquid chromatography (RP-HPLC) with ultraviolet (UV) or diode-array detection (DAD)
- Gas chromatography with mass spectrometric detection (GC-MS)
- Liquid chromatography with mass spectrometric detection (LC-MS)[7]

HPLC with UV/DAD is a relatively rapid way of measuring phytoestrogens compared to MS-based methods.[27,35,36] The advantage of this method is that only hydrolysis and extraction of the samples are needed before analysis.[37]

For soy foods, the main analytical techniques are HPLC with UV or DAD using reversed-phase C_{18} stationary phases with gradient elution.[21] On the other hand, since isoflavonoid levels in nonsoy foods are much lower than in soy, UV detection has been considered not sufficiently sensitive for the analysis of these expected low levels of isoflavonoids.[38,39] In general, if food samples containing phytoestrogen concentrations that are greater than 50 ppm (this is largely restricted to soybean or red clover products) are to be analyzed, HPLC with DAD-UV detection is the method of choice. However, in the case of concentrations less than 50 ppm, HPLC-UV is not adequate.[24]

In general, the mobile phases employed with RP-HPLC columns have been acetonitrile and/or methanol in combination with water containing small amounts of an acid.[24] Merken and Beecher[40] reported that most of the different classes of phytoestrogens and their metabolites are separated by RP-HPLC using elution with a gradient of methanol or acetonitrile in an acidic (0.1 to 1% acetic, formic, or trifluoroacetic acids) or neutral (10 m*M* ammonium acetate or ammonium formate) solvent.

Franke et al.[22,41] quantified daidzein, genistein, and biochanin A in legumes by HPLC-DAD on a C_{18} reversed-phase column using a gradient solvent

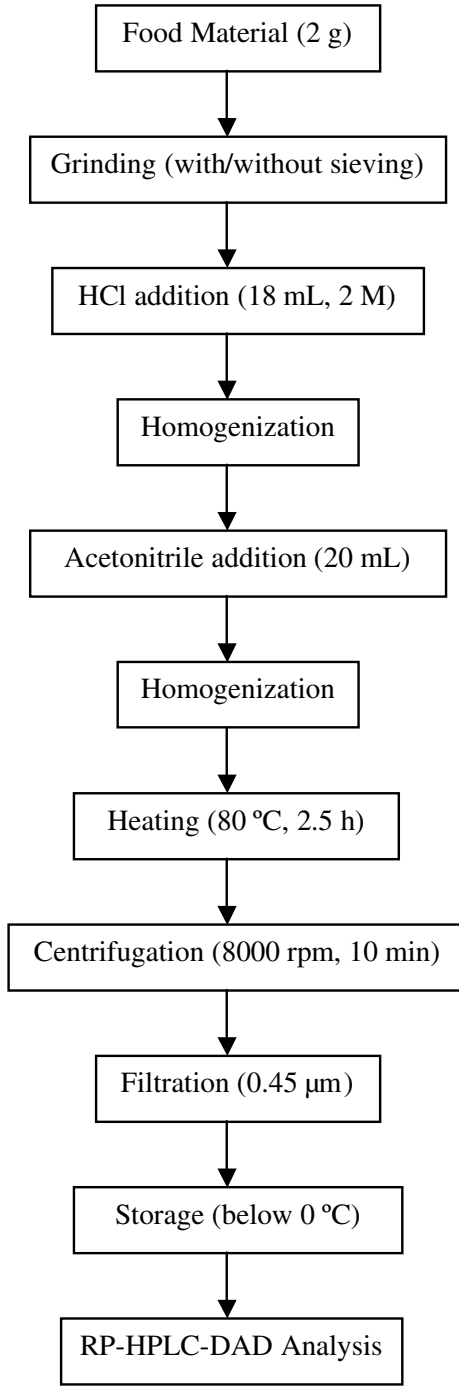

FIGURE 1.4
Outline of an experimental procedure for isolation of phytoestrogens in food samples.

system consisting of A (acetic acid–water [10:90, v/v]) and B (acetonitrile; increased from 23 to 70 B% in 8 min followed by holding at 23 B% for 12 min). Meksem et al.[30] and Lee et al.[31] conducted an HPLC-DAD analysis on a YMC-Pack column according to the method of Wang and Murphy.[24,42] Solvent A was 0.1% glacial acetic acid in distilled water, and solvent B was 0.1% glacial acetic acid in acetonitrile. Solvent B was increased from 15% to 35% over 50 min and then held at 35% for 10 min. The USDA–Iowa State University Isoflavones Database[29] used the analytical method described by Murphy et al.[25] as the reference method for evaluating analytical methodologies for isoflavones in soy products. A linear gradient was composed of 0.1% aqueous acetic acid and 0.1% acetic acid in acetonitrile with a total elution time greater than 45 min.

All the phytoestrogens and their metabolites contain at least one aromatic ring. This means that they absorb UV light with a maximum wavelength in the range from 250 to 270 nm.[20] Maximum absorption of daidzein was achieved at a wavelength of 249 nm with a shoulder at 302 nm, genistein at 259 nm, and biochanin A at 260 nm.[21,43,44] Franke et al.[22,41] monitored daidzein, genistein, formononetin, and biochanin A at or very near their absorption maximum with DAD at 260 nm. Hsu et al.[45] detected daidzein, genistein, and biochanin A in *Psoralea corylifolia* at 260 nm. Griffith and Collison[28] monitored isoflavones at 260 nm. Lee et al.[31] measured elutions of daidzein and genistein at an absorbance of 254 nm, while Mitani et al.[46] selected a wavelength of 259 nm for detection of these compounds.

1.4 Reported Phytoestrogen Content of Foods

The precise phytoestrogen content of many individual foods is not known, and it differs according to variety, location, and season.[5]

The major dietary sources of isoflavonoids for humans are soybeans and soy-based foods.[17,47] Mitani et al.[46] reported that daidzein and genistein were detected at high concentrations in dried soybeans. Mazur and Adlercreutz[39] stated that soybeans proved to be the richest source of genistein.

Isoflavone content data of soybeans from previous studies are summarized in Table 1.2. The variety, the crop year, and the location affect the isoflavone content of the soybeans[24] and contribute to the large variability in the isoflavone content of soybeans.[29]

Isoflavone content data of chickpeas (Table 1.3), lentils (Table 1.4), beans (Table 1.5), and fruits and nuts (Table 1.6) from previous studies are collected and summarized.

TABLE 1.2

Daidzein, Genistein, and Biochanin A Contents of Soybeans (mg/100 g)

Soybean Type	Daidzein	Genistein	Biochanin A	Reference
Soybeans (Centennial)	25.2	34.3	0.0147	48
Soybeans (INIAP Bolivia)	10.5	26.8	nd	
Soybeans (Santa Rosa)	56.0	84.1	0.015	
Soybeans (Chapman)	41.3	46.4	tr	
Soybeans (Brazil, raw)	20.16	67.47		29
Soybeans (Japan, raw)	34.52	64.78		
Soybeans (Korea, raw)	72.68	72.31		
Soybeans (Taiwan, raw)	28.21	31.54		
Soybeans (green, mature seeds, raw)	67.79	72.51		
Soybeans (mature seeds, dry roasted)	52.04	65.88		
Soybeans (mature seeds, raw) (U.S., food quality)	46.64	73.76		
Soybeans (mature seeds, raw) (U.S., commodity grade)	52.20	91.71		
Soybeans			0.01	
Soybeans	49	71.3		49
Dried soybeans (U.S.)	30.8	72.3		32
Dried soybeans (Indonesia)	127.7	83.4		
Dried soybeans (McKenzie's, Australia)	96.4	61.4		
Bowyer dried soybeans (Riverina, NSW, Australia)	65.4	72.0		
Fresh soybeans (Indonesia)	19.8	7.6		
Dried soybeans	7.45	26.77		46
Dried soybeans	15.82	29.56		50
Soybeans (Oriental diet)	10.5–85.0	26.8–102.5		6
Soybean seeds	6.8–100.6	1.8–138.2		7
Soybeans (mature)	0.5–91	1.1–150		47
Powdered soybean chips	80	50		17

TABLE 1.3

Daidzein, Genistein, and Biochanin A Contents of Chickpeas (mg/100 g)

Chickpea Type	Daidzein	Genistein	Biochanin A	References
Chickpeas (Bengal gram) Goya (garbanzo)	0.0342	0.0693	1.42	48
Chickpeas (garbanzo bean)	0.192	0.214	3.08	
Chickpeas	0.0114	0.0763	0.838	
Chickpeas (garbanzo bean, Bengal gram, raw)	0.04	0.06		29
Chickpeas	0.011–0.192	0.069–0.214		39
Chickpeas	0.01–0.2	0.07–0.2		47

TABLE 1.4

Daidzein, Genistein, and Biochanin A Contents of Lentils (mg/100 g)

Lentil Type	Daidzein	Genistein	Biochanin A	References
Lentils (Jack Rabbit)	0.0104	0.0188	tr	48
Lentils (Masoor dahl)	0.0033	0.0071	0.0071	
Lentils (raw)	0.00	0.00	0.00	29

TABLE 1.5

Daidzein, Genistein, and Biochanin A Contents of Beans (mg/100 g)

Bean Type	Daidzein	Genistein	Biochanin A	References
Navy beans (haricot)	0.0137	0.408	0.0044	48
Navy beans (dry)			0.00	29
Navy beans (raw)	0.01	0.2		
Beans (Great Northern, raw)	0.00	0.00		
Beans (small, white, raw)	0.00	0.74		
Kidney, navy, and pinto beans	0.008–0.04	0.007–0.5		47

TABLE 1.6

Daidzein and Genistein Contents of Fruits and Nuts (mg/100g)

Fruit and Nut Type	Daidzein	Genistein	Reference
Apricots (dried)	0.005	nd	51
Currants	0.056	0.2167	
Dates (dried)	0.0018	0.0054	
Figs (dried)	0.0019	0.0045	
Prunes (dried, raw)	0.0052	0.0104	
Raisins (California)	0.069	0.1458	
Chestnuts (raw)	0.0079	0.0059	

1.5 The Future Trends of Phytoestrogen Research

Estrogen deficiency in postmenopausal women can lead to unpleasant symptoms such as hot flashes, sleep deprivation, forgetfulness, and vaginal dryness, with a long-term increased risk of bone loss in addition to cardiovascular disease. Doctors have recommended hormone replacement therapy (HRT) for relief of these symptoms; however, because of the realization that HRT is not as safe or effective as previously thought, interest in phytoestrogens has significantly increased.

Applications of phytoestrogens in industry are becoming prevalent. Phytoestrogen therapy is applied as a natural alternative to the use of postmenopausal HRT. There is a global movement toward increased consumption of foods rich in phytoestrogens (phytoestrogen-rich diets) and tablet formulations of concentrated isoflavone extracts. Phytoestrogen creams and phytoestrogen capsules are being heavily promoted.

In this book, raw materials for the phytoestrogen industry were investigated. Food items belonging to different groups, including fruits, nuts, herbs, and especially legumes, were researched for their daidzein, genistein, and biochanin A contents.

Extraction with acetonitrile and HCl is highly recommended. Acid hydrolysis should be included if aglucones are intended to be detected. Molarity of HCl may be 1 to 2 *M*, 2 *M* being more effective. Heating may be done at 80 or 100°C, 80°C eliminating the possibility of degradation of aglucones.

As for chromatographic analysis, RP-HPLC with UV/DAD is the method of choice if food materials with high phytoestrogen contents are investigated. However, if phytoestrogen contents below 1 mg/100 g are to be detected, MS-based methods will provide better results.

As a result of screening of numerous research results with different food materials, it was concluded that soybeans contain high amounts of daidzein (91.36 mg/100 g) and genistein (85.57 mg/100 g), and chickpeas contain relatively small amounts of genistein (0.89 mg/100 g) and biochanin A (0.95 mg/100 g). The remaining 18 food materials (haricot beans, green lentils, red lentils, licorice root, yarrow, dried chestnuts, prunes, raisins, currants, black cumin, dried apricots, dried parsley, dried dates, dried figs, sage [from Aegean region of Turkey], sage [from Mediterranean region of Turkey], grapevine leaves, and gilaburu) were found to contain none of daidzein, genistein, and biochanin A.

Soybeans are still an important source of daidzein and genistein. Only chickpeas were found to contain genistein among all other analyzed food materials. Because the genistein content of soybeans is nearly 100 times that of chickpeas, it is difficult for chickpeas to be an alternative to soybeans. In the case of biochanin A, only chickpeas were found to contain this compound, not ignoring the fact that the concentration of the compound is too low.

It appears that phytoestrogens, being a very popular subject of interest for the research field of bioactive foods, will remain on the agenda of many countries as a priority for the coming years. Therefore, it is highly recommended that different components of diet, particularly different varieties of legumes, be analyzed for their phytoestrogen contents and estrogenic activities that will contribute to the food, medicine, and cosmetics industries. However, at high concentration, toxicity must be avoided as explained in Chapter 6 of this book.

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2

Biosynthesis of Polyphenol Phytoestrogens in Plants

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CONTENTS

2.1 Secondary Metabolism

2.1.1 Historical Background

The chemistry of plant secondary metabolites can be considered in its different aspects an ancient discipline. From time immemorial, the human race has employed plants as a source of dyes, toxins, fibers, stimulants, and medicines. In particular, finding healing powers in plants is a primordial idea. Since prehistory, man has been applying poultices and imbibed infusions from thousands of indigenous plants.[1] There is evidence that Neanderthals living 60,000 years ago in present-day Iraq used plants such as hollyhock (*Alcea rosea* L.): this species is still widely used in ethno-medicine around the world.[2] In the late fifth century B.C., Hyppocrates mentioned from 300 to 400 different medicinal plants.[3] The Holy Bible offers the descriptions of approximately 30 healing plants. In the first century A.D., Dioscorides wrote *De Materia Medica*, a medicinal plant catalogue which is considered the prototype of modern pharmacopoeia.[1] The fall of Roman empire and the barbarian invasions forestalled Western advances in the understanding of phytochemicals, with a relevant part of the available documentation being destroyed or lost.[2] In Europe, the Renaissance years saw a revival of ancient medicine and an increasing interest in phytochemicals. However, until the nineteenth century the knowledge on phytochemicals was based only on the observed effects on human healing. Only with the development of biochemistry and plant physiology in the nineteenth century did the investigation on phytochemicals assume the feature of a modern science based on experimental and causal approach. The pioneers of modern plant physiology and biochemistry began to separate, purify, and isolate the compounds produced in plant cells. Between 1817 and 1820 the French chemists Pelletier and Caventou isolated in crystalline form the first three plant alkaloids (strychnine, brucine, and quinine).[4] In the mid-nineteenth century, several British chemists extensively investigated numerous flavones extracted and purified from different plants employed for dyeing.[5] New separation methods were developed, and natural products chemistry has brought great stimuli to the development of the refined techniques now available, such as the various

analytical and preparative chromatographic methods (gas-chromatography, thin-layer chromatography, high-performance liquid chromatography, paper chromatography, electrophoresis).[6] Structural elucidation was typically carried out by degradation to smaller fragments of known structure combined with elementary analysis of the compounds. However, without any available methods in spectroscopy, it was difficult to assign a definite chemical structure to the major proportion of isolated phytochemicals. Although the detailed chemical structure of most phytochemicals was unknown, at the end of the nineteenth century, it had become clear that plant cells contained compounds with no obvious function.[7] In 1883 in a lecture on plant physiology Sachs[8] devoted a special chapter to lacticiferous ducts, concluding that lattices contain not only valuable compounds (starch, proteins, carbohydrates) but also other compounds defined "waste products" (resins rubber, terpenes, and alkaloids). In 1891 the plant physiologist Albrecht Kossel was the first to designate the term "secondary" for the phytochemicals without a clear physiological and biochemical role.[9] He affirmed, "Now chemistry must attempt to separate those compounds which are present, without exception, in a protoplasma capable of developing, and to recognize the substances which are either incidental or not absolutely necessary for life. . . . I propose calling the essential components of the cell primary compounds and those which are not found in every cell capable of developing, secondary." In addition, Pfeffer,[10] another pioneer of plant physiology, was convinced that secondary compounds had no essential physiological function for plant life. The thoughts of the pioneer plant physiologists deeply influenced plant chemistry and for a long time the term "secondary metabolite" was associated with inessentiality. Earlier in the twentieth century it was argued that secondary metabolites arise spontaneously or with the aid of "nonspecific" enzymes.[11] The subject of plant chemistry was revolutionized after the Second World War by the implementation of sophisticated chromatographic techniques coupled with a variety of spectral methods (UV-VIS spectrometry, infrared spectrometry, electron-impact mass spectrometry, fast-atom-bombardment mass spectrometry, chemical-ionization mass spectrometry, nuclear magnetic resonance, X-ray diffraction) for purifying plant constituents and for their rapid identification. In addition, in the next period, around 1950, besides the discovery of several hormones in plants, the progress of histology and cytology stimulated the researches on the localization of secondary compounds in cells and tissues. Hence, the rate of structural identification has been increased exponentially each decade since 1950 (Figure 2.1). At the present, approximately 100,000 structures have been described,[12] with many more yet to be discovered. It has been estimated that the total number of secondary metabolites in the plant kingdom alone exceeds the 500,000 marks.[13]

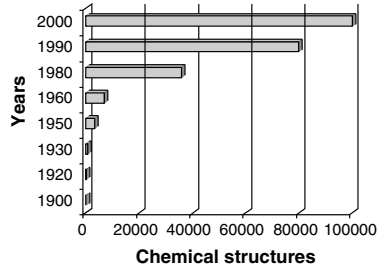


FIGURE 2.1
The trend of structural identification of plant secondary metabolites in the 20th century.

2.1.2 Secondary Metabolism and Metabolomic Approach

The two volumes of Bonner[14] and Paech,[15] published in 1950, contributed to the widespread usage of the term "secondary metabolites," although a univocal definition for these compounds and a clear elucidation for distinguishing them from primary metabolites were not given. Traditionally plant chemicals are classified as primary or secondary constituents, depending on whether or not they have an essential role in plant metabolism and are universally present in all plants. For this reason, nucleic acids and related breakdown products, proteins and their precursors (amino acids), certain carbohydrates, carboxylic acids, and ubiquitous fatty acids are generally considered as products of primary metabolism.[16] Secondary metabolites, produced by pathways derived from primary metabolic routes, include all the other phytochemicals. However, the distinction between primary and secondary metabolites is not sharp. In particular, at present it is clear that not all primary metabolites occur in every plant species and some secondary metabolites, acting as cosubstrates or coenzymes, are involved in primary metabolism. For example, erucic acid is a fatty acid typical of the *Cruciferae* family with a restricted distribution in the plant kingdom. However, this secondary metabolite represents an important storage form of energy as a major acid of the *Cruciferae* seed, which is involved in primary metabolism during the seedling development.[17] Another often-used example is the nonprotein amino acid canavanine.[18] Canavanine is predominantly accumulated in many legume seeds and is toxic to a wide range of herbivores. During germination, the nitrogen released by canavanine is made available to the growing seedling.[18] Several plant hormones (gibberellic acid, auxins, ethylene, abscisic acid) and several structural compounds of plant cell wall (cinnamic acid and related polymers, lignin) are intermediate between primary and secondary metabolism.[19] Shikimic acid and squalene, which were initially considered as secondary metabolites, are important precursors in the formation of primary metabolites (amino acids and steroids, respectively). In addition, the term "secondary," associated with inessentiality, represents an unfortunate choice,[20] especially considering the well-documented

physiological and ecological role of the great proportion of secondary metabolites. Plant genomes are variously estimated to contain 20,000 to 60,000 genes and probably 15 to 25% of these genes encode enzymes for the secondary metabolism.[21] This evidence suggests that secondary metabolites are not less important to the plant than primary metabolites. If secondary metabolites are "waste products"[16] or "expressions of shunt and overflow metabolism,"[22] plants producing these compounds would have a considerable selective disadvantage with respect to the plants with a limited synthesis of secondary metabolites. In particular, biosynthesis, sequestration (several secondary metabolites are synthesized in the cytoplasm or in the cell organelles but are stored in the vacuole), transcription, and translation of genes encoding for the enzymes of secondary metabolisms and their cellular turnover are processes requiring substantial amounts of energy (ATP or reduction equivalents). Considering that the less-efficient organisms are eliminated in the process of natural selection, the relevant energetic cost associated with the production of secondary metabolites suggests a selective advantage for the plants synthesizing these chemicals. Otherwise, the tremendous diversity of secondary metabolites in the plant kingdom would be in contrast with the law of natural selection. In order to avoid the negative implications of the term "secondary," in the last three decades several authors have proposed new terminologies such as "byways of metabolism,"[23] "special metabolism,"[24] "natural products,"[25] "idiolytes,"[26,27] "allelochemicals,"[28] and "dispensable metabolites."[29,30] None of these definitions has found a widespread acceptance, and the term "secondary metabolism" is still the most employed, in particular for the classification of plant biochemicals. However, from a purely formal point of view, the distinction between primary and secondary metabolism is meaningless and has been abandoned.[30] Today, a secondary metabolite is defined as a compound whose biosynthesis is restricted to a selected plant group.[31] In addition, Hartmann[29] suggested to use "primary" or "secondary" in the sense of the function. Hence, erucic acid or canavanine, as carbon or nitrogen sources during seedling growth, are primary metabolites, and as toxic defense chemicals, secondary metabolites. The recently introduced term "metabolome" encompasses primary and secondary metabolism. In the metabolomic approach, any bias against certain classes of chemicals is avoided.[32]

2.1.3 The Main Pathways and Classification of Secondary Metabolism

As previously mentioned, plants are capable of synthesizing an overwhelming variety of low-molecular-weight organic compounds, defined as secondary metabolites, usually with unique and complex structures. Originally, secondary metabolites were thought to occur exclusively in higher plants. Several researches demonstrated the synthesis of these compounds by bacteria, lower plants, and fungi. The discovery of penicillin G, a potent antibiotic first isolated in 1929 by Alexander Fleming from the mold *Penicillium*

notatum, stimulated research on detecting antibiotics in microbial organisms.[33] Today, we know that secondary metabolites occur in all groups of living organisms.[20] Animals, besides sequestering phytochemicals from food plants, are also able to synthesize them *de novo* (e.g., the sesquiterpene cantharidine produced by the hemolymph of blister beetles). Despite the enormous diversity of secondary metabolites and the several thousands of enzymes involved in their synthesis, the number of corresponding basic biosynthetic pathways is restricted and distinct (Figure 2.2). Precursors usually derive from basic pathways of primary metabolism, such as glycolysis, fatty acid biosynthesis, Krebs cycle, and shikimate pathway. The products of secondary metabolism are grouped in the following categories.

2.1.3.1 Acetate-Malonate–Derived Compounds

This wide group includes fatty acids (jasmonic acid and related compounds), acetylenic compounds, waxes, and polyketides (or polyacetate compounds). The synthesis of fatty acids occurs in the chloroplast but involves mitochondria and the cytosol.[34] The synthesis of malonyl-CoA from acetyl-CoA, which occurs in the stroma, catalyzed by acetyl-CoA carboxylase (ACCase), is the first committed step of fatty acid biosynthesis.[34] The fact that radioactive-labeled acetate and malonate label the aforementioned compounds in the same manner as fatty acids strongly suggests their common origin. However, jasmonic acid and related compounds, acetylenic compounds, and plant waxes essentially derive from primary fatty acids. The main precursor of jasmonic acid and related compounds is linolenic acid, while oleic and linoleic acids are the main precursors for acetylenic compounds in plants and microorganisms. Plant waxes are complex mixtures of hydrocarbons, alcohols, aldehydes, ketones, esters, and acids, and combinations of these are deposited in a layer outside the epidermal cells.[35] Most components of the waxy layer, synthesized in the epidermal cells and exuded on surface, are derived from long-chain fatty acids (C_{28} to C_{34}). In contrast, polyketides are formed by the secondary metabolic routes, which are defined as "polyketide pathways." These pathways are similar to the primary fatty acid biosynthesis (acetate-malonate pathway); however, in contrast to fatty acid biosynthesis, after the condensation of acetate and malonate units, the carbonyl groups are not reduced, and the intermediates condense to produce aromatic ring systems, usually with phenolic substitutions.[36] In general, the polyketides found in fungi, bacteria, and lichens are synthesized by polyketide pathways, while polyketides of higher plants (including benzoquinones, naphthoquinones, and anthraquinones) commonly arise by the shikimic acid pathway and/or by oxidation of a number of secondary metabolites of varied biosynthetic origin.[37]

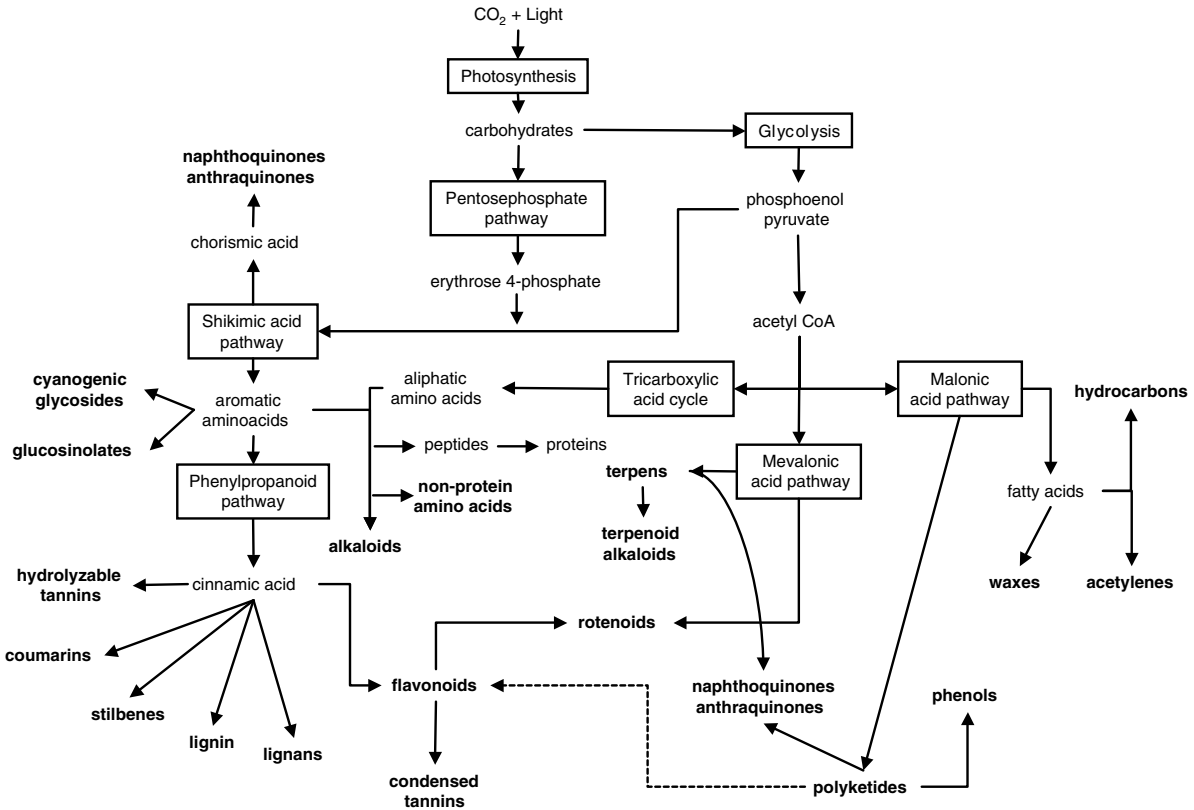


FIGURE 2.2
Plant primary and secondary metabolism.

2.1.3.2 Terpenes (Or Mevalonate Compounds)

These compounds represent the largest group of plant secondary metabolites (at least 15,000 chemical structures described in literature).[38] Terpenes are made up of units of five carbons: C_5 (hemiterpenes), C_{10} (monoterpenes), C_{15} (sesquiterpenes), C_{20} (diterpenes), C_{30} (triterpenes), and C_{40} (tetraterpenes or carotenoids). The common precursor of all terpenes is mevalonic acid, which represents the isoprene five carbon (C_5) units assembled to build up carbon skeletons of terpenes. The first step in the formation of mevalonic acid is the conversion of acetyl-CoA into acetoacetyl-CoA, catalyzed by acetoacetyl-CoA thiolase (AACT). Mevalonic acid is then converted to isopentenyl pyrophosphate (IPP), which is isomerized to dimethylallyl pyrophosphate (DMAPP). Lynen and coworkers[39] were the first to realize that DMAPP is involved in repetitive condensations with IPP, leading to the formations of the different classes of terpenes. Chloroplast seems to contain the entire terpenoid biosynthetic pathway and appears to be autonomous as regard to terpene biosynthesis.[40] Mono-, sesqui-, and diterpenes are biosynthesized by linear condensation of five carbon units, while triterpenes arise via dimerization of two farnesyl pyrophosphate (FPP) units (formed by the union of IPP and DMAPP) to produce the intermediate compound squalene. Squalene is then cyclized to produce the parent skeletons of many different kinds of triterpenes and phytosteroids, which undergo subsequent modifications.[41] Finally, the class of tetraterpenes includes the plastid terpenoids (carotenoids, chlorophylls, plastoquinones, tocopherols, and phylloquinones). The general features of carotenoid biosynthesis are similar in higher plants, algae, fungi, and bacteria. Phytoene, the first carotenoid in the pathway, is derived by dimerization of geranylgeranyl pyrophosphate (GGPP), followed by subsequent rearrangements.[42] The synthesis of GGPP is promoted by geranylgeranyl pyrophosphate synthase, which accepts as substrate IPP, DMAPP, or FPP.

2.1.3.3 Alkaloids

The exact number of these compounds is difficult to estimate, because many alkaloids listed in compendia are not naturally occurring. According to Verpoorte et al.,[43] almost 16,000 alkaloids have been reported from plant, animal, and marine sources. These compounds are widespread in the plant kingdom: approximately 20 to 30% of higher plants accumulate alkaloids. However, some botanical families exhibit a greater attitude to synthesize this class of secondary metabolites: Perhaps 60 to 70% of species of the *Solanaceae* and *Apocynaceae* contain alkaloids.[44] At present, no definition for this class of compounds is widely accepted. The most employed definition describes an alkaloid as a cyclic compound containing nitrogen in negative oxidation state that is of limited distribution among living organisms and able to have physiological activity in animals.[45] The classification of alkaloids is quite complex and was the subject of several publications.[46,47] Now the trend is to classify these secondary metabolites according to both the affinity of

chemical structure and the biosynthetic origin.[47] The alkaloids could be divided into three groups:

1. Alkaloids derived from amino acids, their derivatives (amines and polyamines), or precursors (e.g., anthranilic acid, which is an intermediate in the biosynthesis of tryptophan). The most common compounds involved in the synthesis of these alkaloids are phenylalanine, nicotinic acid, ornithine, lysine, arginine, anthranilic acid, tyrosine, and tryptophan.
2. Alkaloids derived from purine or pyrimidine bases (defined also as pseudo alkaloids).
3. Alkaloids of terpenoid origin.
4. Miscellaneous types of alkaloids.

The alkaloids derived from amino acids include the following subgroups:

1. *Phenylalanine derivatives*. These alkaloids (hordenine, ephedrine) are formed by simple and usually short series of reactions. Phenylalanine is decarboxylated forming the correspondent amine, which is subsequently modified giving rise to, in some instances, quite complicated products.
2. *Both phenylalanine and tyrosine derivatives*. In this subgroup, several types of alkaloids (colchicine and other tropolonic alkaloids), derived from both tyrosine and phenylalanine, are included.
3. *Ornithine and lysine derivatives*. The alkaloids derived from ornithine include pyrrolidine, tropane (cocaine), and pyrrolizidine alkaloids. Ornithine is decarboxylated and converted by amine oxidase to the corresponding aldehyde, which undergoes cyclization to a five-membered ring system. Pyrrolidine alkaloids present a single five-membered ring system. Tropane alkaloids are formed by bridging the five-membered ring with a three-carbon portion derived from acetate-malonate, while pyrrolizidine alkaloids show two fused five-membered rings with a nitrogen at one of the common positions. For pyridine alkaloids (nicotine and related compounds), both ornithine and nicotinic acid serve as efficient precursors. The alkaloids derived from lysine include piperidine, quinolizidine, and indolizidine alkaloids. Lysine is decarboxylated and converted by amine oxidase to the corresponding aldehyde, which undergoes cyclization to a six-membered ring system. Piperidine alkaloids consist of a single six-membered ring system, while quinolizidine alkaloids present two fused, six-membered rings with a nitrogen at one of the common positions. Indolizidine alkaloids are bicyclic compounds with a fused five- and six-membered ring system and are

intermediate to the pyrrolidine (two fused, five-membered rings) and quinolizidine alkaloids (two fused, six-membered rings).

4. *Anthranilic acid derivatives.* Anthranilic acid is an intermediate in the biosynthesis of tryptophan. A number of alkaloids (quinazoline, pyrrolquinazoline, quinolone, and acridone alkaloids) are biosynthesized from this primary metabolite.
5. *Tyrosine derivatives.* These alkaloids, characterized by an isoquinoline moiety in their structure, are among the most common in the plant kingdom. More than 8000 compounds of many structural types but with a similar structural feature are known.[43] The isoquinoline alkaloids (the alkaloids of the peyote, *Lophophora williamsii*, such as anhaladine, lophophorine, and anhalanine) are derived from tyrosine through the intermediacy of 3,4-dihydroxyphenylethylamine (dopamine). Benzylisoquinoline (BIQ) or tetrahydrobenzylisoquinoline alkaloids (laudanosine, papaverine) are formed by condensation of dopamine and 4-hydroxyphenylacetaldehyde. Several BIQ compounds are variedly modified in plant tissues, generating many different alkaloids (aporphine, proaporphine, morphinandienone, morphine, protoberberine, benzophenanthridine) having great medical importance (pain therapy, anticancer agents).

Several alkaloids derive from purine or pyrimidine bases. In general, purine bases appear to be much more common than pyrimidine bases in nature. For this reason, the large majority of the so-called "pseudo alkaloids" derive from the xanthine nucleus of purine bases. The most important of these are caffeine, theobromine, and theophylline. These alkaloids are the major constituents of plant-derived beverages used as stimulants by people throughout the world.

The alkaloids of terpenoid origin include indole, monoterpenoid, diterpenoid, and steroid alkaloids. Indole alkaloids are formed by the condensation of a carbonyl compound (the terpenoid secologanin) and an amine (the corresponding amine of tryptophan, tryptamine). Some authors classify these compounds as alkaloids derived from amino acids (tryptophan derivatives). This class of alkaloids includes several compounds of relevant medical importance (vincamine, strychnine and its relatives, olivacine, vincristine, vinblastine, quinine). A number of alkaloids are known where nitrogen is directly incorporated in monoterpenes, diterpenes, sesquiterpenes, and triterpenes (or steroids). In the major proportion of cases, the source of the nitrogen is unknown. Some representative compounds are the alkaloids of *Valeriana officinalis* (monoterpene alkaloids), aconitine and pseudoaconitine (diterpene alkaloids), and the *Solanum* alkaloids (steroid alkaloids).

Finally, in the miscellaneous class, all the alkaloids not classified in the previous groups are included such as pyrazine alkaloids, betalains, peptide alkaloids, imidazole alkaloids.

2.1.3.4 Amino Acid–Derived Compounds

This group includes nonprotein amino acids (NPAA), cyanogenic compounds, and glucosinolates. Approximately 25 to 30 amino acids are involved in primary metabolism: alanine, valine, leucine, isoleucine, proline, methionine, phenylalanine, tryptophan, cysteine, serine, threonine, asparagine, glycine, tyrosine, glutamine, aspartic acid, glutamic acid, lysine, arginine, histidine, trans-4-hydroxyproline, *N*-methyl-trans-4-hydroxyproline, ornithine, *O*-acetyl-serine, homoserine, and cystine.[48] These primary amino acids usually occur as components of peptides and proteins and are linked by peptide bonds or free usually in small amounts. In addition to these ubiquitous amino acids, as many as 700 amino acids that are not involved in primary metabolism have been reported.[35] About 300 of these are found in plants.[49] The main NPAAs (derived from lysine, homoserine, isoleucine, arginine, asparagine, and glutamic acid) include pipecolic acid, mimosine, canavanine, canaline, hypoglycin, and lathyrine.

The actual cyanogenic compounds have been isolated from more than 475 species of plants.[50] These compounds are glycosides of hydroxynitriles or cyanohydrins. The glycosides are usually accompanied by a β-glycosidase enzyme capable of producing the corresponding aglycone (cyanohydrin) and a sugar.[35] A hydroxynitrile lyase promotes the formation of hydrogen cyanide from the cyanohydrin. The substrate (cyanogenic compound) and the enzymes are stored in different parts of the cells in order to avoid the fortuitous release of the cyanide toxic for the cell itself.[51] The aglycone portion of cyanogenic compounds is derived from a relatively small number of precursors such as tyrosine, phenylalanine, valine, isoleucine, and leucine.

For many properties (in particular for the ecological role), the glucosinolates bear a resemblance to the cyanogenic glycosides, to which they are biosynthetically related. However, with respect to the cyanogenic glycosides, the distribution of glucosinolates is restricted to a small number of botanical families (in particular the *Cruciferae*). The aglycones of glucosinolates are derived from primary amino acids such as alanine, valine, leucine, isoleucine, phenylalanine, tyrosine, and tryptophan.[52] Special β-glucosidases (defined myrosinases or thioglucosidases) catalyze the release of isothiocyanates through a combination of thioglucoside-bond hydrolysis and spontaneous rearrangement of the aglycone.[35] These reactions usually occur after the disruption or damage of plant tissues. In order to avoid autotoxic effects, glucosinolates and β-glucosidases are probably compartmentalized at subcellular level.[53]

2.1.3.5 Compounds Derived from Shikimic Acid and Phenylpropanoid Pathways (Or Phenolic Compounds)

The shikimic acid pathway consists of three different parts: condensation of erythrose-4-phosphate and phosphoenol pyruvate and the subsequent cyclization and production of shikimic acid, alteration of shikimate-3-phosphate to chorismic acid, and the conversion of chorismate into other products

(Figure 2.3). Chorismic acid plays a fundamental role in the pathway, as this compound is the key intermediate and branching point of several plant secondary metabolites. The shikimic acid pathway is located in the chloroplast of higher plants.[54] From chorismic acid, several essential compounds are produced: the aromatic amino acids phenylalanine, tyrosine, and tryptophan; *p*-aminobenzoic acid and the folate group of coenzymes; and the isoprenoid quinones (Figure 2.3). Numerous secondary metabolites in plants and other organisms are formed from intermediates of shikimic acid. Benzoxazinones, responsible for allelopathic effects, are found in several cereal grain species belonging to the *Poaceae*. It has been hypothesized that the precursor of benzoxazinones is anthranilic acid (intermediate in the biosynthesis of tryptophan).[55] Quinic acid, derived from the intermediate 3-dehydroquinate of the synthesis of shikimate, occurs as caffeoyl acid ester (chlorogenic acid), which is produced as a response to wounding in many plants. Gallic acid, which is formed directly from shikimate, is the precursor of several hydrolyzable tannins.[56] However, a greater proportion of secondary metabolites are formed by end products of the shikimate pathway. In particular, the common precursor of these compounds (also defined as phenylpropanoids) is phenylalanine. Phenylpropanoid compounds encompass a wide range of structural classes and biological functions: simple phenylpropanoids, lignin, lignans, coumarins, stilbenes, xanthones, chalcones, and flavonoids (which include several chemical classes such as flavanones, flavones, flavonols, anthocyanins, biflavonoids, isoflavones, coumestans, isoflavans, and pterocarpans). All phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the action of phenylalanine ammonia lyase (PAL), the branch point enzyme between primary (shikimate pathway) and secondary metabolism (phenylpropanoid pathway).[57]

Since the totality of phytoestrogens are phenylpropanoids, particular attention will be devoted (see Section 2.2 and the following paragraphs) to the biosynthesis and ecological and physiological role of these compounds in plant species.

2.1.4 Ecological Role of Plant Secondary Metabolites

The ability to synthesize secondary compounds has been selected throughout the course of evolution in different plant lineages when such compounds addressed specific needs. For examples, floral scent volatiles and pigments have evolved to attract insect pollinators and thus enhance fertilization rates.[58,59] The ability to produce toxic chemicals has evolved to ward off pests (pathogens and herbivores, such as fungi, bacteria, insects, and vertebrates) and to suppress the growth of neighboring plants (allelopathic effects).[60,61] Chemicals found in fruits prevent spoilage and act as signals (color, aroma, and flavor) of the presence of potential rewards (sugars, vitamins, and amino acids) for animals that eat the fruit and help to disperse seeds. Other metabolites are useful to cellular functions that are unique to the plant when

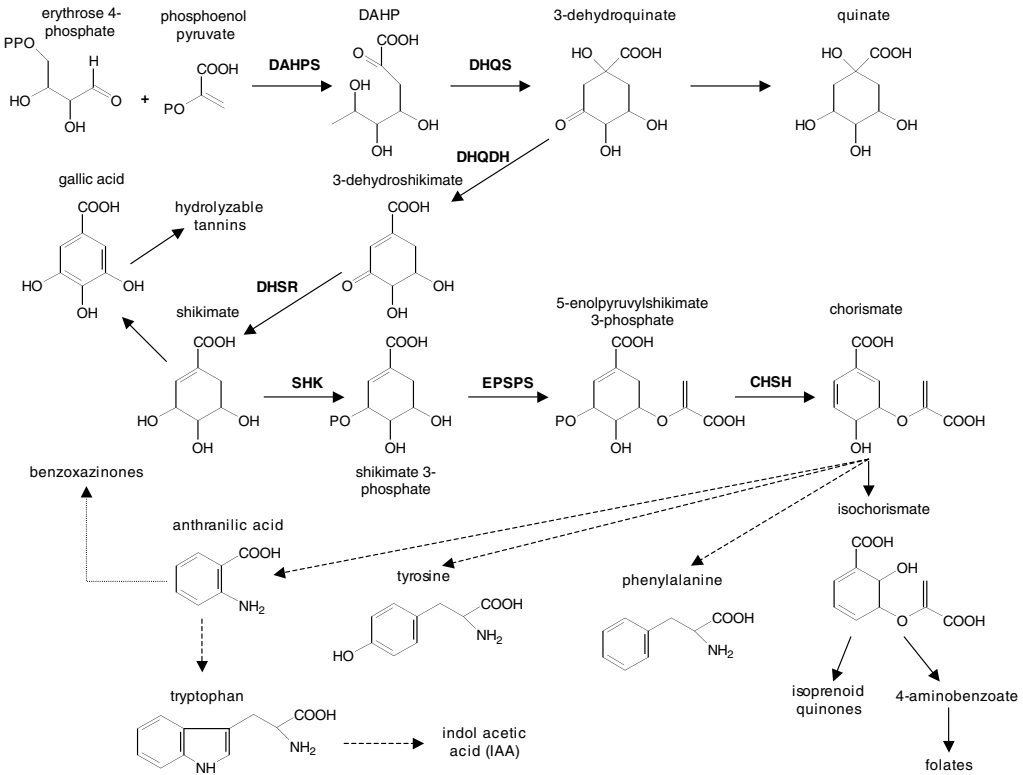


FIGURE 2.3
The shikimic acid pathway. DAHPS = 3-deoxy-*D*-arabino-heptulosonate 7-phosphate synthetase; DHQS = 3-dehydroquinate synthetase; DHQDH = 3-dehydroquinate dehydratase; DHSR = 3-dehydroshikimate reductase; SHK = shikimate kinase; EPSPS = 5-enolpyruvylshikimate 3-phosphate synthase; CHSH = chorismate synthetase.

they occur (e.g., secondary metabolites produced as resistant response to salt and/or drought stressing conditions). Vertebrates have developed sophisticated nervous and immune systems enabling them to detect and respond to danger, and they are capable of escaping from dangerous situations. By contrast, plants cannot take avoidance measures to escape from their attackers, and so they must stay and fight. The production of chemicals that deter or kill pests and pathogens represents means of self-protection.[62]

During the past decades, several studies have determined precise functions of secondary metabolites for the fitness of plants:

- Defense against herbivores
- Defense against fungi and bacteria
- Defense against viruses
- Defense against other plants competing for light, water, and nutrients
- Signal compound to attract pollinating and seed-dispersing animals
- Signal compound between plant and symbiotic microorganisms
- Protection against UV-light or other physical stress

Collectively, plants are capable of synthesizing a diverse array of secondary metabolites.[63] These may be produced constitutively (antimicrobial compounds or phytoanticipins) or in response to biotic stresses (phytoalexins).[64,65] In addition to their direct effects on pests and pathogens, some secondary metabolites may also be important in defense-related signal transduction.[66] The ability of plants to carry out *in vivo* combinatorial chemistry by mixing, matching, and evolving the gene products required for the secondary metabolite biosynthetic pathways is likely to have been the key for their survival and for the generation of diversity at the organism level.[62] At present, it is commonly accepted that plant secondary compounds are mainly synthesized by plants in order to interact with a wide range of animals (mammals, insects, nematodes), plants (higher and lower plants), fungi (beneficial and pathogenical), and bacteria. According to their interactive roles, secondary compounds are classified as allomones (compounds protecting the plants from herbivores, pathogens, or adverse environmental conditions), synomones (compounds involved in pollination and seed and fruit dispersal), and kairomones (compounds used by the interacting organism to locate the plant host).[35] A clear example of the interactive role of plant secondary compounds is given by benzoxazinones. DIMBOA (2,4-dihydroxy 7-methoxy 1,4-benzoaxazin 3-one) and its precursor indol are synthesized in maize tissues as a response after attacks of *Spodoptora exigua* larvae (Figure 2.4). In fact, voliticin (*N*-[17-hydroxylinenoyl]-*L*-glutamine), an elicitor produced by larvae and present in their saliva, induces in maize an increase of indol and DIMBOA synthesis. DIMBOA is not only a phytotoxic substance, but also exerts toxic effects on insect larvae.

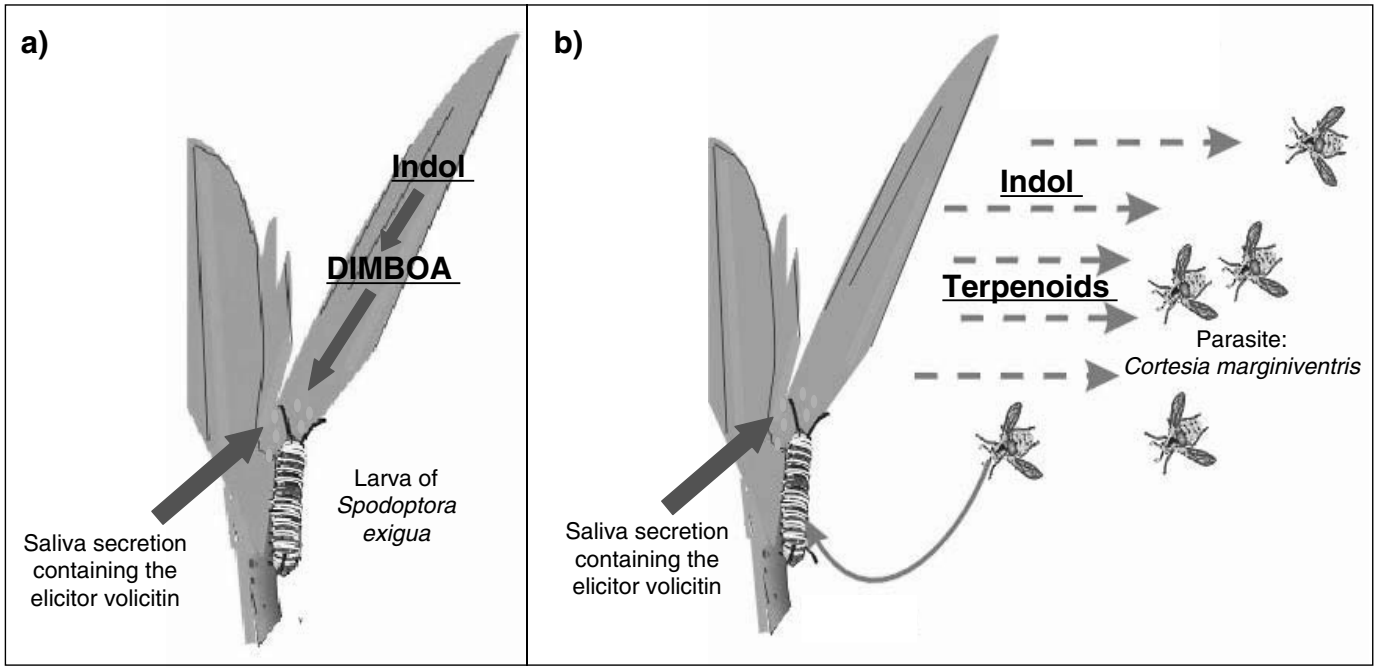


FIGURE 2.4
Tritrophic interaction in maize. The presence of feeding beet army worm caterpillars is recognized by the plant through sensing a substance in the spit of the caterpillar. This substance, identified as volicitin, stimulates the synthesis of indol (a) which is the precursor of DIMBOA, exerting toxic effects on larvae. Volicitin induces the release of volatile compounds (indole, terpenoids), which are attractive to specific parasites (b) using the larvae for oviposition. (Reproduced from Gierl, A. and Frey, M., *Planta*, 213, 493–498, 2001. With permission.)

The defense mechanism is completed by release of indol and terpenes (synthesized in response to voliticin) that specifically attract *Cortesia marginiventris*, a parasite of larvae of *S. exigua*.[197] Another example is given by the isoflavones of legume plants. In particular, isoflavones are almost exclusively present in an appreciable amount in green tissues of legume species such as red clover, alfalfa, soybean, common bean, chickpea, fava bean, and lentil. Today it is common opinion that the ecological meaning of the high content of isoflavones in *Leguminosae* plants is the symbiosis between legume plants and *Rhizobium* or *Bradirhizobium* bacteria. Such a symbiotic relationship requires a delicate balance between partners (bacterium-plant), involving a complex exchange of chemical messengers (including isoflavones) able to ensure correct activation and maintenance in time of the whole process (nodulation, activation of Nod and Nif genes, bacterioid formation, nitrogenase activity)[67] (Figure 2.5).

2.1.5 Regulation of Secondary Metabolism

Many phenylpropanoid compounds are induced in plants by abiotic and biotic stresses and are classified as phytoalexins. They include pterocarpans

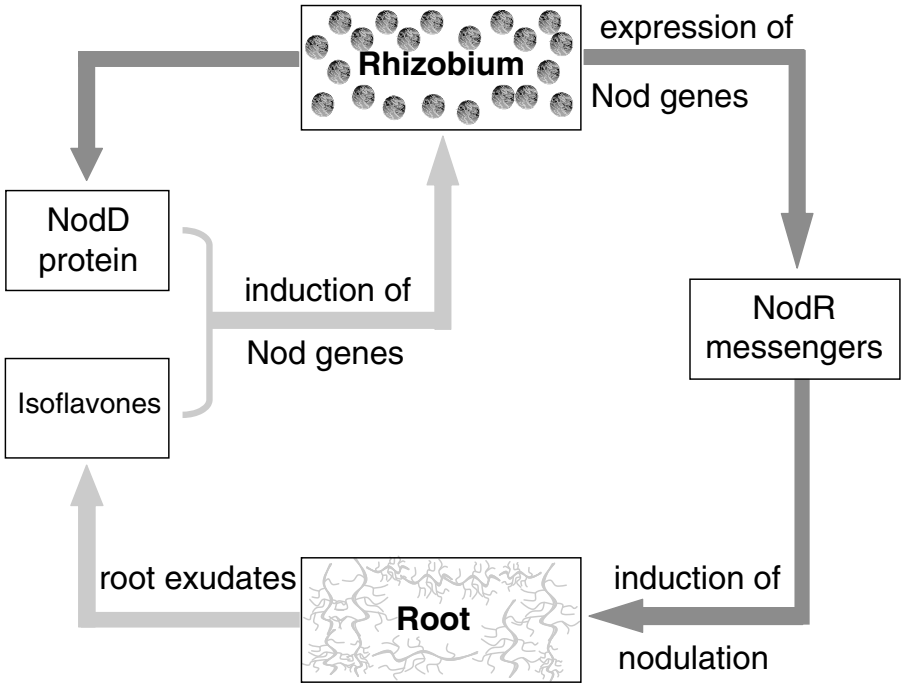


FIGURE 2.5
Molecular messengers involved in the Rhizobium–legume symbiosis.

(glyceollin), isoflavans, prenylated isoflavonoids (kievitone), stilbenes, coumarins, flavonols, and aurones.[68,69] These compounds are phytoalexins, substances involved in plant defense mechanisms against pathogens, and their levels increase greatly around the site of infection.[70–72] In some plant species (tobacco, cucumber, and *Arabidopsis*) after exposure to UV or after infection with pathogens, an increase of salicylic acid concentration in plant tissues was observed.[73,74] According to some authors,[75] salicylic acid is not directly toxic to microorganisms, but this compound is a part of a signaling process that results in systemic acquired resistance, or SAR. SAR is the phenomenon by which pathogen-induced infection causes in the plant a systemic resistance against subsequent infections from the same pathogens or other virulent pathogens by a mechanism not yet clarified. Many experimental papers, however, showed that in the first phases of infection a relevant biosynthetic activity of different secondary metabolites (phytoalexins) in plant tissues takes place.[76] It is thought that phytoalexins subsequently activate specific genes codifying for different substances involved in defense mechanism (hydrolytic enzymes, PR proteins).[67] Today, common opinion is that several abiotic stresses also act as elicitors, inducing defense mechanisms in plants. For example, it has been shown that abiotic stresses (either single or in combination) may induce an increase of the chlorogenic acids in green tissues of tobacco (Figure 2.6).[77] Some molecular studies showed that the transcription of enzymes involved in the synthesis of these compounds is spatially and temporarily coordinated and that enzyme transcripts are induced by either compatible or noncompatible interactions.[78] Such experimental proofs confirm that the expression of phytoalexins is part of a coordinated response framework against abiotic and biotic stresses. The induction of secondary metabolites under stressing condition is the result of increased transcription of genes encoding the corresponding biosynthetic enzymes. Increased transcription rates have been observed at the onset of the phytoalexin response in elicitor-treated cells of parsley.[79] The kinetics of these transcriptional changes have implication for the signal transduction mechanisms involved: the transcription of PAL and chalcone synthase (CHS) genes is rapid and coordinated, while the transcription of other enzymes is delayed, indicating the involvement of multiple signals for the activation of phytoalexin synthesis.[57] This picture is confirmed by the fact that common sequence motifs in the promoters of CHS and PAL genes exist from many plant sources.[80] The coordinate transcriptional control of biosynthetic genes emerges as the major mechanism dictating the final levels of secondary metabolites in plant cells.[81] The transcription factors are proteins binding to specific sequences of DNA and modulating the rate of mRNA synthesis. These proteins are regulated by external signals such as biotic or abiotic elicitors or internal signals such as jasmonic acid.[82] Jasmonic acid and its volatile methyl ester, methyljasmonate, are signaling molecules in biotic and abiotic stresses.[83,84] A large body of evidence indicates the involvement of these compounds in inducing the gene expression leading to synthesis of many proteins and secondary metabolites, some of which are probably associated with the defense

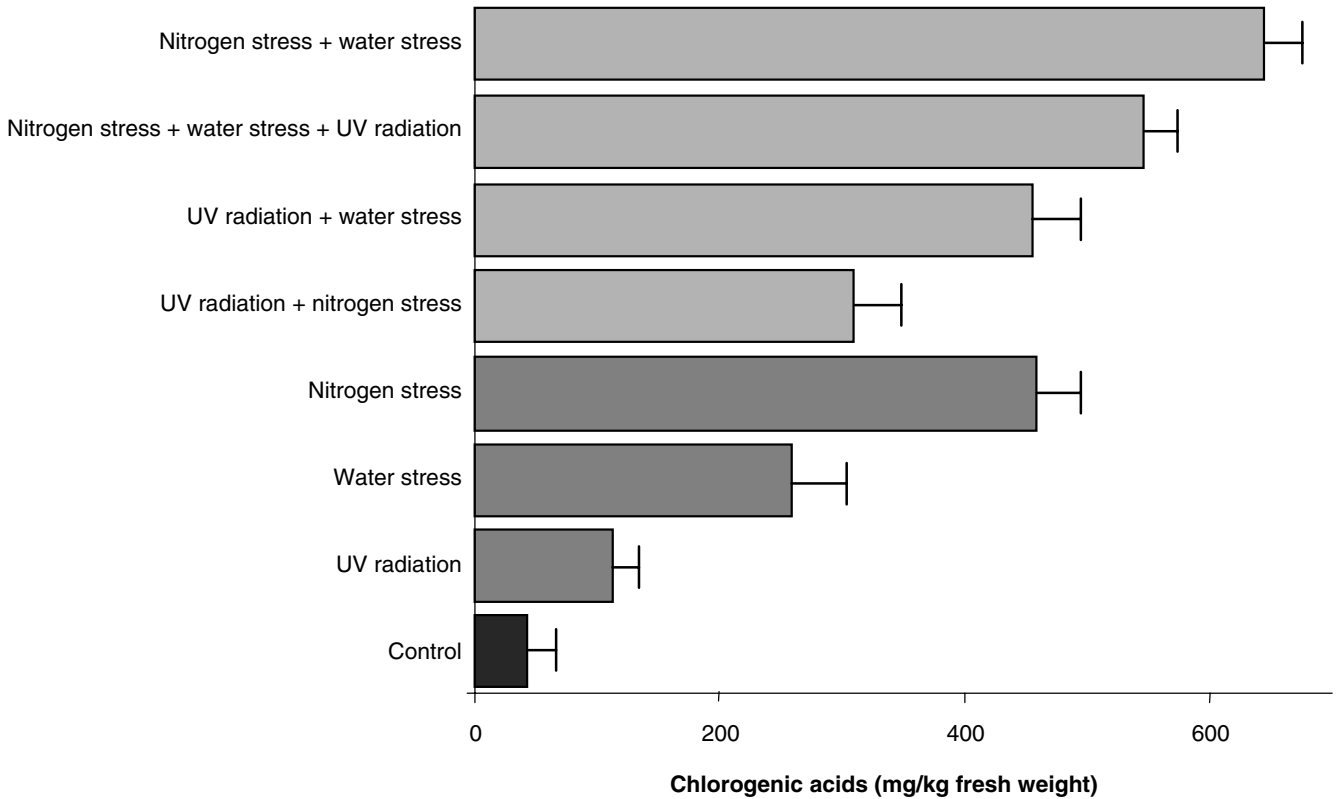


FIGURE 2.6
The "elicitor" effect of different abiotic stresses on accumulation of chlorogenic acids in leaves of tobacco. (Modified from Rizhsky, L., Liang, H., and Mittler, R., *Plant Physiol.,* 130, 1143–1151, 2002. With permission.)

responses of plants.[75] Jasmonates are expected to be the chemical signal compounds in the process of elicitation leading to the *de novo* gene transcription and finally the biosynthesis of natural products in cultured plant cells.[85] However, in many cases jasmonates alone are insufficient to increase secondary metabolites, suggesting that the enhancement of secondary metabolism is mediated by a pool of different substances (including not only jasmonates and salycilic acid but also calcium, polyamines, nitric oxide, ethylene).[75]

Transcription factors have been isolated and characterized for the metabolic pathway leading to flavonoid biosynthesis.[81] In particular the tissue-specific regulation of structural genes involved in anthocyanin biosynthesis (anthocyanins represent the end products of flavonoid pathway) is directly controlled by a combination of two distinct transcription factor family proteins with homology to the protein encoded by the vertebrate proto-oncogene c-MYB and the vertebrate basic-Helix-Loop-Helix (bHLH) protein encoded by the proto-oncogene c-MYC.[81] MYB and bHLH proteins have been mainly studied in *Petunia*, snapdragon, maize, and *Arabidopsis* as regulators of anthocyanin and seed coat tannin biosynthesis.[81] In maize kernel the majority of the structural genes encoding enzymes committed to anthocyanin synthesis are probably coordinated by the bHLH protein-encoding gene R and the MYB gene C1.[86] The expression of P, a MYB-type transcriptional regulator, induces in maize cells the coordinate expression of a subset of biosynthesis genes leading to accumulation of flavonoids different from those regulated by C1/R.[87] On the basis of recent studies, the picture that emerges is that various branches of phenylpropanoid metabolism are regulated by an interplay between branch-specific activating and repressing MYB transcription factors, some of which depend on specific bHLH protein partners.[81]

In conclusion, in the plant cell a wide array of external stimuli are capable of triggering changes that lead to a cascade of reactions, ultimately resulting in the formation and accumulation of secondary metabolites that help the plant to overcome the stress factors.[75] Several biotic and abiotic elicitors can act as external stimuli (Figure 2.7). The stimuli are perceived by receptors, which then result in the activation of secondary messengers (such as jasmonates, salicylic acid, calcium, polyamines, nitric oxide, and ethylene). These messengers then transmit the signals into the cell through the signal transduction pathways leading to gene expression (transcription factor, enzymes) and biochemical changes (accumulation of certain secondary metabolites). Considering that elicitation is probably the most effective approach for increasing yields of plant secondary metabolites, the understanding of the mechanisms involved in the regulation of secondary metabolism is of paramount importance in order to define for different plant species the optimal culture conditions ensuring the high energetic flux into secondary metabolic pathways. In recent decades, our knowledge on this topic has greatly improved, but a further effort to make this knowledge applicable to productive processes (plant cells cultures in large-scale

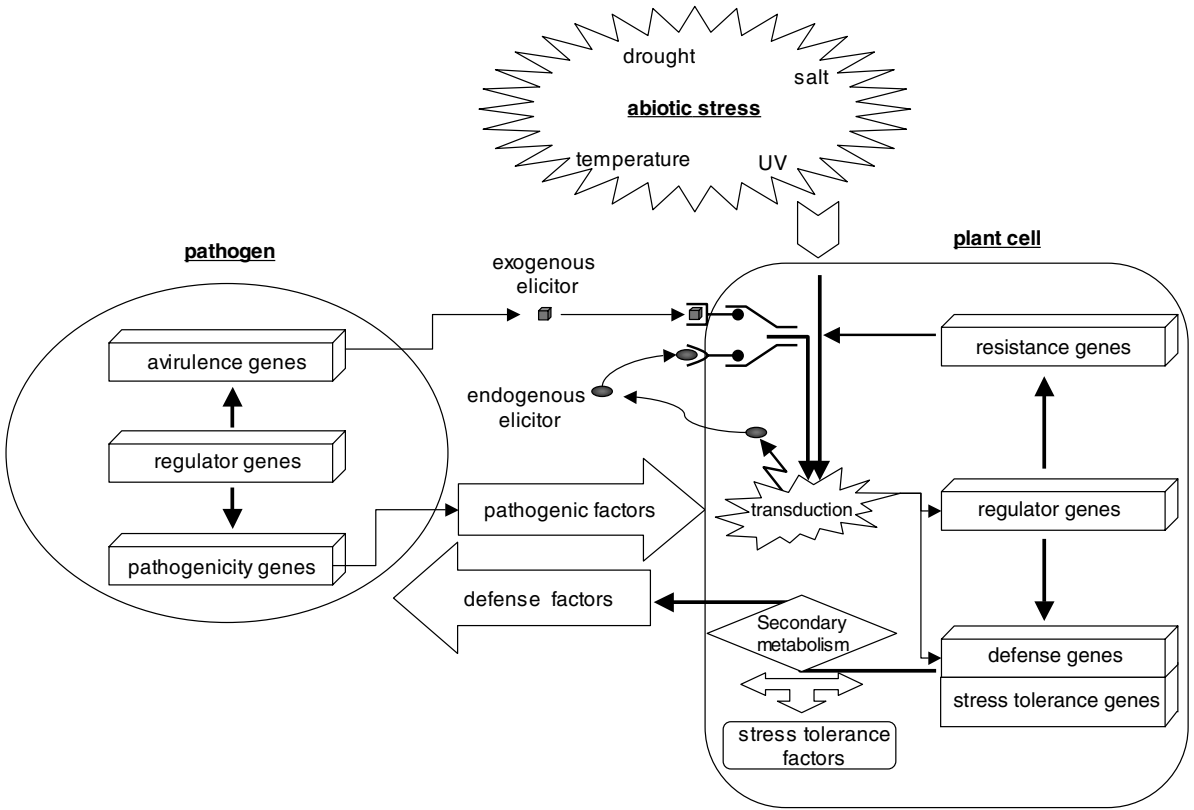


FIGURE 2.7
General scheme of plant–pathogen and plant–abiotic stress relationships.

bioreactors, cultivation of crops in open fields or in controlled environments) will be necessary.

2.1.6 Accumulation and Storage of Secondary Metabolites

The biosynthesis of secondary metabolites exhibits a remarkable complexity.[63] The enzymes are specific for each pathway and highly regulated in terms of compartmentation in space and time. Although all cells of a plant share the genes for the synthesis of certain secondary metabolites, only a limited number expresses them and produces the enzyme needed to synthesize the compound.[35] Organs and tissues important for survival and multiplication, such as epidermal and bark tissues, flowers, fruits and seeds, have distinctive profiles of secondary metabolites, and secondary compounds are abundant in them. However, even if a particular secondary metabolite is found in an organ or tissue, this does not necessarily mean that the compound was formed there.[35] Many secondary metabolites are synthesized elsewhere and transported to the cells, which form an early line of defense for the plant (e.g., epidermal cells).[35] Lupine alkaloids are accumulated in epidermal cells but are synthesized in mesophyll cells and transported to the epidermis via phloem. Usually the distribution of secondary metabolites is different not only at whole plant level but also within single cells. The functional explanation of cell compartmentation is to be found in the fact that allelochemicals and related biosynthetic intermediates are extremely reactive compounds and potentially phytotoxic for the cell. For example, alkaloids can interfere with activities of many intracellular enzymes, while terpenes and quinones can modify the integrity of cell membranes. Hydrophilic compounds are usually stored in the vacuole against a concentration gradient (cyanogenic glycosides, hydrosoluble flavonoids, nonproteic amino acids, alkaloids). In contrast, hydrophobic secondary compounds (terpenes, terpenoids, quinones) are usually stored in extracellular structures protected by solid barriers such as cuticole and trycomes.[35] Flavonoids, in particular the hydrophobic aglycone forms, are toxic endogenous chemicals for the cell due to their high chemical reactivity. Therefore, they need to be removed from the cytoplasm immediately after their synthesis. This can be done either by sequestration in the central vacuole, as for anthocyanins, phytoalexins, and flavonol glycosides, or by excretion into the cell wall, as described for polymethylated flavonol glucosides.[88–92] Another example of cell compartmentation is the biosynthesis of benzoxazinones, a class of hydroxamic acid derivatives playing in grain cereal a relevant role in resistance toward insect pests, fungal, and bacterial diseases, and in allelopathic response. Indol, the precursor of benzoxazinones, is synthesized in the chloroplast (Figure 2.8). Indol is the substrate for cytochrome-P450 enzymes BX2-5, localized in the endoplasmatic reticulum. The benzoxazinoids (such as DIBOA or 2,4-dihydroxy-1,4-benzoxazin-3-one) are readily glucosylated by cytosolic UDP-glucosyltransferases and stored in the vacuole. The specific glucosidase is found

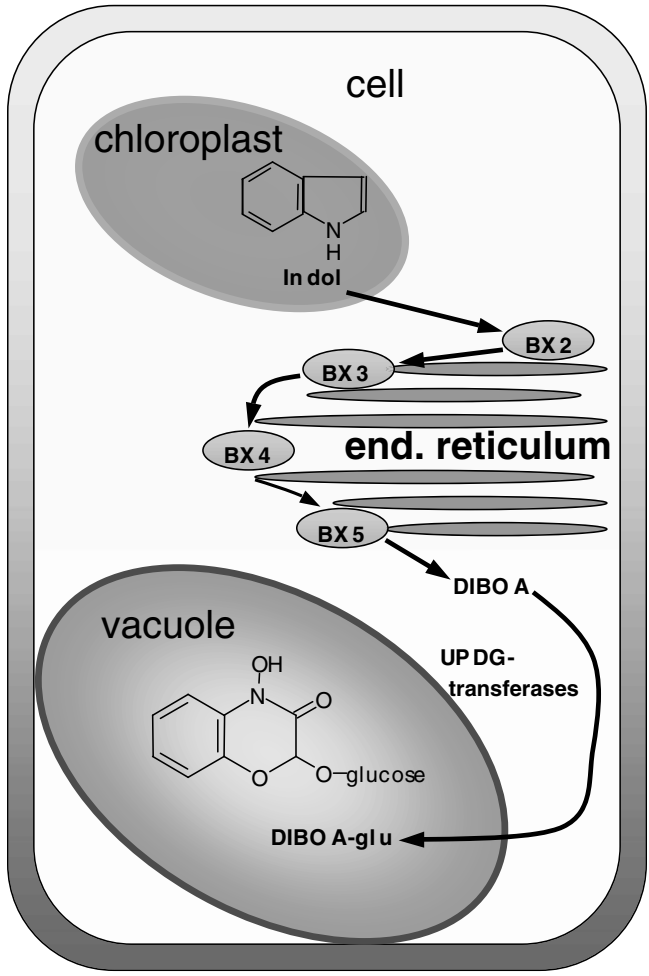


FIGURE 2.8
Cellular compartmentation of DIBOA biosynthesis in maize. (Reproduced from Gierl, A. and Frey, M., *Planta*, 213, 493–498, 2001. With permission.)

in the chloroplast. When the structural integrity of the cell is destroyed, the glucosidase and the glucosides encounter each other and the toxic aglycone is produced. High levels of DIBOA are effective in the control of the European corn borer, a main pest in corn agriculture.[197] Vacuoles offer a larger storage space than cell wall, which is important for flavonoids to reach concentrations high enough to function in the protection against predators and pathogens or as UV light sunscreens or attractants.[93]

Secondary metabolites rarely occur in plants in the free state: they are practically present in conjugated forms and the relative infrequency in plants of simple phenolic is probably due to their significant phytotoxicity. For flavonoids several different conjugated forms are known, especially

glycosides. By increasing the water solubility of secondary metabolites, the glycosylation permits the storage of hydrophobic allelochemicals in the vacuole. In addition, glycosylation of phenolic hydroxyl groups has a functional role in the case of flavonoids involved in plant pigmentation. The variation in the position of sugar attachment on flavonoid ring produces significant shifts in color. In leaves glycosylation of secondary metabolites could increase the deterrent effect on insects. The sugar substitution seems to exert different effects depending on the secondary metabolite. For example, in pea plants, kaempferol 3-glucoside and quercetin-3-glucoside together with their *p*-coumaric derivatives are present. These compounds are involved in growth response of pea, while quercetin acylated 3-glycoside seems to be involved in tendril coiling. *O*-methylation is another important process masking the reactivity of phenolic groups and increasing their solubility and volatility. *O*-methylation of simple phenols makes the substances volatile in a way that they are readily detected by insects and play a role in attracting or deterring insects.

2.2 Biosynthesis and Physiological Activities of Flavonoids, Stilbenes, Lignans, and Lignins

2.2.1 Flavonoids

2.2.1.1 Generalities

Flavonoids, in the broadest sense (including anthocyanins, isoflavonoids proanthocyanidins, catechins, and condensed tannins), are widespread compounds in the plant kingdom with the exception of algae and *Anthocerotales*. More than 4000 structures have been reported and many of them are glycosides of a relatively small number of flavonoid aglycones, which are often accumulated in the vacuoles of the plant cells. These compounds frequently serve as pigments in plants and are involved in many biological interactions.[94] Their basic chemical structure is based on a C_{15} skeleton and includes a chroman ring bearing an aromatic ring in position 2, 3, or 4 (Figure 2.9).[95]

Approximately 200 flavones and 300 flavonols were isolated from plants,[96,97] but only eight (Figure 2.10) are widely distributed in the plant kingdom: kaempferol, quercetin, luteolin, myricetin, apigenin, pelargonidin, delphinidin, and cyanidin. In the plants, the aglycones of these eight compounds can occur in one or some combinations. All of them have the same hydroxylation pattern of the A ring and mainly differ in the oxidation level of the central pyran ring and in the number of hydroxyl groups in the B ring. Flavonoids are divided according to the oxidization level of the C (central) ring in 14 classes, the most common being anthocyanidins (pelargonidin, delphinidin, cyanidin), flavones (apigenin, luteolin), and flavonols (kaempferol, quercetin, myricetin), most of them strongly pigmented and others colorless. Regardless

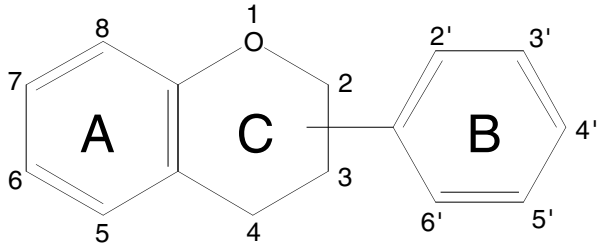


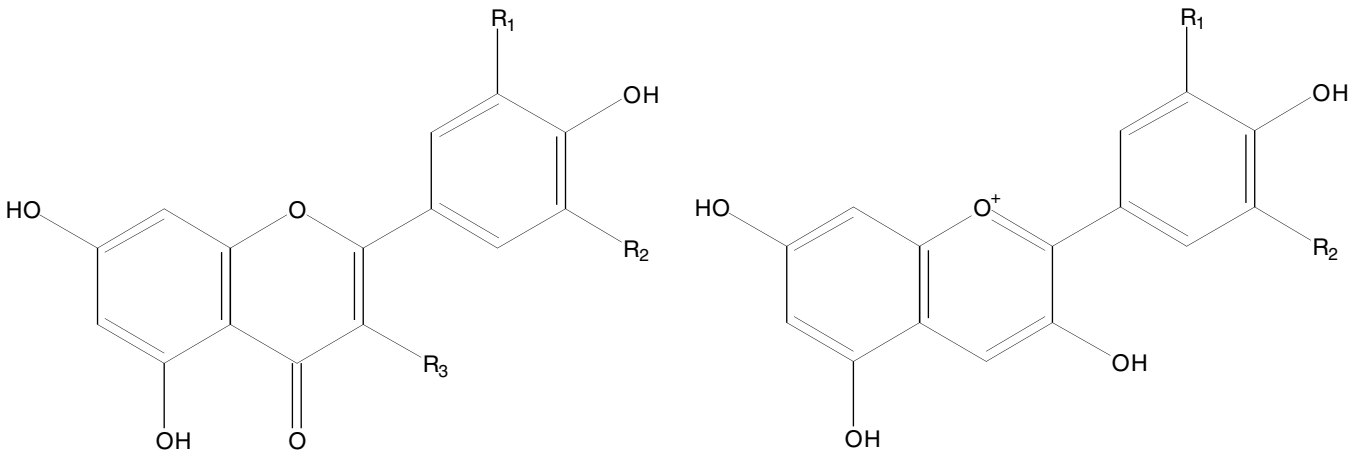
FIGURE 2.9
The basic chemical structure of flavonoids (AC = chromane ring, B = aromatic ring).

of the predominance of glycosidic or other "bound" forms of flavonoids in plants cells, the aglycone forms of flavonoids are often present and occasionally represent a sizeable portion of the total flavonoid compounds in or on the plant. Flavonoid aglycones are frequently methylated or esterified, forming a lipophilic mixture. Some authors evidenced that these compounds are predominantly produced by epidermal glandular trichomes.[98,99] Flavonoids are found in virtually all plants and in many types of biological interactions.[100,101] Plant flavonoid content can be influenced by different factors, such as light, temperature, mineral nutrition, pathogens, mechanical damage, and plant growth regulators. They could act as antioxidants, enzyme inhibitors, pigments for light absorbance, visual attractants for pollination, light screen, promoters of inhibitors of plant growth, chemical signals in nodulation gene induction, and phytoalexins.[100]

2.2.1.2 Biosynthesis

The enzymes of the flavonoid biosynthesis are compartmentalized by membranes surrounding organelles and by those separating cytoplasmic region into different microcompartments. According to Hrazdina and Ibrahim,[102,103] the association of biosynthetic enzymes as loose aggregates and the interaction between constituent enzymes permit the direct transfer of substrates from one enzyme to another and the channelling of intermediates. As a consequence, final products are accumulated into the different sites: hydrophilic derivatives usually compartimentalized in the vacuole and lipophilic compounds in epidermal glandular cells or exuded from roots.

The greater proportion of flavonoids are synthesized from the general phenylpropanoid pathway, and phenylalanine is the common precursor of most flavonoids (Figure 2.11). Phenylalanine, formed in the shikimate pathway, is converted to cinnamic acid by the action of *L*-phenylalanine-ammonia lyase (PAL), a ubiquitous enzyme in the plant kingdom. PAL generally occurs as a tetrameric structure.[104] Several multiple isoforms of this enzyme, differing for their kinetic properties, have been found in angiosperms.[105] The hydroxylation of cinnamic acid to *p*-coumaric acid is mediated by cinnamic acid 4-hydroxylase (CA4H), a mixed-function oxidase first isolated in pea,[35] which requires molecular oxygen, NADPH, and mercaptoethanol.



Kaempferol	$R_1 = H$;	$R_2 = H$;	$R_3 = OH$
Quercetin	$R_1 = OH$;	$R_2 = H$;	$R_3 = OH$
Myricetin	$R_1 = OH$;	$R_2 = OH$;	$R_3 = OH$
Apigenin	$R_1 = H$;	$R_2 = H$;	$R_3 = H$
Luteolin	$R_1 = OH$;	$R_2 = H$;	$R_3 = H$

Pelargonidin	$R_1 = H$;	$R_2 = H$
Delphinidin	$R_1 = OH$	$R_2 = OH$
Cyanidin	$R_1 = OH$;	$R_2 = H$

FIGURE 2.10
The most common flavonoids in the plant kingdom: flavonols (kaempferol, quercetin, myricetin), flavones (apigenin, luteolin), and anthocyanins (pelargonidin, delphinidin, cyanidin).

P-coumaric acid is the precursor of several simple phenylpropanoids (with the basic C_6-C_3 carbon skeleton of phenylalanine) such as caffeic acid, ferulic acid, 5-hydroxyferulic acid, and sinapic acid (Figure 2.11). Salycilic, benzoic, and *p*-hydroxybenzoic acids, characterized by a C_6-C_1 structure, originate from cinnamate and *p*-coumarate.[106] Cinnamic acid, *p*-coumaric acid, and the aforementioned related acids are usually present in plant cells as esters. In plant tissues, several methyltransferases can convert hydroxycinnamates to methylethers.[107] Cinnamic acid and its derivatives (in particular their ethers and their esters) represent the principal constituents of the phenylpropanoid pool in plants. The free acids are usually conjugated to sugars, cell wall carbohydrates, or organic acids.[57] Once formed, these phenolic acids play a central role in the synthesis of lignin, lignans, flavonoids, and a wide range of other secondary phenolic constituents (for example, the umbelliferone, derived from *p*-coumaric acid, is the mother compound for various sets of substituted simple coumarins as well as for furano- and pyranocoumarins) (Figure 2.11). In addition, the main final products of the general phenylpropanoid pathway (i.e., the hydroxycinnamic acids such as *p*-coumaric, caffeic, ferulic, hydroxyferulic, and sinapic acid) are of paramount ecological importance. The ecological functions of these compounds and related conjugated forms include the protection of plant tissues against DNA-damaging ultraviolet light, acylation of anthocyanins and protection of these compounds against water degradation, and defense against pathogen attacks.[108] As regards the latter topic, now it is commonly accepted that the phenylpropanoid pathway plays a basic role in the defense reactions of plants upon microbial infections.[109] It is known that in many cases phenylpropanoids are the direct precursors of several phytoalexins. In addition, hydroxycinnamates, in particular *p*-coumarate and ferulate, and related conjugates are cell-wall incorporated after pathogen induction. The incorporation of these compounds increases the rigidity and decreases the digestibility of the cell wall by pathogenic cell-wall-degrading enzymes.[110] The importance of phenylpropanoid-related defense has been demonstrated by studies carried out with enzyme inhibitors. For example, potato leaves treated with the PAL inhibitor, 2-aminooxy-3-phenylpropionate, before the infection with *Phytophthora infestans* were unable to resist this pathogen.[111]

Cinnamic acid, *p*-coumaric acid, and related acids may be converted to CoA esters by means of a CoA ligase. In particular, *p*-coumaric acid is converted to 4-coumaroyl-CoA by *p*-coumarate CoA ligase (4CL) (Figure 2.11). Some grasses (*Poaceae*) can derive *p*-coumaric acid from tyrosine through a reaction catalyzed by tyrosine ammonia lyase (TAL). It is still unclear if TAL is a distinct enzyme with respect to PAL.[112] Although TAL activity is commonly observed in the grasses, it may result from PAL enzyme with nonstringent substrate specificity. The 4-coumaroyl-CoA and three molecules of malonyl-CoA are condensed under release of three CO_2 molecules by the key enzyme of flavonoid biosynthesis, the chalcone synthase (naringenin-chalcone synthase, CHS), forming the first C_{15}-condensation product of the flavonoid pathway. This enzyme, isolated from cell cultures of several

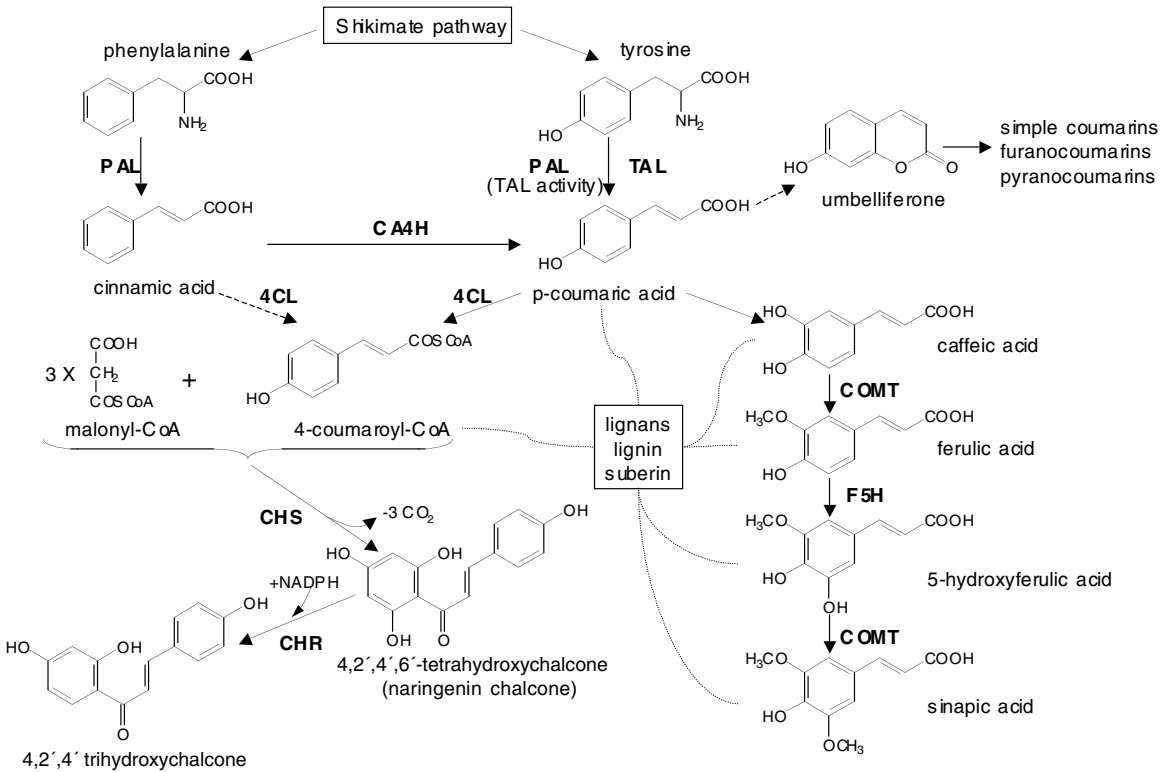


FIGURE 2.11
The general phenylpropanoid pathway: from phenylalanine and/or tyrosine to chalcones. PAL = phenylalanine ammonia lyase; TAL = tyrosine ammonia lyase; CA4H = cinnamic acid 4-hydroxylase; 4CL = 4-coumarate: coenzyme A ligase; CHS = chalcone synthase; CHR = chalcone reductase; COMT = caffeic acid methyltransferase; F5H = ferulic acid 5-hydroxylase.

plant species such as bean (*Phaseolus vulgaris*), parsley (*Petroselinum crispum*), and carnation flowers (*Dianthus caryophyllum*), has a molecular weight of 42,000 and no cofactors.[113] It has been one of the first enzymes characterized at the molecular level.[114] CHS is a cytosolic enzyme probably associated with membranes of endoplasmatic reticulum or the vacuole.[115] Schroder[116] hypothesized that CHS evolution followed an independent path of evolution for a long time. The ancestors of CHS are not known, but the presence of sequence homology in 20 to 30% of the structure in bacteria suggests that the basic protein structure may have existed long before plants adapted it for a key role in flavonoid biosynthesis. Since the reaction catalyzed by CHS is similar to the condensation reaction of fatty acids, a common ancestor for CHS and enzymes involved in fatty acid biosynthesis has been proposed.[117] In legumes, which possess chalcone reductase (CHR) as well as CHS, a trihydroxychalcone is formed by deoxydrilation of naringenin chalcone (Figure 2.11).[118] In several species (such as pine, grapevine, peanut) the condensation of 4-coumaroyl-CoA or cinnamoyl-CoA with three malonyl-CoA molecules can also give rise to stilbenes by the action of stilbene synthase (SS) (see Section 2.2.2).

The first C_{15}-condensation product of the flavonoid pathway (mostly naringenin chalcone or 4,2′,4′,6′-tetrahydroxychalcone) is the key intermediate in the formation of the major groups of flavonoids, isoflavonoids, aurones, anthocyanins, and condensed tannins. In particular, naringenin chalcone and related chalcones represent the direct precursors of both aurones and flavanones (Figure 2.12). The biosynthesis of aurones, usually found as yellow pigments in the flowers of the members of few botanical families (mainly *Asteraceae* and *Scrophulariaceae*), is still unclear at the enzymatic level.[119] In contrast, the key enzyme involved in the synthesis of flavanones is well characterized. The naringenin chalcone has two aromatic rings, A and B, linked by a C_3-bridge (Figure 2.12). In all other flavonoid classes, the chalcone C_3-bridge forms the heterocyclic C ring (Figure 2.9), which with different degrees of oxidization and substitution originates the different flavonoid classes. Although under appropriate conditions the closure of the chalcone C_3-bridge can occur spontaneously, this reaction is usually catalyzed in plant tissues by chalcone isomerase (CHI), a monomeric enzyme with a molecular mass of 24 to 29 kDa[63] (Figure 2.12). This enzyme accepts only aglycone chalcones (6′-hydroxychalcones or 6′-deoxychalcones); consequently chalcone glycosides cannot be transformed by CHI. The homology of CHI from different plant sources is approximately equal to 40% of amino acids residues.[120] The flavanones, formed by the activity of CHI, may undergo different chemical processes. The introduction of a double bond between C_2 and C_3 in the flavanone heterocycle, catalyzed by flavone synthase (FNS), leads to the formation of flavones (Figure 2.12). Two different types of FNS have been characterized. The FNS I is a 2-oxoglutarate-dependent dioxygenase: the enzyme is soluble and probably has a restricted distribution (in particular, members of the *Apiaceae*).[121] The FNS II, firstly isolated from microsomial fraction of *Antirrhinum majus*, is a cytochrome P450-dependent

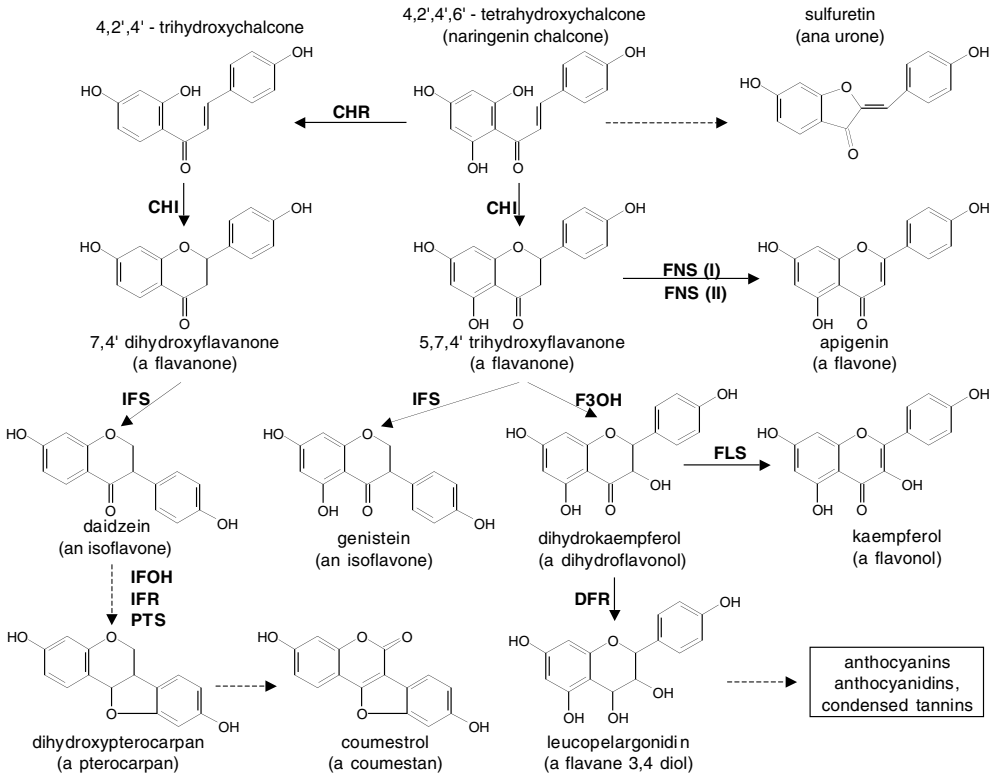


FIGURE 2.12
The general flavonoid pathway. CHR = chalcone reductase; CHI = chalcone isomerase; FNS = flavone synthase; F3OH = flavanone 3β–hydroxylase; FLS = flavonol synthase; DFR = dihydroflavonol 4-reductase; IFS = isoflavone synthase; IFOH = cytochrome P450-dependent microsomial hydroxylase; IFR = isoflavone reductase; PTS = pterocarpan synthase.

monooxygenase: the enzyme, membrane-bound, was observed in members of *Leguminosae, Verbenaceae,* and *Asteraceae*.[122] FNS I and II accept as substrates only flavanones. Another possible reaction involving flavanones is the hydroxylation on C_3 of the heterocycle with the formation of dihydroflavonols (Figure 2.12). The reaction is catalyzed by a 2-oxoglutarate-dependent dioxygenase (flavanone 3β-hydroxylase, F3OH). The F3OH, detected in a variety of botanical families (especially *Asteraceae* and *Leguminosae*), is widespread in the plant kingdom. Dihydroflavonols are the intermediates in the formation of flavonols and anthocyanidins. As regards the biosynthesis of flavonols, the reaction is catalyzed by flavonol synthase (FLS), a 2-oxoglutarate-dependent dioxygenase, which introduces a double bond between C_2 and C_3 of the dihydroflavonol heterocycle. FLS, detected in a number of species belonging to different families,[120] catalyzes the reaction in a similar manner to FNS (type I and II) but accepts as substrates only dihydroflavanols. As concerns the anthocyanidin biosynthesis, the carbonyl group in position 4 of dihydroflavonols can be reduced by NADPH-dependent enzymes, such as dihydroflavonol 4-reductase (DFR), to OH-group.[63] DFR promotes the reduction of dihydroflavonols to the respective flavan-3,4-diols. The flavan-3,4-diols are the precursors of proanthocyanidins, condensed tannins, and anthocyanidins (Figure 2.12). Isoflavone synthase (IFS), an enzyme particularly abundant in the green tissues of *Leguminosae*, can rearrange the carbon skeleton of flavanones (i.e., migration of the B-ring from C_2 to C_3 of the heterocycle), leading to the formation of a wide range of isoflavones, coumestans, and pterocarpans, important compounds for plant–microbe interactions. The IFS, an enzyme requiring NADPH and molecular oxygen, has been isolated from the microsomial fraction of elicitor-challenged soybean cell suspension cultures.[123] Probably the IFS accepts as substrates only flavanones (with or without the 5-hydroxyl group) and not chalcones.[124] The precursor of genistein and related isoflavones is 5,7,4′-trihydroxyflavanone (naringenin), while for daidzein and related isoflavones the common intermediate is 7,4-dihydroxyflavanone (Figure 2.12). The hydroxylation of 5-deoxyisoflavonoids (as daidzein) in position 2′ or 3′ opens the pathway toward the pterocarpans. This reaction is catalyzed by a cytochrome P450-dependent microsomial hydroxylase (IFOH). The 2′-hydroxyl isoflavones are further converted to the respective isoflavanones by isoflavone reductase (IFR), a NADPH-dependent enzyme. The closure of the C-O-C bridge between the isoflavanone heterocycle and B-ring produces the first pterocarpans, such as 3,9-dihydroxypterocarpan (Figure 2.12). This reaction is catalyzed by NADPH-dependent pterocarpan synthase (PTS), a soluble enzyme first isolated from different *Leguminosae* species.[125] Recently it has been shown in alfalfa that the formation of pterocarpans appears to be catalyzed by two enzymes, a reductase and a dehydratase, acting together.[126] In several legume species, after hydroxylation, prenylation, and cyclization, different species-specific pterocarpan phytoalexins are formed (i.e., glyceollin I in *Glycine max*). Finally, pterocarpans are the precursors of coumestans. Several elicitors stimulate the synthesis of isoflavones, coumestans, and

pterocarpans either at plant level or at cell culture level, facilitating biosynthetic studies.

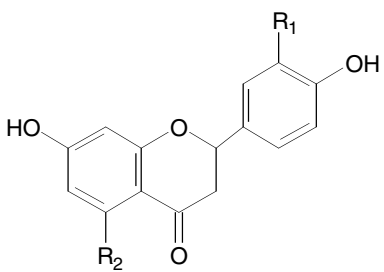
The enzymes involved in the synthesis of the different classes of flavonoids are mostly known and characterized: both their cDNA and genomic clones have been isolated. In addition, their expression and regulation have been studied at the biochemical and molecular level.[63] It is important to underline that the phenylpropanoid and flavonoid pathways, described in Figure 2.11 and Figure 2.12, must be considered valid only from a didactic point of view. In particular, not all cells, tissues, or vegetal species use the whole pathway. Usually plants utilize only partial segments of the whole pathway directing to specific secondary metabolites. In addition, in many cases these metabolites are expressed only in specific tissues of the plant. At present, researchers do not yet fully understand metabolic flux and compartmentalization in the phenylpropanoid and flavonoid pathways.[127] Elucidation of these processes will be a basic step in order to define the control mechanisms in the two pathways.

2.2.1.3 The Main Classes

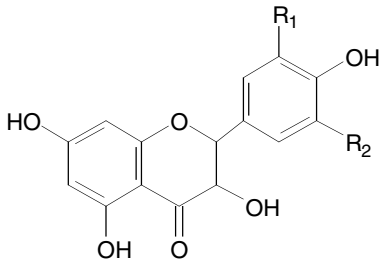
Flavonoids vary widely in their biological properties; it is among the colorless flavonoids that most compounds with significant physiological activity have been reported.[35] Hereafter, for the main flavonoid groups, the most relevant compounds characterized by pronounced biological activities are mentioned.

2.2.1.3.1 Chalcones and Flavanones

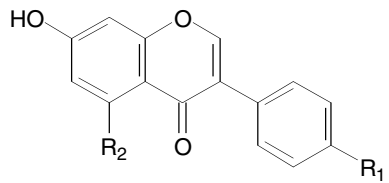
Chalcones and flavanones are easily interconverted in plant cell: the reciprocal isomerization of these compounds can be catalyzed by CHI or under appropriate conditions may occur without any enzyme. Since chalcones, such as naringenin chalcone and 4,2′,4′-trihydroxychalcone (Figure 2.11 and Figure 2.12), are the precursors of all the flavonoids, they are widespread in the plant kingdom, detected in at least 37 botanical families.[128] Usually these phenolic compounds are not accumulated in plant tissues and occur mainly as free aglycones (approximately 20 aglycone structures reported in literature).[128] As biosynthetic intermediates, flavanones are also present as trace compounds in several botanical families, but they are not accumulated in quantity in plant tissues. Approximately the chemical structures of 320 flavanones are known and these phenolic compounds have been reported in at least 60 families.[128] Naringenin, liquiritigenin, and eriodictyol are the flavanones found in plants with some frequency (Figure 2.13). These compounds usually are present in glycosidic form (glycoside typically attached to the C_7) and are characterized by fungistatic and fungitoxic properties. Naringenin exerts multiple biological effects that could contribute to the human health protection ascribed to vegetable consumption.[129] This flavanone exerts an antiestrogenic activity[130,131] and cholesterol-lowering action



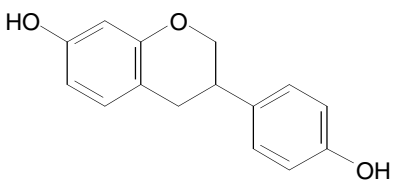
Naringenin $R_1 = H$; $R_2 = OH$
Liquiritigenin $R_1 = H$; $R_2 = H$
Eriodictyol $R_1 = OH$; $R_2 = OH$



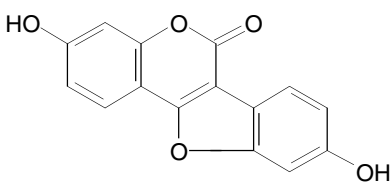
Dihydrokaempferol $R_1 = H$; $R_2 = H$
Dihydroquercetin $R_1 = OH$; $R_2 = H$
Dihydromyricetin $R_1 = OH$; $R_2 = OH$



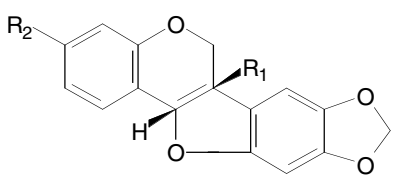
Daidzein $R_1 = OH$; $R_2 = H$
Genistein $R_1 = OH$; $R_2 = OH$
Formononetin $R_1 = OCH_3$; $R_2 = H$
Biochanin A $R_1 = OCH_3$; $R_2 = OH$



Coumestrol



Equol



(-) pisatin $R_1 = OH$; $R_2 = OCH_3$
(-) maackiain $R_1 = H$; $R_2 = OH$

FIGURE 2.13
Some representative flavanones (naringenin, liquiritigenin, eriodictyol), dihydroflavonols (dihydrokaempferol, dihydroquercetin, dihydromyricetin), isoflavones (daidzein, genistein, formononetin, biochanin A), isoflavans (equol), coumestans (coumestrol), and pterocarpans (pisatin, maackiain).

and seems to affect oxidative processes associated with chronic degenerative diseases.

2.2.1.3.2 *Flavones*

Flavones derive from the oxidation of flavanones. Approximately the chemical structures of 650 flavones (including 200 aglycone forms) are known. The two most common flavones, found in several plant species, are apigenin and luteolin (Figure 2.10). Both compounds are usually accumulated in plant tissues as *C*-glycosidic (orientin or 8-*C*-glucosyl-luteolin, vitexin or 8-*C*-glucosyl-apigenin) or *O*-glycosidic derivatives. Luteolin is found in high concentrations in celery, green pepper, and chamomile. *In vitro*, this phenolic compound is among the most potent and efficacious flavonoid inhibitors of LPS-induced TNF-, α-interleukin-6 production, and inducible nitric oxide expression. *In vivo*, luteolin has been reported to exhibit antifertility, antiallergic, radioprotective, and antioxidant properties. In addition, the introduction of this compound with the diet assures general health benefits, in particular preventing vascular disorders, diabetes, and obesity.[132–135]

2.2.1.3.3 *Dihydroflavonols*

Dihydroflavonols play a central role in the biosynthesis of flavonoids, as they are the precursors of flavonols and anthocyanidins. The chemical structures of approximately 110 dihydroflavonols (including aglycone and glycoside forms) are known. This class of phenolic compounds is found in several families, especially *Asteraceae* and *Leguminosae*.[136] The most common dihydroflavonols are the compounds corresponding to the flavonols quercetin, kaempferol, and myricetin (Figure 2.13).

2.2.1.3.4 *Flavonols*

Harborne[94] reported the existence of approximately 1030 flavonols (including approximately 300 aglycone forms). In flowers, these compounds are associated with anthocyanidins as copigments. However, they are almost universally present in green tissues and seeds.[137,138] Kaempferol, quercetin, and myricetin are the flavonols more frequently found in plant species (Figure 2.10). A survey carried out on 1000 angiosperms evidenced that 48% of species had kaempferol, 56% quercetin, 10% myricetin, and approximately 60% all the three flavonols.[139] Kaempferol and quercetin are characterized by a powerful antioxidant activity, and their role in the prevention of several degenerative diseases has been postulated.[137]

2.2.1.3.5 *Isoflavonoids (Isoflavones, Isoflavans, Coumestans, Pterocarpans)*

The chemical structures of approximately 234 isoflavone aglycones, 139 pterocarpans, 31 coumestans, and 51 isoflavans are reported in literature.[140] Isoflavones are mainly present in plant cells as glycosidic forms, and many of them have prenyl groups attached to the phenolic ring. In contrast, isoflavans, coumestans, and pterocarpans typically occur as aglycones and only

rarely as glycosides. The most common isoflavones are daidzein, genistein, formononetin, and biochanin A, while coumestrol is one of the most abundant coumestans found in green tissues of alfalfa and clover (Figure 2.13). Equol, formed in animals from the metabolism of isoflavones, is a representative member of the isoflavan chemical class (Figure 2.13). Pisatin and maackiain can be considered typical pterocarpans (Figure 2.13). The distribution of isoflavonoids is more restricted with respect to the other groups of flavonoids: the great proportion of isoflavonoids is prevalently found in *Leguminosae*.[140] In particular, isoflavonoids are present in an appreciable amount in green tissues of several forage crops (red clover and alfalfa) and in seeds of legume crops (soybean, common bean, chickpea, fava bean, lentil). Soybean differs from other grain legumes: it is the only one able to accumulate huge quantities of isoflavones in seeds (from 1 to 5 mg of isoflavones per gram of seed fresh weight). In other grain legumes, the seed isoflavone content is approximately from five- to tenfold lower than that observed in soybean (from 0.1 to 0.5 mg of isoflavone per gram of seed fresh weight). Nevertheless, it is to underline that several studies on various soybean genotypes were performed, while for other grain legumes available information is quite scarce. Another interesting aspect is the variability of isoflavone production in soybean. Experimental studies performed on the same soybean genotype showed how isoflavone yield could fluctuate (± 50%) year after year as a function of growth environmental conditions and adopted cropping techniques.[141–143] Tsukamoto et al.[143] and Carrao-Panizzi et al.[144] demonstrated that seed isoflavones content increased when seed development occurred at moderate temperatures. On the contrary, high temperatures during seed development seemed to cause the opposite effect. Wang and Murphy[142] detected a strong influence of climatic conditions on seed isoflavones content, whereas the qualitative composition of isoflavone was basically influenced by soybean variety. A valid hypothesis to justify this phenomenon has not been postulated yet, in that the physiological role of isoflavones has not been completely clarified. Isoflavonoids are no steriodal phenolic compounds (or better *bis*-phenolic) with a molecular arrangement similar to natural oestrogen, such as estradiol. By means of such a structural arrangement, isoflavonoids can specifically bind to oestrogen receptors in animal tissues and act as weak oestrogens. Coumestans are more active estrogenic compounds than simple isoflavones. For example, coumestrol is approximately 30 times more active than genistein and formononetin. However, coumestans occur in plant tissues at lower concentrations and have less total effect than isoflavones.[145] Isoflavonoids appear to protect human health against hormone-related diseases such as breast cancer. Their action in this sense is by competing with the body's own estrogen for the same receptor sites on cells. In this way, some of the risks of excess estrogen can be lowered. On the other side, isoflavones can also have estrogenic activity, compensating the dropping of natural estrogens' level during menopause in women, by binding to the same receptor sites, easing menopause's symptoms. Other beneficial effects of these substances encompass

the reduction of heart disease risk, protection against prostate cancer, and improving bone health. Different *in vitro* surveys evidenced bacteriostatic and fungistatic properties of isoflavonoids extracted from different *Leguminosae*. It is commonly thought that these compounds interfere with cell-division signal transduction linked to the prevention of fungi and bacteria proliferation. Pharmacological properties of pterocarpans have been the subject of studies, in particular with regard to gangetin from *Desmodium gangeticum*.[146] This pterocarpan exhibited anti-inflammatory and analgesic activities in rats.

2.2.1.4 Physiological and Ecological Properties

There is still considerable uncertainty about the physiological role of flavonoids and derivatives in plant growth and metabolism. Many compounds are able to exert significant effects on growth processes when applied to plant tissues at physiological concentrations. Due to their extreme variability in structure, they could not have one particular role in plant growth and development. Rather, it is possible that individual classes or individual substances can possess significant activity in certain physiological processes. Flavonoids can exert an important function on IAA oxidase, the enzyme responsible for auxin activity in plants. Quercetin, apigenin, and kaempferol can bind to cell membrane receptors, inhibiting auxin transport from one cell to another. It has been demonstrated that this action takes place only with quercetin, while the common quercetin glycoside, rutin, is ineffective.[35] As regards the hypothesized role of these compounds as endogenous hormones in the plant kingdom, from most considerations the answer could be negative for a lot of them, even if interactions with compounds of auxin classes have been discussed.[147] Besides direct effects, it has been observed that flavonoids may affect polar transport of auxins.[148] Flavonoids probably play an important role as internal physiological and chemical messengers in plants, and according to some authors, this could be one major physiological significance for these compounds.[149] In addition, some flavonoid aglycones such as luteolin, daidzein, and genistein are effective in inducing nodulation in *Leguminosae* plants, by activating nod genes (Figure 2.5).[150]

Although it is still difficult to identify the precise physiological role of flavonoids in plant metabolism, it is easier to explain the ecological roles of these compounds. Most flavone and flavonol aglycones are present on plant surface or are associated with secretory structures. This fact indicates the role of these compounds either in the plant defense mechanism against pathogens and predators or in allelopathic responses. Finally, the chemical class of flavonoids is one of the most important group of compounds for floral pigmentation. In particular, flavonoids provide cyanic colors (from orange and red to blue) as well as yellow and white. Often flavones and flavonols form a complex with anthocyanidins and are able to generate a wide range of colors. Even if the yellow color of flowers is mainly due to carotenoids, often chalcones are responsible for this type of color, especially in *Asteraceae*.

2.2.2 Stilbenes

2.2.2.1 Generalities and Biosynthesis

This class of phenolic compounds includes approximately 200 aglycone and glycoside forms.[151] Stilbenes are mainly constituents of the heartwood of the genera *Pinus* (*Pinaceae*), *Eucalyptus* (*Myrtaceae*), and *Maclura* (*Moraceae*).[152] Although stilbene aglycones are common in heartwood, plant tissues may contain stilbene glycosides.[152] Stilbenes are characterized by two aromatic rings linked by a C_2 bridge (Figure 2.14). Resveratrol, detected in *Picea, Pinus, Myrtaceae, Leguminosae,* and *Vitaceae,* is the most widespread stilbene in nature (Figure 2.14).[153] In contrast, pinosylvin is restricted to the genus *Pinus*, while lunularic acid is found in liverworts and mosses. Stilbenes are considered phytoalexins induced by various abiotic and biotic stressors such as pathogen attack, UV radiation, or ozone exposure.

The stilbene pathway is a branch of the general phenylpropanoid pathway (Figure 2.15). The formation of stilbenes is controlled by stilbene synthases (STSs), which comprise a small gene family in most species examined[154]: three malonate units are added to cinnamic or *p*-coumaric acid and cyclized via an aldolic condensation.[151] STSs are classified into either a *p*-coumaroyl-CoA specific type such as resveratrol synthase (RS) or a cinnamoyl-CoA specific type such as pinosylvin synthase (PS). The latter type has been

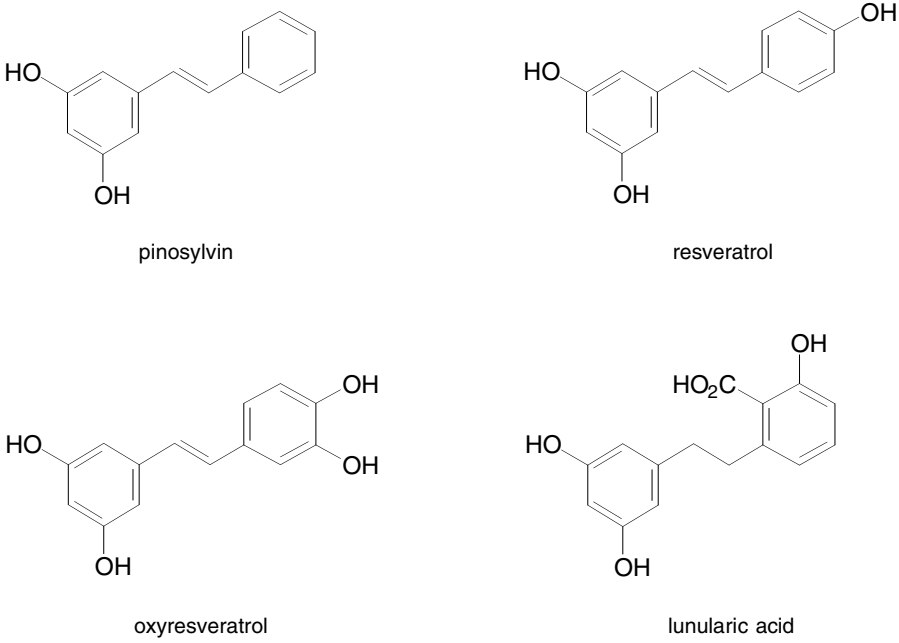


FIGURE 2.14
Some representative stilbenes.

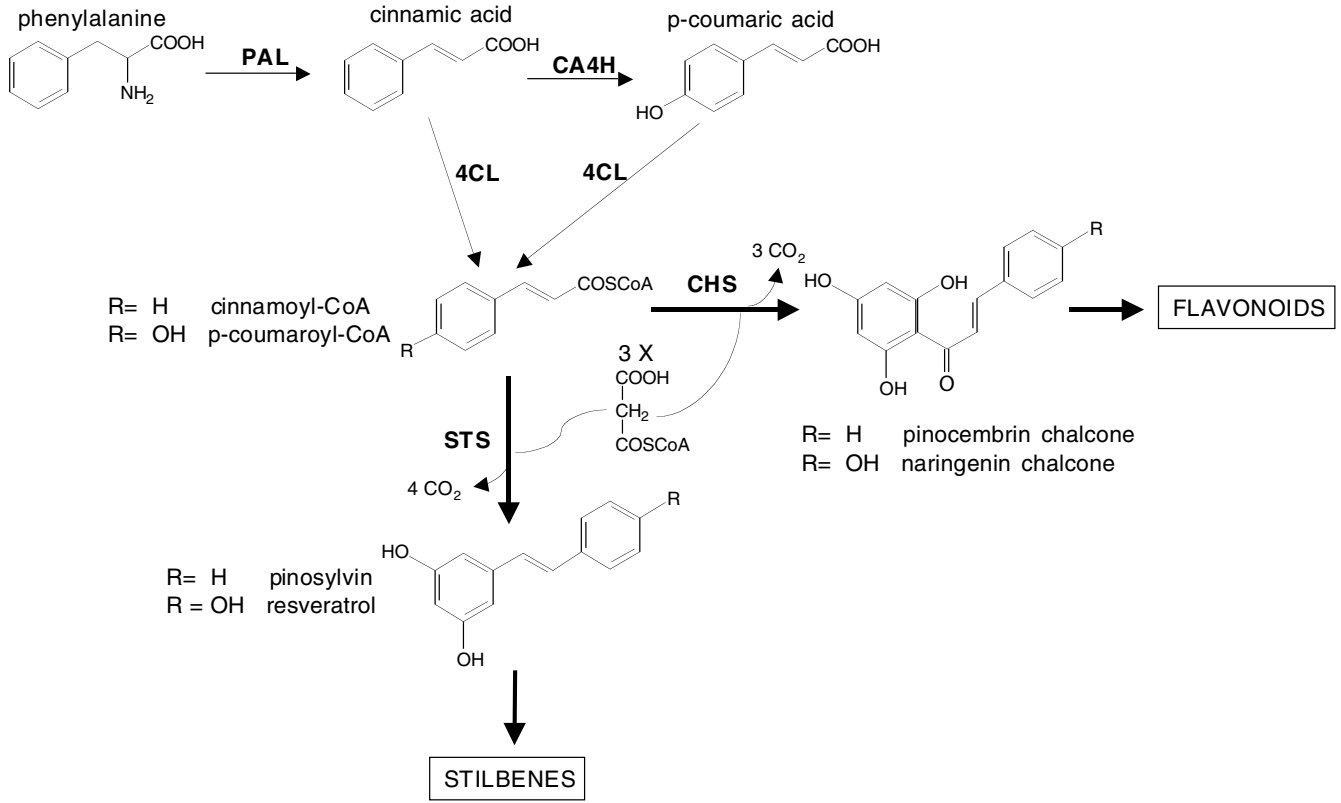


FIGURE 2.15
The pathway for stilbene biosynthesis and its relationship with the flavonoid pathway. PAL = phenylalanine ammonia-lyase; CA4H = cinnamate 4-hydroxylase; 4CL = 4-coumarate: coenzyme A ligase; CHS = chalcone synthase; STS = stilbene synthase.

reported in *Pinus sylvestris* and *Pinus strobus*, while the former has been found in *Arachis hypogaea* (peanut) and *Vitis vinifera* (grape). These enzymes are structurally and functionally closely related to CHS, the key enzyme for the synthesis of flavonoids.[155] STSs and CHS use CoA-esters (such as 4-coumaroyl-CoA, cinnamoyl-CoA) as starter units and perform three sequential condensation reactions. However, the cyclization catalyzed by STSs occurs via an aldol condensation, while CHS catalyzes a Claisen-type condensation.[155] STSs appear to have evolved from CHS during land plant evolution. STSs and CHS share more than 65% of amino acid homology.[156] These enzymes are also characterized by a similar protein structure and sequence: CHS and STSs are homodimers of identical subunits and share in the same position a strictly conserved residue of cysteine, which represents the active site of the condensing reactions.[157] CHS and STSs have no significant similarities to other eukaryotic condensing enzymes, thus representing an independent evolution line.[156]

2.2.2.2 Physiological and Ecological Properties

Usually woods characterized by a high content of stilbenes are highly resistant to decomposition. In addition, the presence of these compounds in a number of tree species causes several difficulties in paper manufacture. It is common opinion that these facts are due to the relevant antifungal and antibacteric properties of stilbenes.[158] In general, stilbenes are considered phytoalexins produced by plant in response to either pathogen attacks or environmental stresses. The synthesis of a number of antifungal stilbenes has been demonstrated after elicitation with fungal preparations or other factors such as UV light. For example, in peanut (*Arachis hypogaea*) and in young pine seedlings microbial infections stimulate the synthesis of resveratrol and pinosylvin, respectively. α-viniferin and ε-viniferin, which are oligomers of resveratrol, are produced in grape (*Vitis vinifera*) after infection with the cryptogam *Botrytis cinerea*. In sugarcane (*Saccharum officinarum*) the synthesis of the stilbene piceatannol is elicitated by the infection of the red hot fungus (*Collecotrichum falcatum*).[35]

As regards the biological activities of stilbenes, the last few years have brought a steadily increasing number of publications on the effects of resveratrol and its derivatives on human health. As it is often the case, the complexity of the potential effects in humans makes it difficult to estimate with some certainty whether the benefits are as high as claimed. It has been known for a long time that a low-calorie leads in many organisms to retardation of the aging processes and to longer life. The molecular mechanisms, at least in higher organisms, are still a matter of debate. However, there are good points to be made for a hypothesis that the principles are not so fundamentally different in all organisms. This made some fairly recent findings with baker's yeast (*Saccharomyces cerevisiae*), so exciting because yeast has often been used as a model system to understand aging in humans.[159] In particular, cells with a low-calorie supply activate a specific set of genes

that leads to longer life for the individual cell. One of the effects of this activation is that DNA repair processes operate more efficiently in cells living in long-term calorie restriction. The exciting new finding is that this effect apparently can be mimicked by certain low-molecular-weight plant natural products, as some flavonoids (e.g., quercetin) and the most efficient resveratrol. The principle apparently works also in fruit flies, and related genes are also known in humans and other mammals. Naturally, it is not that easy to prove this with humans, but the hopes are high, and experiments with other mammals and also rhesus monkeys are under way. Resveratrol has been found only in plants. However, it is not present in most of our important crops, and grapes and wine are the most noticeable exceptions. Actually, the presence of resveratrol in many wines (especially red wines) may well provide the explanation for the "French paradox," i.e., that French people have less heart problems than, for example, North Americans, despite living on a high-fat diet and with high cholesterol levels. The explanations given for yeast may also help explain reports that resveratrol protects against heart disease, raises the "good" HDL cholesterol, inhibits blood clots, stops viral replication, and blocks cancer at every stage of development. Unfortunately, not all the vegetal species contain resveratrol. In fact, the enzyme necessary for its biosynthesis (resveratrol synthase) is present in a limited number of botanical genera. Interestingly, a single additional enzyme is sufficient to synthesize resveratrol because the substrate for its biosynthesis (*p*-coumaric acid) is present in all plants. The first cDNAs and genes for a resveratrol synthase (from peanuts, *Arachis hypogaea*) were identified already in 1988,[160] and thus the basis for the biosynthesis has been known for a long time. cDNAs/genes for stilbene synthases from trees, Scots pine (*Pinus sylvestris*), and Eastern white pine (*Pinus strobus*) were also described.[161]

2.2.3 Lignans

2.2.3.1 Generalities and Biosynthesis

The number of isolated and identified lignans has grown exponentially since the 1930s. In a review of lignans dated 1936, only 14 compounds were described; it took nearly two decades to double that.[127] The number of identified lignans jumped to 164 in 1978 and to 440 in 1987.[162] At present the approximately 450 chemical structures have been isolated from at least 55 families (especially from *Asteraceae, Pinaceae,* and *Rutaceae*). Lignans are related closely to intermediates in the biosynthesis of lignin and are formed from analogous pathways.[35] The term *lignan* describes a class of dimeric phenylpropanoid (C_6-C_3) compounds linked by way of 8-8′ bonds (Figure 2.16).[127] Recently, the term *neolignan* was coined: this class includes all the related dimers in which the two C_6-C_3 units are joined by dimerization of the aromatic rings (e.g., 8-5′, 8-*O*-4′ linkage) instead of by the propanoid tail (e.g., 8-8′ linkage) (Figure 2.16).[35] It is interesting to note that the majority of lignans occur in distinctive chiral forms, while no optically

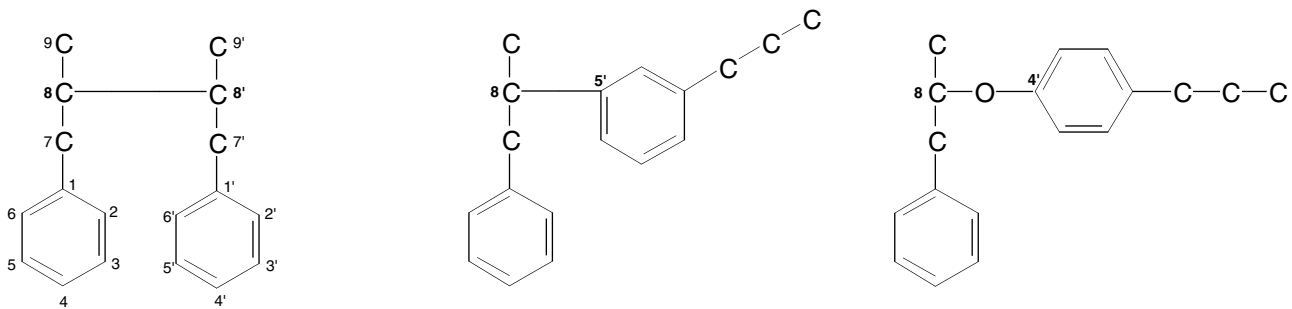


FIGURE 2.16
Examples of different lignan linkages: 8-8′ (e.g., pinoresinol, secoisolariciresinol, matairesinol), 8-5′ (e.g., licarin), 8-O-4′ (e.g., eusiderin, virolin).

active degradation product of lignin has yet been isolated. A major reason for the increasing interest for lignans is that these compounds exhibit a remarkable range of biological activity: fungal enzyme and growth inhibition; insect antifeeding properties, and a wide range of hormonal and nonhormonal activities *in vivo* and *in vitro*. These properties of lignans suggest plausible mechanisms for potential health effects of diets rich in these compounds in humans.[162]

Monolignols are the direct precursors of lignans and lignin. Monolignols, which include the *p*-coumaryl, sinapyl, and coniferyl alcohol, are derived from the general phenylpropanoid pathway (Figure 2.17). Hydrocinnamic acids (*p*-coumaric, caffeic, ferulic, hydroxyferulic, and sinapic acid) are converted to the corresponding CoA thioesters by ligases collectively called *p*-coumarate-CoA ligase (4CL). In different species, this enzyme may preferentially activate the hydrocinnamic acids in a specific order, thus controlling subsequent phenylpropanoid branch pathways. After the formation of CoA esters, two sequential NADPH-dependent reductions produce the monolignols. In particular, cinnamoyl-CoA reductase (CCR) converts CoA-activated cinnamic acids to the corresponding aldehydes by using NADPH as reductant. The substrate preference is slightly different as a function of different sources: usually feruloyl-CoA is the preferred substrate followed by sinapoyl-CoA and *p*-coumaroyl-CoA. The second reduction step of the monolignol synthesis is catalyzed by cinnamoyl alcohol dehydrogenase (CAD), which uses NADPH for the reduction of cinnamoyl aldehydes to the respective alcohols.[108] The specificity of the substrate varies according to plant species and different CAD isoforms within the same species: in general, coniferyl aldehyde is preferred followed by *p*-coumaroyl and sinapyl aldehydes. CAD is expressed mainly in lignifying tissues, but in some cases CAD activities are observed in tissues with little or no lignin content (i.e., in pods of *Phaseolus vulgaris*).[108] These data suggest that probably the biosynthesis of lignans and lignin occurs in plant tissues as parallel pathways sharing common precursors (monolignols). Only in recent years was the biochemistry of lignan formation identified. In particular, research was mainly focused on biosynthesis of 8-8′-linked lignans. These compounds usually occur as stereochemically pure (+) or (–) enantiomers in different plant species; for example, in *Forsythia* spp. (+)-pinoresinol is found, while flax (*Linum usatissimum*) seeds accumulate (–)-pinoresinol.[127] These data suggest that the dimerization of monolignols is stereospecific. As observed in *Forsythia* species (+)-pinoresinol synthase catalyzes the stereospecific dimerization of two coniferyl alcohol molecules (Figure 2.18). (+)-Pinoresinol synthase (PRS) is an enzymatic system characterized by two proteins: an oxidase (laccase or laccase-like enzyme) and a dirigent protein.[127] The oxidase promotes the oxidation of the two coniferyl alcohol molecules with the formation of free radicals, while the dirigent protein orients the free radicals, preventing their random dimerization. The gene encoding for the *Forsythia* dirigent protein has been cloned and the functional recombinant protein expressed.[127] This protein is devoid of an active catalytic center, acts specifically for the

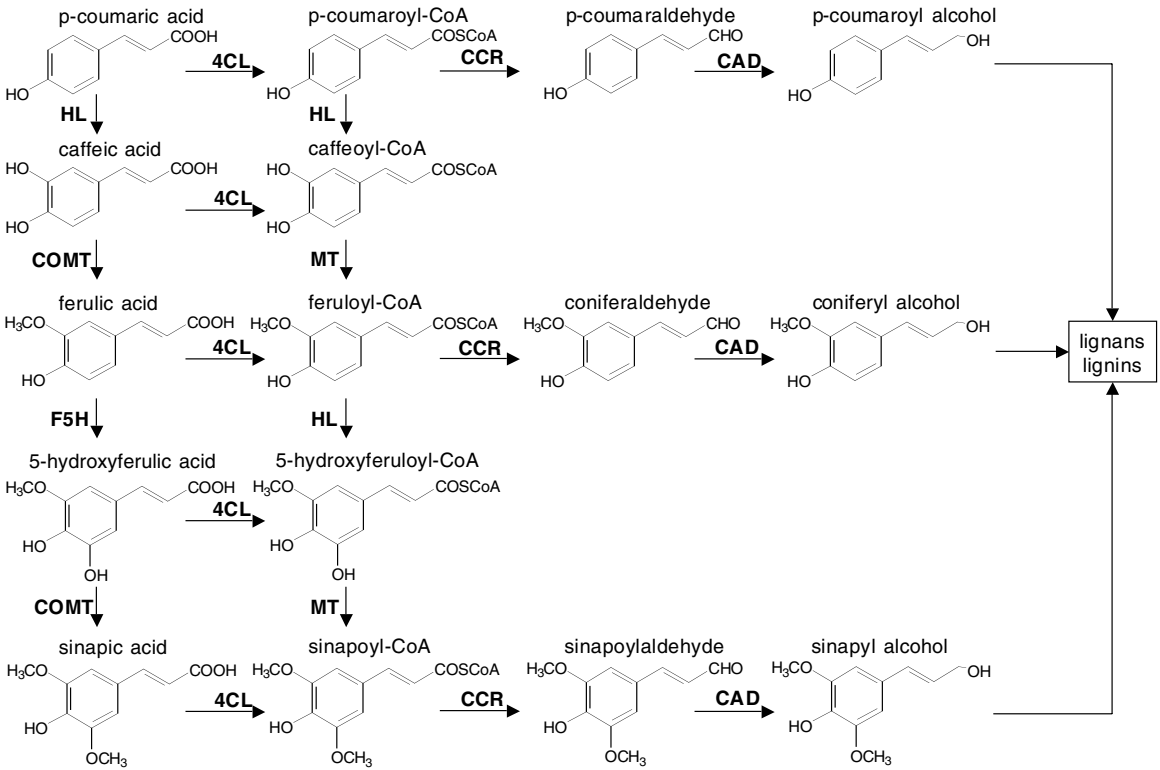


FIGURE 2.17
The biosynthesis of monolignols (coumaryl, conifery, sinapyl alcohol). HL = hydroxylases; 4CL = 4-coumarate: coenzyme A ligase; COMT = caffeic acid methyltransferase; F5H = ferulic acid 5-hydroxylase; MT = O-methyltransferases; CCR = cinnamoyl-CoA reductase; CAD = cinnamoyl alcohol dehydrogenase.

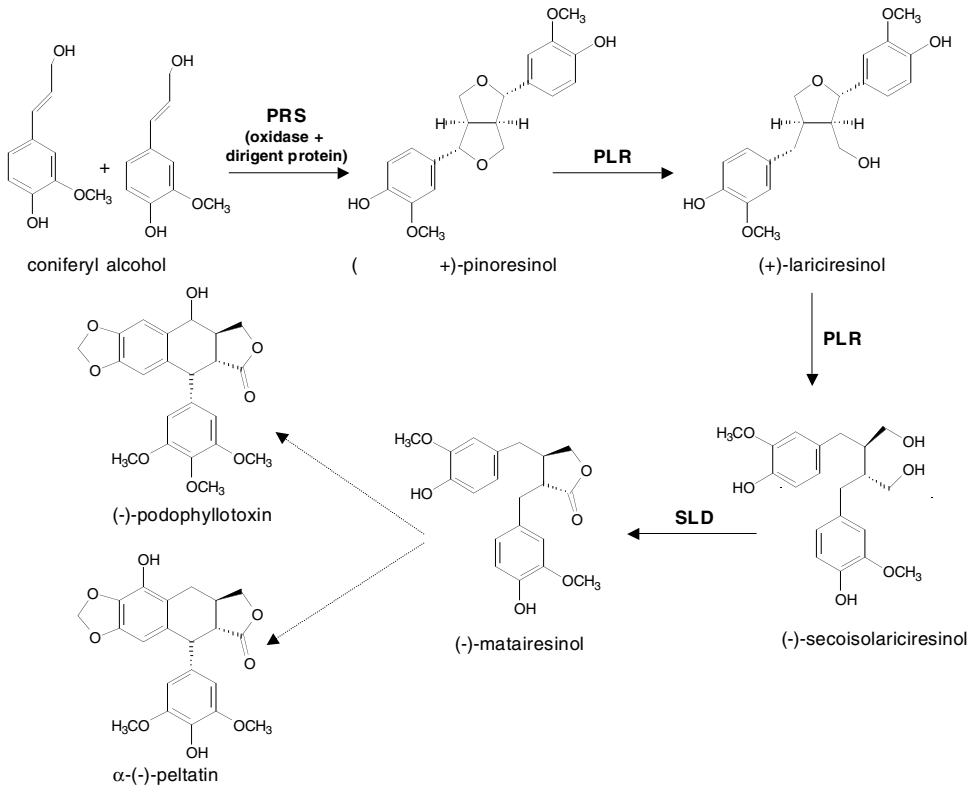


FIGURE 2.18
Proposed pathway for the synthesis of various lignans (8-8′ linked). PRS = (+)-pinoresinol synthase; PLR = pinoresinol/lariciresinol reductase; SLD = secoisolariciresinol dehydrogenase.

formation of (+)-pinoresinol, and is not homologous to any other protein. Once formed, pinoresinol can be subjected to different conversions depending on plant species. In *Forsythia* species, (+)-pinoresinol is reduced to give (+)-lariciresinol and then (–)-secoisolariciresinol[163] (Figure 2.18). Both reductions are catalyzed by pinoresinol/lariciresinol reductase (PLR), which accepts either (+)-pinoresinol and (+)-lariciresinol as substrates. A considerable homology exists between PLR and isoflavonoid reductases (such as IFR), which are involved in the formation of phytoalexins. This sequence similarity could be more than a coincidence, as both lignans and isoflavonoids are derived from the general phenylpropanoid metabolism and are characterized by similar roles in plant defense. Since lignans are present in the pteridophytes, hornworts, gymnosperms, and angiosperms, and the distribution of isoflavonoids is restricted to higher plants, some authors speculate that isoflavone reductase could be evolved from PLR.[163] (–)-Secoisolariciresinol can be further dehydrogenated to form (–)-matairesinol: the reaction is catalyzed by secoisolariciresinol dehydrogenase (SLD). Even if little information is still available on the precise biosynthetic steps, matairesinol is regarded as the key intermediate leading to two separate subgroups of lignans: those with 3′,4′,5′-trimethoxy (i.e., podophyllotoxin and related compounds) and those with 4′-hydroxy-3′,5′-dimethoxy (i.e., α-peltatin and related compounds) substitution pattern in the pendant ring (Figure 2.18).

2.2.3.2 Physiological and Ecological Properties

In several plant species, lignans play an important role in the defense mechanisms against pathogen attacks. In particular, these compounds can exert antimicrobial, antifungal, and antifeedant activities. In addition, lignans have been proposed as cytokinins[164] and as intermediates in lignification,[165] suggesting a critical role in plant growth and development. It is widely accepted that the development of lignan biosynthesis was essential for the successful evolution of aquatic plants to their vascular dry land counterparts, which occurred approximately 480 million years ago.[166,167] Based on existing taxonomic data, lignans are present in "primitive" plants, such as ferns[168] and the hornworts.[169] Interestingly, evolution of both gymnosperms and angiosperms was accompanied by major changes in the structural complexity and oxidative modification of the lignans.[167,170]

As regards the beneficial effects for human health, lignans have a long and fascinating history, beginning with their use in popular medicine by many different cultures. Kelly and Hartwell[171] dated back the first medicinal use of lignans over a thousand years. Many reports from early English medical books, such as the Leech book of Bald, indicated the use of wild *Anthriscus cerefolium* for treating cancer.[172] Historical reports from Himalayan natives or American Indians reported contemporarily the employment of a resin produced by roots and rhizomes of *Podophyllum* as a cathartic compound.[171] Chinese and Japanese physicians have been employing lignan-rich plant

products for centuries, but unfortunately a lot of them were not tested scientifically. The widespread use of these compounds as folk medicines suggests that lignans have strong potentialities for the development of new classes of drugs. In these last few years, the interest in the role of lignans for the treatment and prevention of several pathologies has increased dramatically. At least 40 lignans are known to have antitumor and antiviral activity.[162,173] Podophyllotoxin, extracted from *Podophyllum* species (*Berberidaceae*), is one of the most powerful lignans and has been known for many years to have therapeutic properties. It was such popular to be included in U.S. Pharmacopeia from 1820 to 1942.[162] Currently podophyllotoxin remains an effective treatment for venereal warts[174] and may also be effective in nasal papillomas.[175] Interestingly, other members of the *Berberidaceae* family have been used for the treatment of venereal warts and solid tumors in China and India since the second century A.D.[176] Podophyllotoxin and related compounds (i.e., α-peltatin, β-peltatin, deoxypodophyllotoxin, picropodophyllotoxin) exhibit activity against measles and herpes simplex type I viruses. The semisynthetic derivative of podophyllotoxin, named teniposide, is widely used in cancer treatment. Compounds structurally related to podophyllotoxin often have antimitotic activity probably due to specific binding with tubolin.

The lignans (–)-secoisolariciresinol and (–)-matairesinol have shown to have important nutritional functions in health protection. During digestion, intestinal bacteria convert (–)-secoisolariciresinol and (–)-matairesinol to enterodiol and enterolactone, respectively (Figure 2.19). These "mammalian" lignans are believed to be responsible for preventing the onset of and substantially reducing the rate of incidence of prostate and breast cancer. The protection is higher in individuals following a diet rich in grain, vegetables, and berries. In contrast, a diet poor in these foods does not ensure comparable protection.[127]

2.2.4 Lignins

2.2.4.1 Generalities and Biosynthesis

Lignin is a polymer that serves as a matrix around the polysaccharide components of the cell wall, providing rigidity and strength, as well as hydrophobicity and impermeability.[177] Despite the importance of lignin for the plant growth, not all plant cells accumulate lignin during their development. Cells devoted to the accumulation of lignin such as xylem elements and sclerenchymatic cells undergo some irreversible modifications. In particular, before lignin biosynthesis is initiated, the cells destined to form secondary xylem experience a programmed expansion of their primary walls, followed by so-called secondary thickenings, which involve ordered deposition of cellulose, hemicellulose, pectin, and structural proteins.[127] Finally these changes lead to cell death and the formation of conducting elements (i.e., tracheids).[127] These data indicate that the architecture of plant cell wall

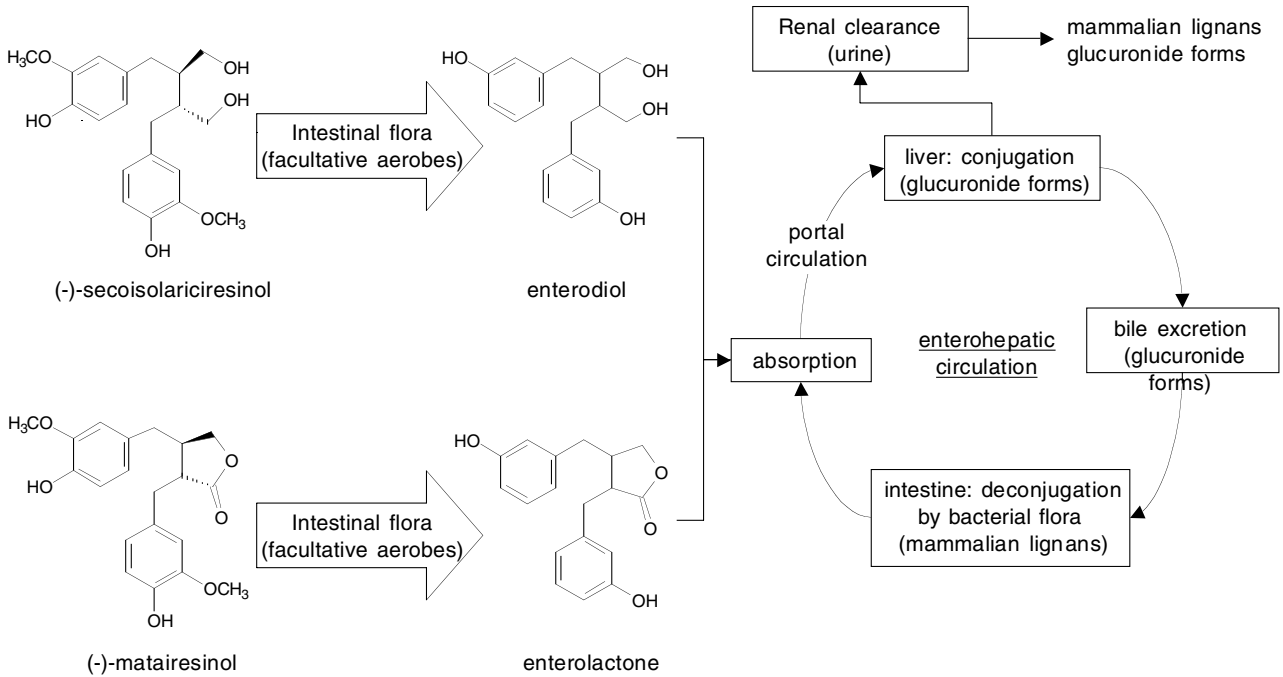


FIGURE 2.19
The "mammalian" lignans: enterodiol and enterolactone are formed during digestion by intestinal bacteria and undergo enterohepatic circulation.

is determined before lignification takes place. The biosynthesis of lignin can be induced by wounding or disease, indicating a gene response to development and environmental factors.[177]

Lignin is difficult to isolate in pure form and to degrade chemically. As a consequence, the definite chemical structure of this polymeric amorphous plant material is still puzzling (Figure 2.20).[35] However, in literature there is a general agreement on the principal structural features of lignin. The concept that lignin is derived from polymerization of monolignols dates from the late 19th and early 20th centuries.[178] The lignification process encompasses three different steps: the biosynthesis of monolignols, their transport to the cell wall, and the polymerization into the final molecule. The general outline of the pathway that gives rise to coniferyl alcohol and other monolignols was proposed 30 years ago (Figure 2.17).[179,180] Three are the monolignols (*p*-coumaryl, coniferyl, and synapyl alcohol), differing only in the

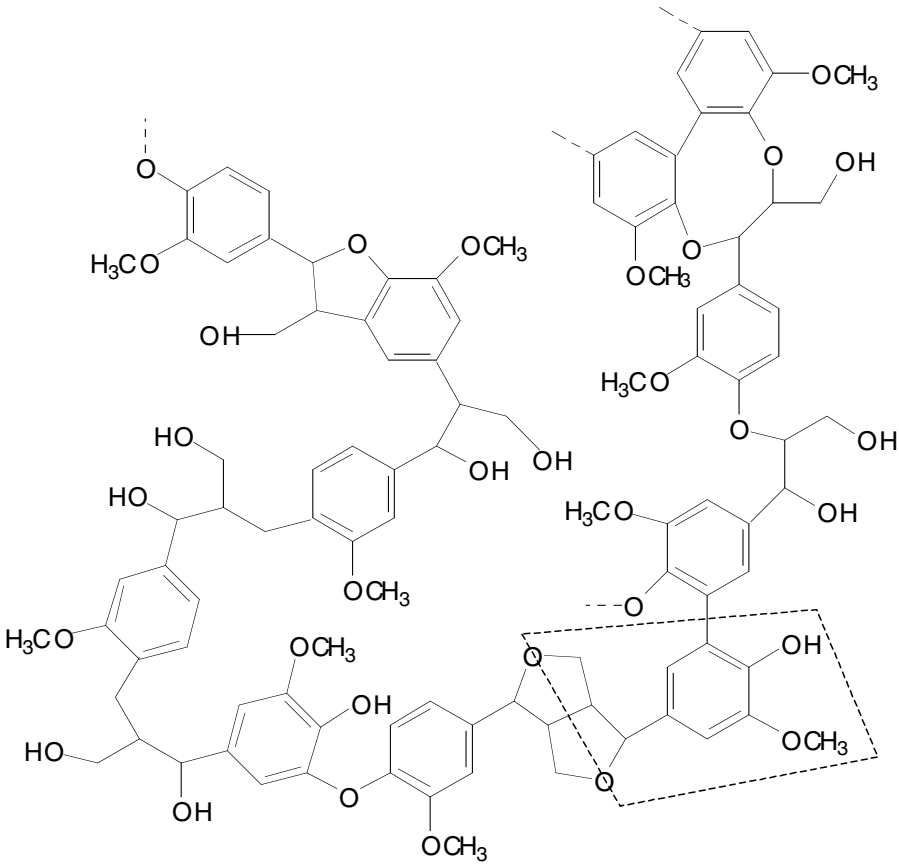


FIGURE 2.20
Hypothetical lignin structure. As an example, one monomer residue (coniferyl alcohol) is boxed.

substitution pattern on the aromatic ring, usually polymerized into lignin (Figure 2.17). It was suspected that this view might be too simplistic, and there are now many examples showing that other phenolics (than the previously mentioned three monolignols) can be incorporated into lignins.[181] In contrast to many of the lignans, monomers of lignin are racemic and optically inactive compounds. In addition, no optically active degradation product of lignin has been yet isolated. Gymnosperm lignin is primarily derived from coniferyl alcohol, and to a lesser extent from *p*-coumaryl alcohol, whereas angiosperms contain coniferyl alcohol and sinapyl alcohol in roughly equal proportion.[127] The relative abundance of the different monolignols residues in lignin varies between and within species, as does the total lignin content.[177] The model of lignin formation has been the subject of many disputes among researchers. The pathway leading to the formation of monolignols for lignin is quite the same to that of lignans. There are strong indications that lignans are synthesized in the cytoplasm and stored in the vacuole, whereas lignin is an extracellular compound.[108] These data suggest that lignin and lignans biosyntheses seem to be separate biosynthetic pathways only using the same precursors.[182] In general, the levels of monolignols in plants are small and they occur in glycoside forms (Figure 2.21). Specific glucosyltransferase (UDP glucose: coniferyl alcohol glucosyltransferase) catalyzes the formation of glucocoumaroyl, syringin, and coniferin, transferring the glucose unit from UDP-glucose to the aromatic hydroxyl group in the paraposition of the side chain of *p*-coumaryl, sinapyl, and coniferyl alcohol, respectively.[183] It was supposed that the glucosylated monolignols are transported across the plasma membrane and deglycosylated prior to polymerization in the cell wall. Cell-wall-associated β-glucosidases (i.e., coniferin β-glucosidase) have been found mainly in lignifying cells. Some authors suggested that the rate and the location of lignification are controlled by these β-glucosidases.[184] However, it is still unclear whether coniferin and other glucosylated monolignols are obligate precursors of lignin. In 1933, Erdtman[185] proposed that monolignols are polymerized into lignin by an oxidative mechanism involving free-radical intermediates. Peroxidase enzymes were thought to be involved in this step (Figure 2.22).[177] Despite the 50 years of studies, the exact mechanism of lignin formation is still unclear. Some reviews about evidence for and against the involvement of peroxidases exist.[186,187] In addition, the mechanism involved in the formation of functional lignin macromolecules is still the subject of debates. The oxidative coupling between monolignols can result in the formation of several different interunit linkages (Figure 2.22). In the wood of several species, 8-*O*-4 linkages are the most abundant (more than 50% of total monomers). Currently there are two models for coupling radicals to produce lignin. One, the random coupling model, centers on the hypothesis that the lignin formation occurs in a near-random fashion.[188] According to this hypothesis, the amount and type of individual monolignols available at the lignification site and normal chemical coupling properties regulate lignin formation.[181] The mechanisms of controlling different proportions of monolignols subunits in lignin are not well understood,

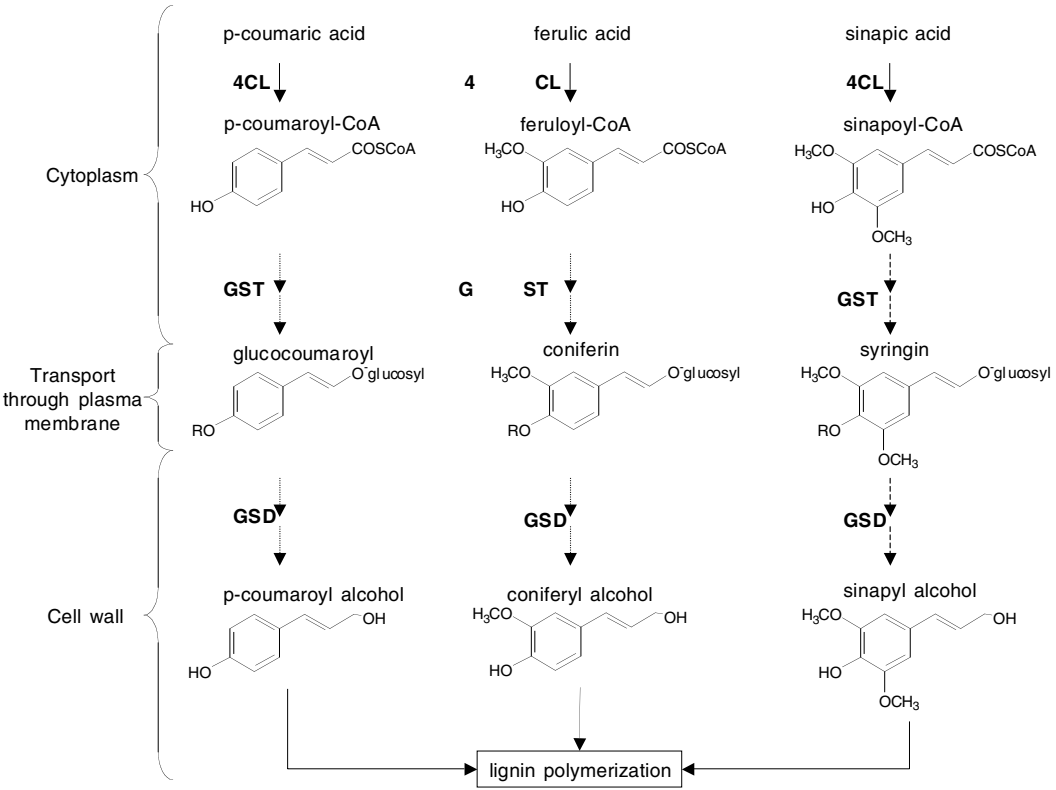


FIGURE 2.21
Transport of lignin monomers (monolignols) from cytoplasm to cell wall. 4CL = 4-coumarate: coenzyme A ligase; GST = glucosyltransferases; GSD = β-glucosidases.

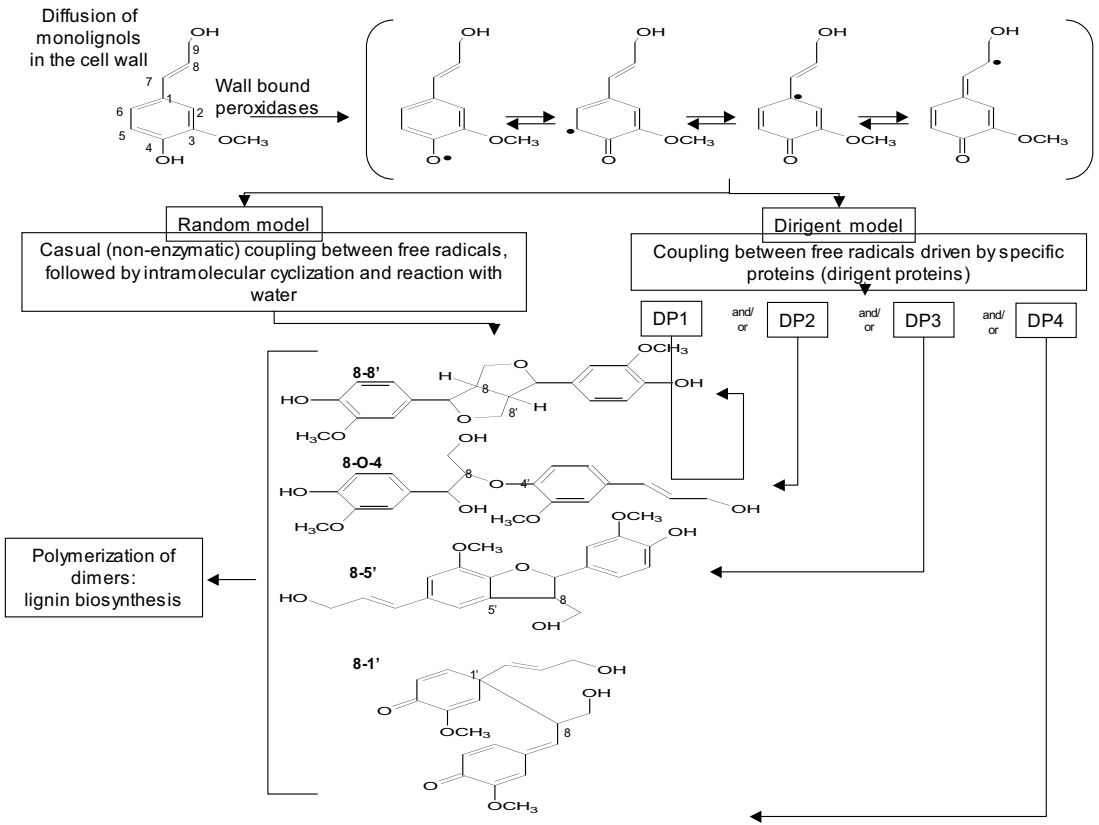


FIGURE 2.22
The "random" and "dirigent" models for lignin biosynthesis.

although hypotheses were formulated about the role of the different enzymes involved in formation of monolignols (i.e., *O*-methyltransferases, coumarate:CoA ligases, coniferyl alcohol deydrogenases).[177] The differences observed in lignin monomer composition of angiosperms and gymnosperms have been attributed to these differences in enzyme substrate specificity.[189] However, considering that often monomer composition is different between cell types within plants and between different regions of the cell wall, it seems unlikely that substrate specificity is the only causal factor.[177] The "random coupling" hypothesis was historically the first formulated to justify the formation of lignin structure, and it is still the most accredited. Recently a second model, the dirigent protein model, based on the hypothesis that lignin polymerization is a highly organized step, has emerged. This model is based on the hypothesis that lignin polymerization is a highly organized step.[190,191] Some observations corroborate this view. For example, phenyl rings of lignin are aligned in the plane of cell walls, while some studies have shown that monolignols polymerization is not a random process.[173,192,193] According to the dirigent protein model, the lignification occurs under the strict regulation of specialized proteins controlling the formation of individual bonds.[174,194] This new hypothesis for lignin formation is based on the concept of dirigent proteins.[194] As previously observed for lignans (see Section 2.2.3), dirigent proteins direct the coupling of two monolignol radicals, producing a dimer with a single regio- and stereo-configuration.[181] The rationale for this new model is the belief that nature would not leave the formation of such an important molecule as lignin to chance.[174] It is argued that the only way to explain the high proportion of 8-*O*-4 linkages in lignin would be through regulation by specific dirigent proteins.[174] The issue of bond specificity in lignin is complex. At present, the debate on the process involved in lignin formation is still open. The random model for lignification is not invalidated by the dirigent protein model. Claims that the latter model is the obvious correct one and replaces the random coupling model are therefore premature.

2.2.4.2 Physiological and Ecological Properties

From a functional point of view, lignin imparts strength to cell walls, facilitates water transport, and prevents the degradation of wall polysaccharides, thus acting as a major line of defense against pathogens, insects, and herbivores.[181] Terrestrial plants therefore may have appeared after the evolution of lignin biosynthesis, as the structural support and water transport functions of this polymer are pivotal for the biology of higher plants. Only with lignified cell walls was it possible to build the rigid stems of woody terrestrial plants and the conducting elements for water transport.[195] From 15 to 36% of wood dry weight is lignin; for this reason, this compound is the most abundant polymer, together with cellulose in higher plants.[196]

Concerning the healthy properties of lignin, the beneficial effects of this polymer have been outlined by several investigations. Dietary fiber consists

of the structural and storage polysaccharides and lignin in plants that are not digested in the human stomach and small intestine. However, interest in fiber is not a recent development. Hippocrates, the father of medicine, recommended making bread from whole grain flour for its "beneficial effect on the bowels." Fibers will not cure or prevent all diseases, but they should be part of a healthy diet. Consumption of dietary fibers that are viscous lowers blood cholesterol levels and helps to normalize blood glucose and insulin levels, making this kind of fiber part of the dietary plans to treat cardiovascular diseases and type 2 diabetes. Fibers that are incompletely or slowly fermented by microflora in the large intestine promote normal laxation and are functional in treating constipation and preventing the development of diverticulosis and diverticulitis. An adequate fiber-rich diet is also usually rich in micronutrients and nonnutritive ingredients that have additional health benefits and is associated with a lower risk of colon cancer. A fiber-rich meal is processed more slowly, which promotes earlier satiety, and is frequently less calorically dense and lower in fat and added sugars.

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3

Chemistry and Mechanism of Action of Phytoestrogens

Rakesh Kumar Rishi

CONTENTS

3.1 Introduction

There are thousands of naturally occurring substances including vitamins, antioxidants, glucosinolates, and flavonoids that exhibit structural, hormonal, attractant, and chemoprotective actions.[1] These phytochemicals are of great importance as they are present in a large number of plant foods consumed by humans as a result of their introduction into the food chain from the environment, and they play important roles in disease prevention. Over the past few decades, population-based studies have suggested important clues to possible etiological factors responsible for disease states.[2] Epidemiological evidence of numerous phytochemicals with potential biological

activity is rapidly increasing, which continues to indicate strong associations between diet and disease states.[2]

Phytoestrogens make up a broad group of naturally occurring plant-derived nonsteroidal polyphenolic compounds with estrogen-like biological activity that are found in many foods.[3,4] Phytoestrogens emerged from their peculiar role in human health based on the hypothesis that the Western diet is relatively deficient in these substances as compared with populations that consume large amounts of plant foods and legumes.[1]

In addition to hormone-related diseases and bowel cancers, phytoestrogens are under active investigation for their protective effects on human health for other hormone-related conditions such as menopausal symptoms, osteoporosis, and heart disease.[1] It is now generally well accepted that many common diseases of Western nations are related to diet[5] and could be avoided if significant modifications in the diet are made. Phytoestrogens, consumed by Asiatic societies, appear to be biomarkers of a "healthy" diet, as they protect against so-called Western diseases such as breast, endometrial, prostate, and bowel cancer, osteoporosis, menopausal symptoms, and cardiovascular disease.[4,6]

3.2 Classification of Phytoestrogens

Major classes of phytoestrogens of current interest from a nutritional and health perspective are flavonoids, lignans, and coumestans (see Figure 3.1). These phytoestrogens have been shown to bind with estrogen receptors. Some terpenoids and saponins have also been reported to exert similar effects, but scientific data for classifying such compounds as phytoestrogens is rather inadequate. Apart from plant sources, compounds with estrogenic activity are also present in animals (ovarian steroids), molds that contaminate cereal crops (mycoestrogens), and compounds made synthetically (such as bisphenol A and nonylphenol).[7,8] Various drugs such as diethyl stilboestrol, estradiol benzoate, and so on, also exhibit estrogenic activity.[4] There is a wide range of compounds with estrogenic activity, which needs attention due to their introduction into the food chain. One example is pesticides and insecticides, including DDT, which contains estrogen-like compounds.[9] These compounds and several other environmental estrogens have been classified as xenoestrogens.[9] The long-term effects of xenoestrogens are not fully known, but concern over their potentially deleterious effects on human health is growing.[10] Structurally diverse synthetic estrogens are collectively known as Selective Estrogen Receptor Modulators (SERMs).[11]

Within the flavonoid group are isoflavones and phenols, which are structurally similar to mammalian estradiol.[12] Isoflavonoids are the best-known class of phytoestrogens, and soy protein is the most common source.[13] Several isoflavones have been identified from plant sources, but two are

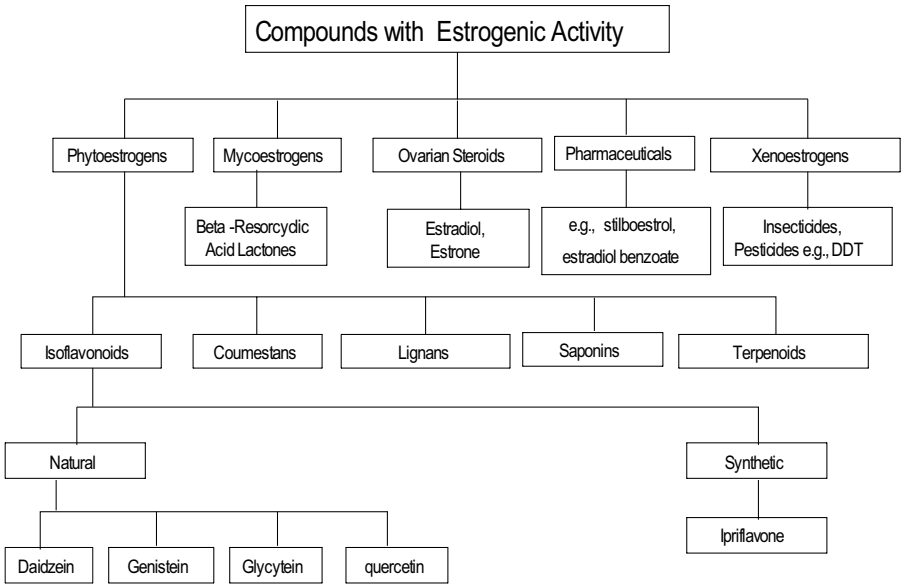

FIGURE 3.1
Classification of phytoestrogens.

principal isoflavones, namely, daidzein and genistein. Daidzein and genistein remain in plants as inactive glycosides (daidzein and genistin, respectively) and their respective 4′-methyl ether derivatives, formononetin and biochanin A.[14] Another major glycoside found in soya beans is glycitein, which also remains as inactive glucose-conjugated compound glycitin.[15] Intestinal microfloral enzymes (beta-glucosidases) present in several groups of bacteria including lactobacilli, bifidobacteria, and bacteroids are responsible for hydrolysis of these inactive glycosides, converting them to their corresponding active aglycones: daidzein, genistein, and glycitein.[16–18] However, another class of polyphenols, the flavonoids (such as rutin and quercetrin), has been shown to be absorbed in their naturally occurring glycosidic forms.[19] Daidzein is further metabolized by the intestinal microflora to equol (an isoflavan) and *O*-desmethylangolensin.[15]

The lignans are a group of dimeric phenylpropanoids.[14] The major lignans are seco-isolariciresinol and matairesinol, which are primary plant precursors and occur in the glycosidic form.[15] These plant precursors are converted after ingestion by intestinal bacterial microflora into biologically active metabolites — enterolactone and enterodiol, respectively.[1,4,14] Enterodiol can be further metabolized to enterolactone in the intestines.[15] Lignans have been shown to bind with estrogen receptors and to suppress estrogen-stimulated responses.[20] Both plant precursors and their metabolites are measurable in various body fluids such as plasma, urine, and feces.[4]

The coumestans comprise another class of phytoestrogens, and among them, coumestrol is the most studied one. Coumestrol has a higher affinity

for the estrogen receptors than the isoflavones, as it is only 10 to 20 times lower than estradiol.[21]

3.3 Sources

Numerous plant foods that contain traces of phytoestrogens have the potential to improve health; however, relatively few foods contribute significant amounts to the human diet.[22] The highest levels of phytoestrogens have been found in people who live in Asiatic nations (e.g., Japanese men and women), and this has been linked to the use of soybean products.[23,24] Isoflavonoids are also found in fruits, vegetables, whole grain products, alcoholic beverages, cow's milk, and meat.[13] Soy isoflavones are found in many products, including isolated protein flour, infant formulas, and specific soy foods such as cheese, drinks, miso, tempeh, tofu, and meat substitutes.[13] Both isoflavones and coumentans are present in a variety of legumes. Soybeans are a very rich source of phytoestrogens. Isoflavones are the most studied phytoestrogens and are found almost exclusively in the family Leguminosae.[25] Soybeans contain approximately 2 g of isoflavones per kg fresh weight; however, it should be noted here that the isoflavone content of different soy products can greatly vary between different soybean varieties and through various ways of processing of soybean.[26,27] The isoflavonoid content in soya bean varies not only with the strain of the soya bean but also with the area in which it is grown.[13] Further, soybean oil does not contain isoflavones, and soy sauce has little or no isoflavones.[14] A phytoestrogen, resveratrol, is found in abundance in red wine. Ipriflavone (7-isopropoxyisoflavone) is a synthetic analogue of isoflavones.[4]

Another class of phytoestrogens is lignans, which are present mainly in oilseeds, such as flaxseed, but they are also present in the cell walls in the fibers of seed, whole cereals, grains, vegetables, fruits, berries, and nuts.[13–15] Lignans are commonly found in flaxseed, pumpkinseed, sunflower seed, cranberry, black or green tea, coffee, garlic, broccoli, bran, and peanuts.[13] Coumestrol is uncommon in the diet and found primarily in lucerne (*Medicago sativa*), clovers (*Trifolium* species), and soya bean sprouts and in high amounts in mung-bean sprouts.[28] Another compound with estrogenic activity is zealalenone, which is produced by molds contaminating cereal crops.[4]

3.4 Chemistry

With a few exceptions, the chemical structures of phytoestrogens contain a diphenolic ring structure, which is a prerequisite for estrogen receptor

binding.[29] This unique diphenolic structure of phytoestrogens provides these compounds an exceptional stability.[15] In foods, isoflavones are present in multiple chemical forms. Isoflavones daidzein and genistein are present either as the aglycone (unconjugated form) or as different types of glycoside conjugates with sugar moiety including 6″-*O*-malonyl-glucosides, 6″-*O*-acetylglucosides, and the β-glucosides of daidzein and genistein.[2] The malonyl and acetyl glycosides are susceptible to heat and readily get converted into more stable β-glucoside.[2] Little is known about the biological activities of individual glycosidic conjugates of isoflavones because they are readily hydrolyzed by the intestinal microflora, making it difficult to directly assess their *in vivo* activity.[30] When steroid hormones are administered orally, conjugates facilitate their absorption. Thus, conjugates of isoflavones may be important in influencing the bioavailability of their respective aglycone structures. In soya bean, major glycosides are daidzein, genistein, and glycetin. These conjugated forms of isoflavones are not estrogenically active and are referred to as precursors. In the stomach and intestines, these glycosides are hydrolyzed to their respective aglycones (nonconjugated form), which are estrogenically active and are referred to as primary isoflavones molecules.[22] In the intestines, this hydrolysis is caused by microflora.

Lignans are a group of dimeric phenylpropanoids that occur in plants as secoisolariciresinol and matairesinol, with glucose residues attached to the phenolic or side chain hydroxyl groups. Glucose groups and methyl groups are removed during the process of fermentation by the colonic bacterial microflora to form the diphenols enterodiol and enterolactone, which are structurally similar to estradiol.[1]

Due to their structural similarity to the human female hormone 17-beta estradiol, phytoestrogens have the ability to bind with estrogens receptors.[31] Phytoestrogens share several features in common with the estradiol structure, including a pair of hydroxyl groups separated at a similar distance; one hydroxyl group is a substituent of an aromatic A ring while the second hydroxyl group lies at the opposite end of the molecule.[22] The estrogen-like structure of phytoestrogens explains the ability of these ligands to interact with estrogen receptors. Therefore, phytoestrogens can display agonistic or antagonistic activity competing for estradiol at the receptor complex.[32–34] However, this interaction is not equivalent to endogenous estrogens as well as among individual phytoestrogens because (1) occupancy time and affinity for estrogen receptors are significantly reduced for phytoestrogens, and (2) there are small differences in the structures of individual phytoestrogens, which dramatically alter their estrogenicity.[22] Daidzein and genistein share identical chemical structures except for an additional hydroxyl group on the A ring of genistein. In the structure of glycitein, there is an extra methoxy group on the A ring as compared to daidzein (see Figure 3.2 and Figure 3.3).

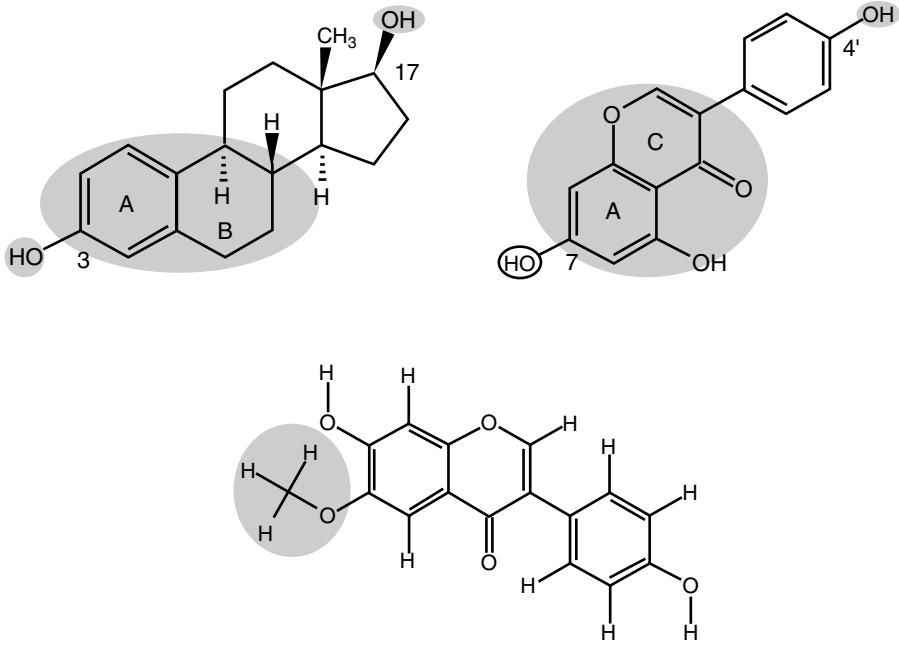

FIGURE 3.2
Structural similarities of genistein, daidzein, and glycitein (CH_3-O-, methoxy).

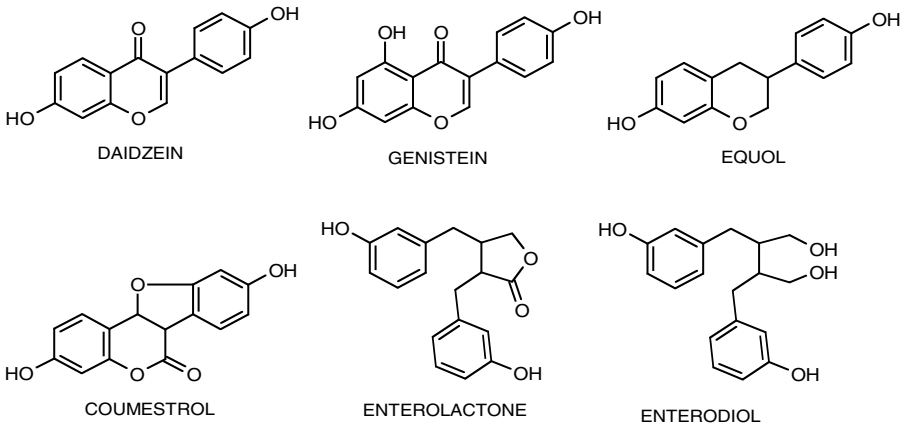

FIGURE 3.3
Structure of phytoestrogens.

3.5 Mechanism of Action

In the early 1940s, it was observed that sheep became infertile after grazing on pastures containing clover in Australia, which gave the first indication that phytoestrogens can have estrogenic effects.[35] Later on, this was termed "clover disease" and was attributed to the consumption of rich amounts of formononetin present in the clover, which is converted to diadzein in the rumen.[36] A theory regarding the inhibitory effects of phytoestrogens against certain types of cancers was postulated in the 1980s due to their similarity in structure to the human female estrogens.[12] It is well known that steroid hormone estrogen is involved in the growth, differentiation, and functioning of many target tissues.[13] Because of a structural similarity between phytoestrogens and endogenous estrogens, many of these actions are mediated through estrogen receptors.[13] Their actions at the cellular and molecular levels are influenced by many factors, such as concentration dependency, receptor status and its site, presence or absence of endogenous estrogens, and the type of effector organ or tissue.[2]

3.5.1 Relative Estrogenic Activity

Estrogen receptors (ERs) are members of the steroid/thyroid hormone receptor superfamily. Human ERs are of at least two types — ER-α and ER-β. Both subtypes differ in the *C*-terminal ligand-binding domain and in the *N*-terminal trans-activation domain.[37] Phytoestrogens have been shown to have greater affinity toward ER-beta than toward ER-alpha.[38] The binding affinities of genistein, dihydrogenistein, and equol were comparable to 17-estradiol, and equol induced transcription most strongly with ER-α and ER-β.[37] Although isoflavones can bind both to ER-α and ER-β, binding affinity of genistein for ER-β is 7- to 30-fold greater.[13] Coumestrol induces transcription as strongly as genistein.[39] The concentration required for maximal gene expression was much higher, and maximal activity induced by these compounds was about half that of 17β-estradiol.[37] Coumestrol binds as strongly as 17β-estradiol to both ER-α and ER-β.[39] Biochanin A, 5-OMe-genistein, formononetin, and tectorigenin bind well to ER-β, but significant binding to ER-α is observed only with 5-OMe-genistein, formononetin, and tectorigenin.[39] The binding of 7-MME-genistein and irisolidone is poor to both ER-α and ER-β.[39] These binding differences suggest that distinct clinical effects may occur when various phytoestrogens selectively trigger ER-β-mediated transcriptional pathways in their own way.

There are also estrogen-related receptors (formerly known as "orphan receptors"), which are expressed to a variable extent in different tissues. The biological effects of phytoestrogens are very complex, as they are large in number and have in common their capacity to perform like human female estrogens by interacting, to a large extent, with estrogen receptors. However, this is not

the only mechanism by which phytoestrogens exert their biological effects, as many of these effects are unrelated to the estrogenic properties of these compounds.[15] Apart from "conventional" ER-mediated response, estrogens have also been shown to act through other pathways in which binding to the receptors is not necessary or in which an estrogen-responsive element in the genome is not involved.[40] Therefore, phytoestrogens will also interact at places where estrogens interact. Combination of responses of endogenous estrogens and compounds present in food and the environment having estrogenic activity make such effects extremely variable between individuals. Therefore, there is a possibility of a vast spectrum of biological effects among different individuals and different organs and tissues in the same individual.[40]

3.5.2 Role in Cancer Prevention

Magee et al. (2004) reviewed many *in vitro* studies that demonstrated the effects of phytoestrogens on the proliferation of both ER (+) (mainly MCF-7) and ER (–) breast cancer cell lines.[15] Genistein is the most studied, as it is present abundantly in soya foods and has been shown to have anticancer effects in animal models. Genistein and lignan metabolite enterolactones exert biphasic effects on the proliferation of ER (+) cell lines, stimulating growth at lower concentrations.[15] Phytoestrogens at higher concentrations, however, were unable to stimulate the growth of ER (–) cell lines; instead, as with the ER (+) cell lines, they inhibited cell proliferation.[15] In another study, stimulation of ER (+) cell lines has been shown by genistein and equol, which correlates with the binding affinities of these compounds to the ER.[41] These studies suggest differential mechanisms for phytoestrogens on cell proliferation. At low concentration, they appear to act via an ER-mediated mechanism, whereas at higher concentrations, a different mechanism of action is exerted on the cells as both ER (+) and ER (–) cell growth is inhibited.[4]

Genistein also induces G_2/M cell cycle arrest in breast cancer cells, which is associated with an increased expression of the cell-cycle inhibitor p21WAF/CIP1 followed by an increase in apoptosis.[42,43] It is also suggested that genistein's mechanism of action is modulated via ER- and p53-independent mechanisms. Thus, genistein may exert the antitumor effects by arresting critical points in the control of the cell cycle and by the induction of apoptosis.[15] Genistein has been shown to inhibit the invasion of MCF-7 cells and ER(–) cell lines, which is independent of ER and p53 status and is characterized by the down-regulation of the 92 kDa type IV collagenase matrix metalloproteinase (MMP)-9 and up-regulation of tissue inhibitor of metalloproteinase-1.[44] It is postulated that the inhibitory effect of genistein on tumor cell invasion is due to its potent inhibitory action on tyrosine kinases, and this theory is supported with preliminary studies that demonstrate that other tyrosine kinase inhibitors also inhibit tumor invasion.[45] In the half-maximal concentrations, phytoestrogens and structurally related

flavonoids (e.g., 3-hydroxyflavone) have been found to potently inhibit angiogenesis.[46] The ability of genistein for growth inhibition was postulated by Kim et al., who suggested that genistein modulates transforming growth factor β-1-signaling pathways *in vitro*.[47]

The phytoestrogens have been shown to influence cell behavior by regulating and altering the metabolism and availability of endogenous estrogens, which are responsible for growth and development of hormone-dependent tumors.[15] Phytoestrogens are potent inhibitors of key enzymes that are involved in metabolism and biosynthesis of estrogens such as sulfotransferases, aromatase, 5α-reductase, and 17β- and 3β-hydroxysteroid dehydrogenase.[15] Genistein inhibits DNA topoisomerases I and II. It also inhibits tyrosine kinases and several other enzymes, including gastric H^+, K^+-ATPase.[48] Phytoestrogens increase the length of the follicular phase of the menstrual cycle in premenopausal women and also suppress midcycle surges of leuteinizing hormone and follicle-stimulating hormone.[49] This suggested that phytoestrogens are protective against breast cancer, as an increase in menstrual cycle length would result in a decreased lifetime exposure to estrogens. Phytoestrogens interfere with the tyrosine kinase activity of activated growth factor receptors and cytoplasmic tyrosine kinases, which are essential for transduction of mitogenic signals, and thereby inhibit tumor cell growth.[3] The isoflavones inhibit intestinal epithelial cell proliferation and induce apoptosis, which is presumed to be the basis of their anticancer effects.[50]

3.5.3 Effects on Bone

Phytoestrogens have been proposed to be anabolic to bone, based on specific effects on osteoblasts and osteoclasts. Phytoestrogens increase bone mass by either increased bone formation by osteoblasts or decreased bone resorption by osteoclasts. Therefore, the phytoestrogens can help in the prevention of osteoporosis. Genistein stimulates proliferation of MC3T3-E1 osteoblast-like cells and protects cells from oxidative free-radical damage.[51] Phytoestrogens increase DNA synthesis, alkaline phosphatase activity, and collagen synthesis and prevent apoptosis in MC3T3-E1 osteoblast-like cells.[52] Overall benefit of bone mass is observed; however, phytoestrogens have controversial effects on calcium content and alkaline phosphatase activity. Various phytoestrogens have different effects on calcium content: Genistein increases, whereas resveratrol decreases, the calcium content in the bone. Some other phytoestrogens, such as glycytein, quercetin, or catechin, have no effect on calcium content in bone.[53] It is now well known that osteoprotegerin (OPG) and receptor activator NF-B ligand (RANKL) can have a pronounced effect on bone formation and resorption.[13] Phytoestrogens have been shown to suppress interleukin-6 and enhance the production of OPG in a dose-dependent manner.[54]

Osteoclasts are formed from hematopoeitic cells of the monocyte/macrophage lineage and are responsible for bone resorption. It is proposed that phytoestrogens inhibit the formation of osteoclasts and that topoisomerase II is involved in the mechanism of action because phytoestrogens have been recognized to inhibit the activities of topoisomerase II and protein tyrosine kinases.[13] In rat osteoclasts, phytoestrogens have been shown to stimulate protein tyrosine kinase activity, inhibiting the inward rectifier K^+ current.[55] It is reported that phytoestrogens inhibit the bone resorption, but studies are not conclusive. Animal studies suggest that phytoestrogens exert a protective effect against bone loss after ovariectomy. Epidemiological studies suggest that phytoestrogens increased bone mineral density.[4] However, it is too early to state that phytoestrogens prevent the bone loss in ovarian hormone deficiency.

3.5.4 Effects on Cognition

The phytoestrogens can significantly improve cognitive performance. The estrogens are involved in the induction of synaptogenesis in the hippocampus, direct effects on excitatory amino acids, and a trophic effect on cholinergic neurons.[56] The estrogen receptors have been localized in glial cells, which influence neuronal functions such as regulation of extracellular ion concentrations and neuronal glucose supply.[57] In menopause, there is a decline in estrogen levels, and in old age, cognitive impairment is common, leading sometimes to Alzheimer's disease. Therefore, a diet containing phytoestrogens can be useful in these circumstances. Dietary soy isoflavones have influence on certain aspects of brain structure, learning, memory, and anxiety along with the brain androgen-metabolizing enzyme, aromatase.[58] Phytoestrogens may influence mental abilities controlled by the frontal lobes.[57] Apart from positive effects on cognitive performance, phytoestrogens also have some significant effects on mood.[57] Several well-controlled studies have found that estrogen replacement therapy significantly improves episodic and semantic memory.[59,60] Belcher et al. (2001) recently reviewed estrogenic actions of phytoestrogens in the brain.[61]

3.5.5 Antioxidant Effects

Phytoestrogens exhibit antioxidant properties, much like any other polyphenols.[62] Moreover, there is a possibility of positive synergy between phytoestrogens and other antioxidants.[63] This may be important in disease processes involving oxidative stress — e.g., reduction of lipid carrying proteins (LDL-cholesterol) in atherosclerosis and oxidation of critical enzymes in the signal transduction pathways through protection of cysteine groups.[64] Isoflavonoids act as free-radical scavengers and prevent DNA damage.[65] Genistein stimulates several antioxidant enzymes, including catalase, superoxide dismutase, glutathione peroxidase, and reductase.[15] This protection

was found to be greater than 17β-estradiol. Cardioprotection by isoflavones at cellular level is due to inhibition of vascular smooth muscle cell migration and proliferation, mitogen activated protein (MAP) kinase activity, antioxidant effect, platelet activation and aggregation, and reduction of platelet serotonin uptake.[13,22]

3.5.6 Other Effects

Isoflavones have been reported as substrates for thyroid peroxidase, which led to speculation that there would be either a fall in conversion from T_3 to T_4 or overcompensation by the thyroid gland, resulting in increased thyroid activity.[66] These studies suggested that thyroid cancer may be prevented via diet containing phytoestrogens, but this idea warrants further research at this time. Isoflavones also inhibit human aldehyde and alcohol dehydrogenase isozymes, raising the possibility that they might be useful in the treatment of alcohol abuse.[67,68] Phytoestrogens modulate the synthesis and release of sex hormone–binding globulin, which binds to endogenous estrogens and prevents their action.[69] Some phytoestrogens have antiandrogenic activities.[4]

3.6 Conclusion

The effects of phytoestrogens on humans are diverse and complex. Various molecular mechanisms responsible for exerting effect of phytoestrogens are being supported by animal and *in vitro* studies. These studies support the role of phytoestrogens in lowering the risk of various types of cancers (especially breast and prostate cancer) and cardiovascular disease. Moreover, epidemiological data also supports the evidence. However, contradicting reports are also coming up simultaneously, which is creating confusion. The exact mechanism of action of phytoestrogens is still unclear and more research is required to establish the role of phytoestrogens in the previously discussed conditions.

Among various phytoestrogens, isoflavones (genistein and diadzein) have been the most extensively studied. Studies on lignans are few, and for coumestans, very few. Study of effects of individual compounds at molecular level is the need of the hour. Based on dietary phytoestrogens, structure–activity relationship studies can be carried out, and more synthetic and semisynthetic compounds (like ipriflavone) can be evolved.

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4

Pharmacokinetics of Phytoestrogens

Devarakonda R. Krishna and Masuram Somashekhar

CONTENTS

4.1 Introduction

Phytoestrogens are plant compounds (diphenols) that can bind to the mammalian estrogen receptor, and they have a wide range of biological effects. Bradbury et al.[1] published a list of 53 plants in 1954 that possessed estrogenic activity. Later in 1975, the list was expanded to over three hundred.[2] Phytoestrogens include isoflavones, coumestans, other bioflavonoids, lignans, and phytosterols, which occur in food in much higher amounts than steroid hormones. The structures of various phytoestrogens are shown in Figure 4.1. *In vitro* studies showed that the affinities of these phytoestrogens to estrogen receptor (ER) range from 10^{-2} to 2×10^{-5} compared to 17β-estradiol. The dietary estrogen equivalents are estimated to be 10^{-2} to 10^{-3}. The isoflavones, genistein (4′,5,7-trihydroxyisoflavone) and daidzein (4′,7-dihydroxyisoflavone), inhibit the growth of human breast cancer and prostate cancer cell lines.[3,4] Genistein suppresses osteoclastic activity and reduces bone loss.[5] Daidzein has been shown to enhance several immunologic functions and

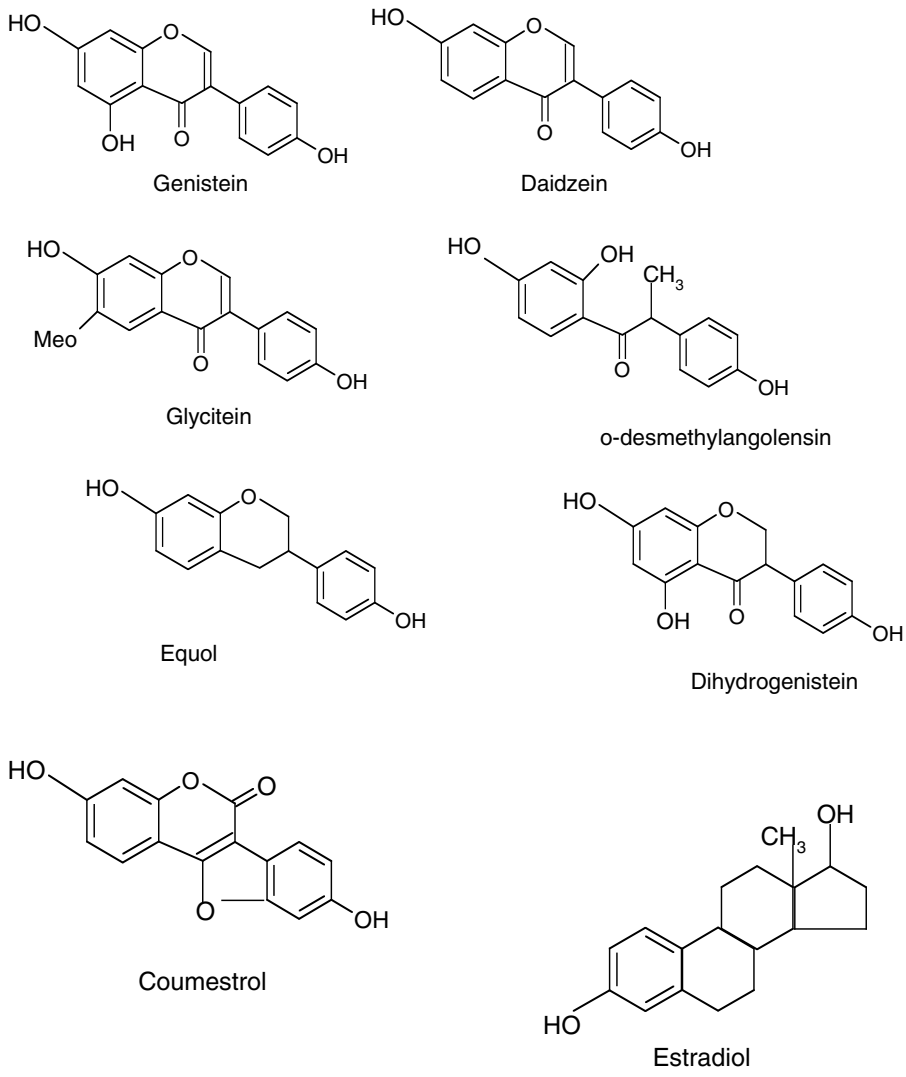


FIGURE 4.1
The structure of phytoestrogens, their metabolites, and estradiol.

lymphocyte activation in animal (mice) and *in vitro* studies.[6,7] Coumestans are biosynthetically related to the isoflavones, and few of them possess uterotropic activity. Coumestrol, the most common of coumestans, acts as an estrogen agonist in the reproductive tract, brain, and pituitary of immature female rats and has antiestrogenic actions such as suppression of estrous cycle.[8] Other flavonoids (chalcones, flavones, flavonols, flavanones) can also stimulate the transcriptional activity of the human ER.[9] Some lignans have antioxidant properties. Similar to other phytoestrogens, phytosterols have been shown to reduce plasma total and LDL cholesterol levels.[10]

4.2 Pharmacokinetics

4.2.1 Absorption

In plants, phytoestrogens occur in at least fifteen different chemical forms. The isoflavonoids, like the lignans, secoisolariciresinol and matairesinol, are conjugated with glucose (to form glycosides), and in soybean, the major glycosides are daidzein, genistin, and glycitin. The complexity of these phytoestrogens is a major barrier in the study of its pharmacokinetics in human subjects. Early studies considered flavonoids nonabsorbable because they occur as conjugates of sugars called glycosides.[11]

The removal of the sugar moiety from the glucoside by β-glucosidase activity is required for the absorption through the intestine into the peripheral circulation.[12] The bioavailability of the isoflavones daidzein and genistein in humans after the ingestion of a single complex meal was efficient compared to that of other flavonoids. However, one study[13] reported that the hydrolysis of glucoside before ingestion does not improve the bioavailability of isoflavones from isoflavone-enriched extracts. Another study[14] also confirmed that previous hydrolysis of glycosides to aglycones does not enhance the bioavailability of isoflavones in humans. Lactase phlorizin hydrolase, a β-glycosidase present in the brush border of the mammalian small intestine, is capable of hydrolyzing isoflavone glycosides, thereby making the free isoflavones available for resorption.[15] Uehara et al.[16] conducted a study to show that fructo-oligo saccharides modified the absorption and the enterohepatic circulation of soy isoflavones. However, in an *in vitro* study,[17] combining *Lactobacillus reutri* strain with soygerm powder in fermented milk, showed that the *Lactobacillus* strain cleaved the β-glycosidic isoflavones, exhibiting advantageous probiotic effects and enhancing bioavailability of the isoflavones. It is yet to be shown whether isoflavone glycosides can be directly absorbed intact in humans. It was shown in an isolated rat small intestinal perfusion model that only 1.4% of genistin crossed the intestinal wall after perfusion of the luminal compartment with pure genistin.[18] Similarly, only 2.1% of genistin and 2.6% of daidzein appeared on the vascular side after luminal perfusion of each of these glycosides in the form of a simulated digested tofu mixture.[19] These are extremely small proportions, and the concentrations measured in these studies are close to the detection limits of the HPLC methods used. It is evident that there is negligible uptake of glycosides across the apical membrane of the rat enterocyte. The use of monolayers of human Caco-2 cells *in vitro* also showed that the intracellular efflux of genistin across the apical membrane to the luminal side is negligible.[20]

Setchell[21] reported that the presence of food matrix may limit the bioavailability of isoflavones present in soybeans. Healthy adults absorb isoflavones like daidzein and genistein rapidly and efficiently. In most adults, the time required to attain peak plasma concentration after ingesting their aglycones

is 4 to 7 h, whereas when the β-glycosides are ingested, the t_{max} shifted to 8 to 11 h, indicating that the rate-limiting step for absorption is initial hydrolysis of the sugar moiety.[22] The early rapid increase in plasma isoflavone concentration could be due to initial absorption of aglycones in the stomach, duodenum, and proximal jejunum. Isoflavones, like estrogens, have been shown to undergo enterohepatic recycling, and when administered orally, they soon appear in bile.[23–25] The systemic bioavailability as determined by comparing dose-normalized AUCs is greater in β-glycosides than for corresponding aglycones.[22]

There are abundant β-glycosides along the entire length of the human intestine sufficient to hydrolyze isoflavone glycosides. It has been shown that the activity of β-glucosidase shows a pattern of developmental expression, as it is present early in life.[26] Therefore absorption of isoflavones contained in soy infant formula is high, resulting in very high plasma concentrations in infants.[27] There are a number of membrane-bound β-glucosidases[28] that are expressed in jejunum. For most dietary isoflavones, the glucose residue is at position 7 of the molecule, and intestinal β-glucosidases show high affinity for such isoflavones.[29] The pKa (negative logarithm of equilibrium constant for association) of isoflavone aglycones is favorable for nonionic passive diffusion from the jejunum. Studies using Caco-2 TC7 monolayers have demonstrated that genistin does not readily penetrate the enterocyte, whereas its aglycone, genistein, is readily permeable. Therefore, it is evident that the bioavailability of isoflavones is contingent on hydrolysis of the glycosidic moiety. The greater bioavailability of isoflavones when the isoflavone glycosides are ingested is because the glycoside moiety acts as a protecting group to prevent biodegradation of the isoflavone structure.[22] Isoflavones undergo significant intestinal metabolism in humans to produce a number of metabolites.[30,31] The protecting groups prevent biotransformation of the parent drug, thereby improving its absorption.

It is evident that isoflavone glycosides are not absorbed across the enterocyte even though daidzein and genistein reach high concentrations in plasma. Some absorption of aglycones, but not β-glycosides, was shown to take place in the rat stomach.[32] This may also be the case with humans, since absorption rates of aglycones are faster than β-glycosides.[22,33] The plasma kinetics, notably the long t_{max} of the isoflavone β-glycosides in healthy humans, suggest that most of the hydrolysis must occur distally and is most likely of bacterial action.[34] However, quercetin glycoside is more bioavailable than its aglycone as measured from the appearance of quercetin in the plasma.[35,36] Therefore, intact flavonoid glycosides can be absorbed, even though the glycoside is not measured in plasma. Part of the hydrolysis of soy isoflavones, like that of the flavonoid glucosides of quercetin,[37] may occur in the proximal intestine, which is consistent with the presence of several cytosolic and brush border membrane-bound β-glycosidases.[28,38–40] *In vitro* intestinal perfusion studies have shown that the hydrolysis of isoflavone glucosides occurs on the luminal side.[29] The role of intracellular

β-glycosidase[39] is difficult to assess, and it could be speculated that it hydrolyzes any isoflavone glycosides that penetrate apical membrane.

The soy isoflavones can be extensively degraded by gut bacteria to a series of metabolic products, including dihydroisoflavones, desmethylangolensin, equol, and *p*-ethyl phenol,[30] but once absorbed, they are not further metabolized, except for the formation of glucuronide and sulfate conjugates.[41] Thus, several factors may influence the bioavailability of the isoflavones, including those that may affect the rate and extent of bacterial deconjugation and degradation as well as factors that may influence the rate and extent of transport of the isoflavones across the gut wall and of excretion in urine and bile. The conjugates of daidzein are more bioavailable than those of genistein in rats.[42] Daidzein has also been shown to be more bioavailable than genistein in women.[43] These results are contradictory to the results published by Setchell et al.[44] that systemic bioavailability and maximum serum concentrations of genistein are significantly greater compared to daidzein. No studies of the absorption of conjugated as compared to unconjugated isoflavones have been conducted in human subjects. The finding of equol, a metabolite of daidzein, in high concentrations in portal venous blood of rats and in bile established enterohepatic circulation for isoflavones and lignans.[23] Recent studies of the pharmacokinetic behavior of genistein in rats also confirmed this commonality with endogenous estrogens.[45,46] Isoflavones undergo biliary excretion,[47] whereas enterohepatic recycling of lignans in humans has not been reported. Zhang et al.[48] studied the bioavailability and metabolism of daidzein, genistein, and glycitein in male and female subjects after consuming soymilk (high in genistein and daidzein) and soygerm (high in daidzein and glycetein). Based on plasma isoflavone concentrations at 6 h after dosing, the bioavailabilities of daidzein and genistein were similar in men and women. At the high glycetein dose (soygerm), plasma concentration at 24 h after dosing suggested a modest gender difference in glycitein bioavailability. Recent studies have demonstrated that deglycosylation of genistin (genistin 7-*O*-glucoside) to genistein already begins in the mouth[49] and then continues in the small intestine.[29]

Lignan glycosides are probably hydrolyzed in part by gastric acid.[43] When consumed, lignan glycosides are not absorbed, or are poorly absorbed, from the small intestine because of their hydrophobic nature, and because they are β-glucosides, they are not easily hydrolyzed by mammalian enzymes but are readily hydrolyzed by bacterial enzymes.[50] It was observed very early that glucosides undergo a sequence of metabolic changes necessary for their conversion to mammalian lignans and subsequent absorption and utilization. The chemical reactions involve hydrolysis of the sugar moiety, dehydroxylation, demethylation, and further oxidation. Therefore, intestinal bacterial metabolism has profound effects on conversion, enterohepatic circulation, and bioavailability of lignans. It was also found that administration of antibiotics almost completely eliminates the formation of enterolactone and enterodiol from plant precursors in gut.[51,52] It has been shown that quercetin, a flavonol, is bioavailable from foods such as onions, tea, and

apples,[53] which are its main dietary sources.[54] Similarly naringenin, a flavanone, is bioavailable from citrus.[55] It is evident that the bioavailability of quercetin and other flavonoids is greatly affected by the type and binding site of the sugar moieties.[56]

4.2.2 Distribution

Limited data is available on the distribution of phytoestrogens. Classical pharmacokinetics of isoflavones have established that the volume of distribution in adults is large, indicating a wide tissue distribution. Peak appearance and disappearance in pre- and postmenopausal women are similar.[57] Although plasma and urine levels of isoflavonoids have been thoroughly investigated, little is known about their tissue distribution, which is particularly important for tissues with high endogenous estrogen and estrogen receptor concentrations. In a recent study, Maubach et al.[58] investigated the concentrations of genistein, daidzein, and equol in human breast tissue homogenate. They found that intake of soy-based food supplements for 5 consecutive days did not result in significantly higher genistein, daidzein, and equol concentrations in breast tissue homogenate when compared with placebo group. The concentrations were in the low nanomolar range, whereas in the corresponding serum samples, concentrations were 100-fold higher. However, in an earlier study by Maubach et al.,[59] equol (less than 1 μmol/l homogenate) was found to be the predominant phytoestrogen in breast tissue, and its concentrations exceeded those in serum.

In rats, the fetal or neonatal serum concentrations of total genistein were approximately 20-fold lower than maternal serum concentrations, although the biologically active genistein aglycone concentration was only fivefold lower. Fetal brain contained predominantly genistein aglycone at levels similar to those in the maternal brain.[60] This study showed that genistein aglycone crosses the rat placenta and can reach fetal brain from maternal serum genistein levels. In rats, endocrine-responsive tissues including brain, liver, mammary, ovary, prostate, testis, thyroid, and uterus showed significant dose-dependent increases in total genistein concentration. Female liver contained the highest amount of genistein (7.3 pmol/mg tissue), and male whole brain contained the least (0.04 pmol/mg).[61]

Adlercreutz et al.[62] showed in humans the free transfer of phytoestrogens from the mother to the neonate based on measurements in maternal and cord plasma. These results suggest that the fetus may be exposed to high circulating levels of phytoestrogens, which may elicit toxic responses that may otherwise be innocuous to the mother. The phytoestrogen levels in the biological fluids of different populations derived from epidemiological studies are presented in Table 4.1. The V_d/F (average volume of distribution normalized to apparent bioavailable fraction; where F is the bioavailability fraction) was large for both daidzein (236.4 ± 35.9 l) and genistein (161.1 ± 44.1 l), indicating extensive tissue distribution.[22] Isoflavones have

TABLE 4.1

Phytoestrogen Levels in the Biological Fluids of Different Populations[a]

	Ethnicity	Biological Fluid	Genistein	Daidzein	Equol	O-dma	Enterolactone	Enterodiol	Matairesinol	Ref.
1.	Americans									
	Long-term soy[b]	Urine[c]								102
	With fiber	(μmol/24 h)	8.8	9.9	2	0.8	4.6	nd	nd	
	Without fiber		10.5	12.9	2.4	1.1	8	nd	nd	
	Short-term soy[b]									102
	With fiber		8.6	14.3	2	2.2	5.5	nd	nd	
	Without fiber		6.7	11.8	1.9	2.6	6.7	nd	nd	
	Vegetarians	Urine (μmol/24 h)	0.22	0.56	0.08	0.13	2.12	0.45	0.07	122
2.	British									
	Omnivores	Prostatic fluid (nmol/l)	nd	44.5	2.1	nd	68	8.6	nd	123
3.	Chinese									
	Omnivores	Prostatic fluid (nmol/l)	nd	95.7	120.7	nd	110	22	nd	123
4.	Japanese									
	Omnivores	Urine (μmol/24 h)	4.9	2.5	0.25	0.35	0.5	0.27	0.01	100
5.	Finnish									
	Omnivores	Plasma (nmol/l)	4.9	4.2	0.8	0.07	29.0	1.4	0.02	124
		Urine (nmol/24 h)	184.4	133.5	37.5	1.45	2,350	161	nd	125, 126
	Vegetarians	Feces (nmol/24 h)	11.6	45.4	14.9	5.67	1,510	147	22.3	95
		Plasma (nmol/l)	17.1	18.5	0.7	0.8	89.1	5.4	0.06	124
		Urine (nmol/24 h)	nd	94.0	72.8	14.4	7,400	436	nd	125

Note: nd = Not determined.

[a] Values are geometric means (urine) and mean values of total (plasma, feces, prostatic fluid).

[b] Long-term soy: Women consuming soy protein in long-term. Short-term soy: Women consuming soy protein in short-term.

[c] Values are back-transformation of log-transformed least-squares means.

been found to appear in nipple aspirates,[63] whereas studies with rats have shown that they rapidly partition into the brain.[64,65]

4.2.3 Metabolism

The metabolism of phytoestrogens has not been studied in detail until recently. The studies on isoflavones have been focused mostly on daidzein and genistein as well as on the principal metabolites of red clover isoflavones, formononetin, and biochanin A. Little attention has been paid to other isoflavones present in soy, such as glycitein. The metabolic studies on metabolism of the red clover isoflavones, formononetin, and biochanin A in humans have not been comprehensive.

One of the metabolites of daidzein, the isoflavan equol, is structurally very similar to the endogenous hormone estradiol.[66] Only one third of the population in humans is capable of producing it,[67] depending on the composition and enzymatic capability of gut microflora. Recently this metabolite has gained more interest because of its particular benefit. Both the phase I and phase II metabolites have been identified in human body fluids after isoflavone supplementation.

4.2.3.1 Phase I Metabolism

4.2.3.1.1 Reduction

The studies on isoflavone metabolism have been focused on reductive metabolism of soy isoflavones daidzein and genistein. Equol[68,69] and O-desmethylangolensin (O-dma)[70] have been identified in human urine as the end products of metabolism of daidzein. An isomer of equol — 3′,7-dihydroxyisoflavan — was reported in human urine.[71]

A comprehensive study on soy isoflavone metabolism of daidzein and genistein was carried out by Kelly et al.[67] The main metabolites reported of daidzein were equol, dihydro-daidzein and O-dma (Figure 4.2). In another study,[31] minor metabolites of daidzein, namely, 2-dehydro-O-dma, and two isomers of tetrahydrodaidzein, better known as 4-OH-equol (isoflavan-4-ol), were also reported. Two novel metabolites of genistein, dihydrogenistein and 6′-OH-O-dma, were identified (Figure 4.3). The presence of the *cis* isomer of 4-OH-equol, dihydrogenistein, and 6′-OH-O-dma in urine samples was also demonstrated with authentic reference compounds.[72] Unlike Kelly et al.,[67] Heinonen et al.[72] found that dihydrogenistein is an abundant metabolite of genistein and only 6′-OH-O-dma is only a minor metabolite of genistein in all investigated urine samples. The discrepancy could be explained by different silylation reagents used for silylation of samples for GC-MS. In addition, Heinonen et al.[72] identified two analogues of O-dma with additional hydroxyl substitution in either phenolic A- or B-ring. One of the metabolites, 5′-OH-O-dma, was assumed to originate from glycitein by demethylation and reduction, but no origin for the other metabolite,

3′-OH-O-dma, was identified. Three new metabolites of glycitein were identified in urine samples collected after soy supplementation. The structures of dihydroglycitein and the equol analogue of glycitein, 6-OMe-equol, were characterized with authentic reference compounds, while the identification of 5′-OMe-O-dma was carried out by interpretation of the mass spectrum of the trimethylsilylated compound.

The presence of 3′,7-dihydroxyisoflavan,[71] an isomer of equol, in urine samples has also been confirmed with an authentic reference compound. In animal studies, two reduced metabolites of formononetin, 4′-*O*-methylequol and angolensin, have been identified in the urine of sheep.[73,74]

Hur et al.[75] detected two strains of bacteria capable of producing primary and secondary metabolites from daidzein and genistein. Both *Escherichia coli* HGH21 and the gram-positive strain HGH6 converted daidzein and genistein to their respective aglycones daidzein and genistein. Under anoxic conditions, strain HGH6 further metabolized the isoflavones daidzein and genistein to dihydrodaidzein and dihydrogenistein, respectively. The reduction of a double bond between C-2 and C-3 to a single bond was isoflavonoid-specific by strain HGH6.

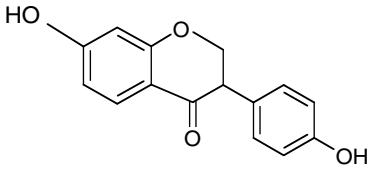
4.2.3.1.2 Demethylation

Isoflavones that have methoxyl groups are demethylated, the extent of demethylation depending on the position of the methoxyl group. The red clover isoflavones formononetin and biochanin A, which have 4′-methoxyl groups at B-ring, are almost completely converted to the demethylated metabolites daidzein and genistein. The demethylation of glycitein, the soy isoflavone that has a methoxyl group at 6-position, occurs to a lesser extent.[76]

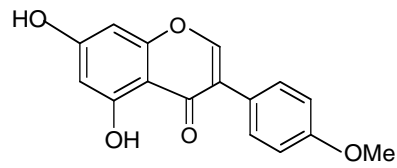
4.2.3.1.3 Hydroxylation

The oxidative metabolism of genistein by rat and human cytochrome P450s was studied.[77] In rat liver microsomes, the metabolism of genistein is NADPH- and time-dependent, and the metabolism of genistein was noticed to be affected by different P450s yielding different product profiles. Five oxidized metabolites were found, one of them being orobol (3′,4′,5,7-tetrahydroxyisoflavone). Two other metabolites were suggested to be hydroxylated at 6- and 8-positions, while structures of the remaining two metabolites remained unknown. Both isoflavones, daidzein and genistein, are good substrates for cytochrome P450 enzymes and are extensively metabolized. Ten new metabolites were reported for daidzein and six for genistein. Most of the metabolites were formed by hydroxylation at an ortho position of the existing hydroxyl group in the phenolic rings yielding mono-, di-, tri-, tetra-, and pentahydroxylated metabolites. One monohydroxylated metabolite daidzein and one monohydroxylated metabolite of genistein were suggested to be hydroxylated at 2-position of the C-ring.[78,79]

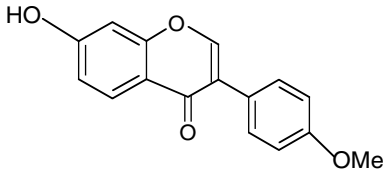
Jacobs et al.[80] has identified nine novel metabolites of enterolactone and enterodiol in the urine of female and male humans ingesting linseed for



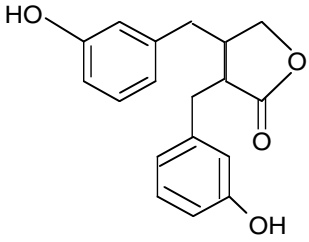
Dihydrodaidzein



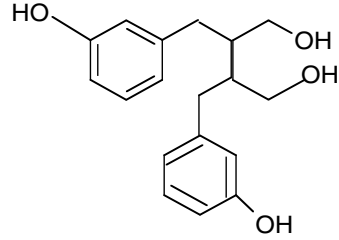
Biochanin A



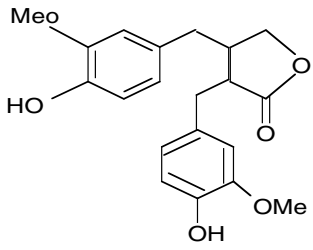
Formononetin



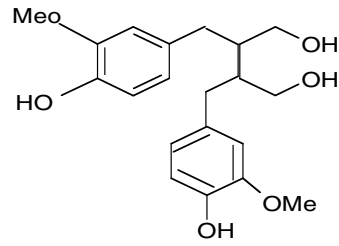
Enterolactone



Enterodiol



Matairesinol



Secoisolariciresinol

FIGURE 4.2
The metabolic pathway of daidzein.
1: Dihydrodaidzein
2: O-dma
3: Equol

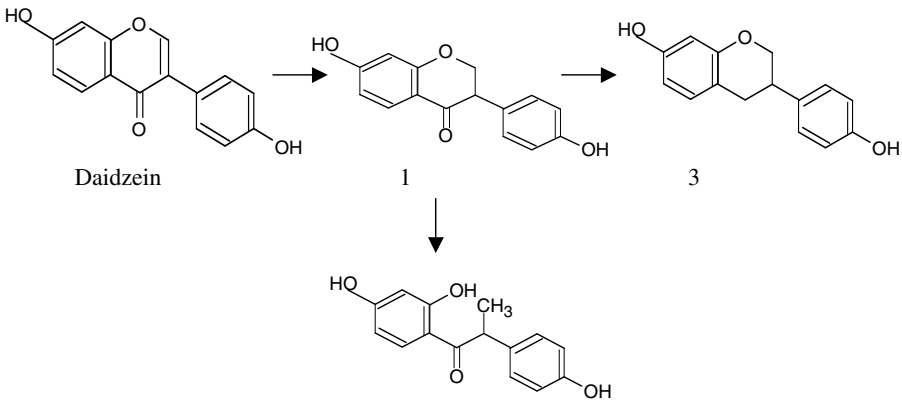


FIGURE 4.2
(Continued)

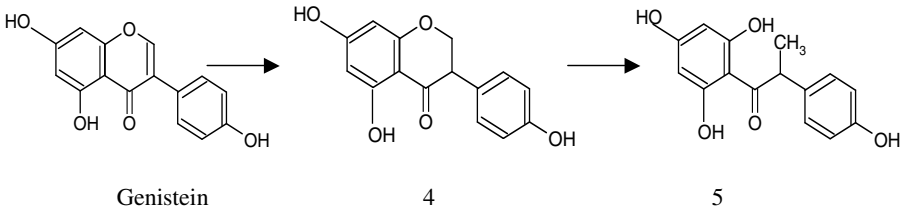


FIGURE 4.3
The metabolic pathway of genistein.
4: Dihydrogenistein
5: 6′-OH-O-dma

5 days. The six identified metabolites of enterolactone were the products of monohydroxylation at the para-position and at both ortho-positions of the parent hydroxy group of either aromatic ring. Similarly, the three enterodiol metabolites were formed through aromatic monohydroxylation at the para- and ortho-positions. However, two of the six compounds among enterolactone metabolites and one of the three among enterodiol metabolites may be intermediates of the bacterial conversion of secoisolariciresinol (SECO) and/or matairesinol (MAT) to enterolactone or secoisolariciresinol to enterodiol, respectively.

4.2.3.1.4 Methylation

Methylation of isoflavones having two vicinal hydroxyl groups seems to be a minor metabolic reaction of isoflavones. Four methylated metabolites with an isoflavone structure in human urine after soy supplementation have been identified.[79] One of the metabolites was suggested to be 3′- or 4′-*O*-methyl-7,3′,4′-trihydroxyisoflavone, and three others, one dimethylated and two

monomethylated metabolites, were proposed to be formed by methylation of 6,7,3′,4′-tetrahydroxyisoflavone.

4.2.3.1.5 C-Ring Fission

The metabolism of flavonoids is known to proceed to metabolites like phenolic acids and resorcinols that do not have an intact flavone skeleton.[81] The metabolism of isoflavones to similar smaller metabolic products has not been fully established. For many years, *p*-ethylphenol was considered the end metabolite of genistein, which was recognized first in urine samples of sheep and later in urine of human subjects. The main metabolite of genistein after 48 h is 2-(4-hydroxyphenyl)-propionic acid, HPPA.[82] The *in vitro* data strongly suggests that *p*-ethylphenol is merely formed from isoflavones other than genistein.[83] The presence of HPPA in human urine has been confirmed, but at lower concentrations than reported for rats.[84]

4.2.3.2 Phase II Metabolism

Only few studies on phase II metabolism of isoflavones in humans have been carried out. Apart from the main glucuronide conjugates, sulfates and sulfoglucuronide conjugates have also been identified.[85] Doerge et al.[86] studied the formation of isoflavone conjugates *in vitro* by using recombinant human UDP glucuronosyl transferase (UGT) and sulfotransferase (SULT) isoforms or microsomes isolated from several human tissues. With purified bovine UGT, two glucuronide conjugates, 7- and 4′-glucuronides, of both daidzein and genistein were formed. The experiments carried out with SULT enzymes showed that genistein was readily converted to sulfate conjugates. None of the tested enzymes had any activity toward daidzein. However, the identification of sulfate conjugate of daidzein in human blood samples led the authors to suggest the participation of other SULT isoforms *in vivo.* For daidzein, genistein, and glycitein, two isomeric glucuronides and one putative sulfate conjugate were identified in plasma samples of volunteers who had ingested soy-based dietary supplements. A number of different glucuronide conjugates of soy isoflavones and their main phase I metabolites have been identified in rat urine samples.[87] In plasma, isoflavonoids and lignans occur as free compounds and as mono- and disulfates, as mono- and diglucuronides, and as sulfoglucuronides.[88,89] As shown for Finnish postmenopausal women, the free sulfate fraction is low for genistein (3.8% of total), but for enterolactone and enterodiol, it is as much as 21 to 25%.[90] The efficiency of conjugation of isoflavones is high, and consequently the proportion of circulating free isoflavones is small.[91] The finding that enterolactone, enterodiol, and equol are predominantly conjugated to glucuronic acid in portal venous blood[92] suggests that conjugation of phytoestrogens may occur in the intestinal wall during absorption from the gut. This has been subsequently confirmed in rats by using averted intestinal sac preparations.[25] The metabolism of coumestans has not been characterized.

4.2.4 Excretion

Lignans and isoflavonoids are mainly excreted as monoglucuronides in urine, but small amounts of diglucuronides and sulfates also occur.[23,69,92–94] In feces, these phytoestrogens are excreted as free forms (more than 90%).[95] Most studies of the metabolism of phytoestrogens have focused on their urinary excretion. Human metabolism and excretion of isoflavonoids after soy consumption showed individual variation.[43,96,97] Early studies reported urinary equol excretion after a soy challenge[34,94] and urinary enterolactone excretion after a linseed challenge.[98] Equol formation may depend on diet; a high fat/meat content diet increases equol production.[94,99] Japanese men and women who consumed more fat and meat showed significantly higher urinary excretion of equol than the other subjects.[100] However, in another study by Lampe et al.,[101] it was shown in women that equol excretors consumed a significantly higher percentage of energy as carbohydrate and greater amounts of plant protein and dietary fiber compared to nonexcretors. Such differences were not observed in men, though they had higher fiber intakes than women. Therefore, in women, dietary fiber or other components of a high-fiber diet may promote the growth or the activity of bacterial populations responsible for equol production in the colon. Similarly, good equol producers excreted about 200-fold more equol in their urine than the poor equol producers excreted, and they consumed significantly less fat and more carbohydrate, and also greater amounts of nonstarch polysaccharides, compared with the poor equol producers.

Lampe et al.[102] showed that a daily 16-g dietary fiber dose as wheat bran and the addition of soy protein do not significantly modulate the capacity of colonic microflora to produce equol. Furthermore, in 74 premenopausal women, no differences in usual diet were detected between equol excretors and nonexcretors. These results suggest that equol excretor status is a relatively stable phenotype that is not strongly associated with diet and not readily altered by diet.

Dietary fiber intake correlates with the urinary excretion of lignans[68,103,104] and also with the excretion of isoflavones.[105] The intake of total fiber from berries and fruits, vegetable fibers, and legume fiber correlated significantly with the urinary excretion of lignans and isoflavones.[105] In 19 premenopausal women who consumed 10 g of ground linseed per day for three menstrual cycles, both urinary[106] and fecal[107] lignan excretion increased and varied greatly among subjects.

The recovery of genistein in urine, expressed as a percentage of the dose, from humans has been variable — as low as 1%[96] and as high as 39%.[43] In rats,[108] 0.73% of a 500 mg/kg dose of daidzein was recovered in urine. In a study conducted by Zhang et al.,[48] the average 48-h urinary excretion of glycitein, daidzein, and genistein was about 55, 46, and 29% of the dose ingested, respectively. The excretion of these three isoflavonoids differed significantly from each other in men and women.

The fecal bacteria could rapidly degrade genistein in humans with average half-life of 5.0 h and daidzein of 17 h.[103] In another study,[48] the mean fecal degradation half-lives of genistein and daidzein were 8.9 and 15.7 h, respectively, in healthy men and women. Less fecal degradation would result in the appearance of greater amount of isoflavones in the circulation and greater urinary isoflavone excretion. The average urinary excretion of daidzein (46.4%) was nearly twofold that of genistein (28.7%), which agreed with the average fecal metabolism results.[48] Dietary patterns may be able to alter gut motility and fecal isoflavone degradation. For example, increasing insoluble dietary fibers would increase fecal bulk and decrease gut transit time[110] and also decrease microorganism populations, which would influence isoflavone metabolism and absorption. Karr et al.[111] conducted a diet-controlled study in 14 subjects in four 9-day diet treatment periods. Urinary excretion of genistein varied by as much as 12-fold and daidzein by as much as 15-fold within diet treatments.

In a study conducted by Lu et al.,[112] the isoflavone metabolism and disposition were affected by the duration of soy ingestion and by sex. Women excreted more of their daidzein intake (as daidzein glucuronide and sulfate) than did men. This was also observed in 15- to 17-year-old subjects, where females excreted 69 ± 14% of their daidzein intake as daidzein glucuronide and sulfate and males excreted 40 ± 7% in urine ($p = 0.02$).[113] No significant differences between males and females in urinary recoveries of genistein glucuronide and sulfate were observed after acute soy ingestion.[112] Thus the elimination $t_{1/2}$ of both isoflavones was longer in women (4.4 ± 0.7 h for daidzein, 6.7 ± 0.8 h for genistein) than in men (2.9 ± 0.5 h for daidzein, 3.8 ± 0.7 h for genistein; $p < 0.001$).[112] It should be noted that in addition to mixed sulfoglucuronide conjugates, genistein and daidzein form both mono- and disulfate and mono- and diglucuronide conjugates.[85] It was reported that 2.5 ± 1.9% of genistein is present in human female urine as the monosulfate, compared with 5.7 ± 2.5 for disulfates. For daidzein, the percent is 2.8 ± 1.6 compared with 2.0 ± 1.1, respectively.

Urinary isoflavone concentrations in soy-fed 4-month-old infants are highly variable (range: 5 to 1300 μg/l), which may reflect individual differences in the maturation of digestive capability.[114] The urinary isoflavone concentrations observed by Irvine et al.[115] in infants were higher and averaged 2.9 ± 0.3 mg/l for daidzein and 1.5 ± 0.2 mg/l for genistein. This variability in the excretion of isoflavones may be due to the spot collection of urine. The mean percentage of the daily isoflavone intake recovered in the infants' urine was 38 ± 4% for daidzein and 13 ± 3% for genistein.[115] These values are similar to those measured in adults (21% and 9%, respectively,[116] and 46.9% and 14.6%, respectively[117]). Furthermore, adults and infants both seem to excrete relatively more of their intake of daidzein than of genistein.

The prevalence of equol excretors in the study group of 60 subjects was 35%, and there was no significant difference in prevalence of equol excretion in men (43%) compared with women (27%).[118] Elimination half-lives were

4.7 ± 1.1 and 5.7 ± 1.3 h for daidzein and genistein, respectively.[119] Peak rates of urinary excretion occurred between 6 and 12 h after the meal, with more than one-half of total excretion occurring during the first 12 h.[119] The higher urinary excretion of daidzein compared with genistein suggests a greater fractional excretion of latter via the bile.[116,120] The ratio of the total amount of daidzein excreted in urine to that in feces ranged from 5 to 30, that of genistein ranged from 4 to 50, that of O-dma ranged from 0.5 to 11.8, and that of equol ranged from 0.7 to 6.4 in the individual subjects.[121] In the same study, 2.9% of the genistein and 4.6% of the daidzein were found in the feces. In a study conducted by King et al.,[42] urinary excretion of daidzein over the 48-h postdose period was 17.4 ± 1.2% of the dose, but only 11.9 ± 1.1% of the genistein dose was excreted in urine. Equol excretion was 5.0 ± 1.5% of the genistein dose. Equol excretion was 5.0 ± 1.5% of the daidzein dose, but 41.9 ± 5.0% of the genistein dose was excreted as 4-ethyl phenol. Fecal daidzein accounted for 2.3 ± 0.5% and fecal genistein for 3.4 ± 0.4% of the respective doses.

Intake of total dietary fiber and fiber from grains was most strongly associated with lignan excretion.[122] Compared with omnivores, vegetarians excreted significantly higher amounts of lignans in urine and feces.[95,103] Urinary enterolactone levels in the high vegetable and fruit intake groups were 2.53 μmol/day,[122] in omnivores in Boston and Helsinki, the levels were 2.05 and 2.46 μmol/day, respectively, and in women eating an omnivorous diet containing <10g fiber/day, the levels were 2.55 μmol/day.[106] These levels were lower than for Bostonian lactovegetarians (4.17 μmol/day) and macrobiotics (17.68 μmol/day).[103] The low vegetable and fruit intake group had the lowest enterolactone level (1.77 μmol/day). Enterodiol excretion followed a similar pattern.[122]

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5

Phytoestrogens in Cell Signaling

Ozgur Kutuk and Huveyda Basaga

CONTENTS

5.1 Introduction

Phytoestrogens are polyphenolic nonsteroidal plant-derived compounds that structurally and functionally mimic mammalian estrogen 17-β-estradiol (E2) and therefore are considered to play an important role in the prevention of cancer, heart disease, menopausal symptoms, and osteoporosis. They bind to ERs and exert the characteristics of endogenous steroidal estrogen signaling. Cell cycle arrest, apoptosis, cell proliferation, and cell differentiation are all mediated through signal transduction processes, and many transducer proteins, such as protein kinases, phosphatases, and proteases, are involved in these important cellular functions. Therefore, many laboratories are

currently investigating the effects of estrogens and phytoestrogens on the activities of these transducer proteins. Considering the large spectrum effects that estrogens have on the human body, it is important to determine the potential of phytoestrogens for human health as they mimic endogenous estrogens and cause estrogenic effects. The outcome of numerous studies reported will be selectively described and discussed in this chapter.

5.2 Cell Signaling and Its Importance

The cell is the absolute target for all physiological or pharmacological stimuli. All cells receive and process signals not only from the plasma membrane but also from different compartments within the cells. These signals involve alterations in the availability of nutrients, growth factors, and cytokines as well as oxidative, heat, or mechanical stress. Specific pathways receive signals simultaneously and transmit them to their corresponding targets; this refined type of cellular information transfer is not through linear freestanding pathways but through strictly controlled signaling networks.[1,2] This dynamic characteristic of cells enables them to interpret and respond to environmental or intracellular changes, a phenomenon that is fundamental to life (Figure 5.1).

Cellular signaling is mediated by signaling molecules, which are expressed on signaling cell in the case of cell–cell interactions or secreted directly to the extracellular environment. Most of these stimuli consist of chemical compounds transporting biological information, the so-called first messengers or ligands. They are synthesized and released by signaling cells and produce a specific response in target cells that have a receptor for that particular signal. These signaling molecules involve proteins, amino acids, steroids, fatty acids, and gases such as nitric oxide, and most of them are hydrophilic and therefore cannot pass through the plasma membrane directly; instead they bind to receptors on the target cell surface.

Receptors are the gatekeepers for cellular signaling and they exhibit specific and high-affinity binding to their corresponding ligands. Virtual receptors are transmembrane proteins on the cell surface, and once activated by an extracellular ligand, they initiate a series of signaling events that result in various cellular responses depending on the nature of the stimuli. However, some receptors are located inside the cells and they are targets for small, hydrophobic first messengers, which are able to diffuse across the plasma membrane.

In general, there are three types of cellular signaling models:

- Endocrine signaling, in which the ligands are usually hormones and they act on relatively distant sites from where they are produced and secreted

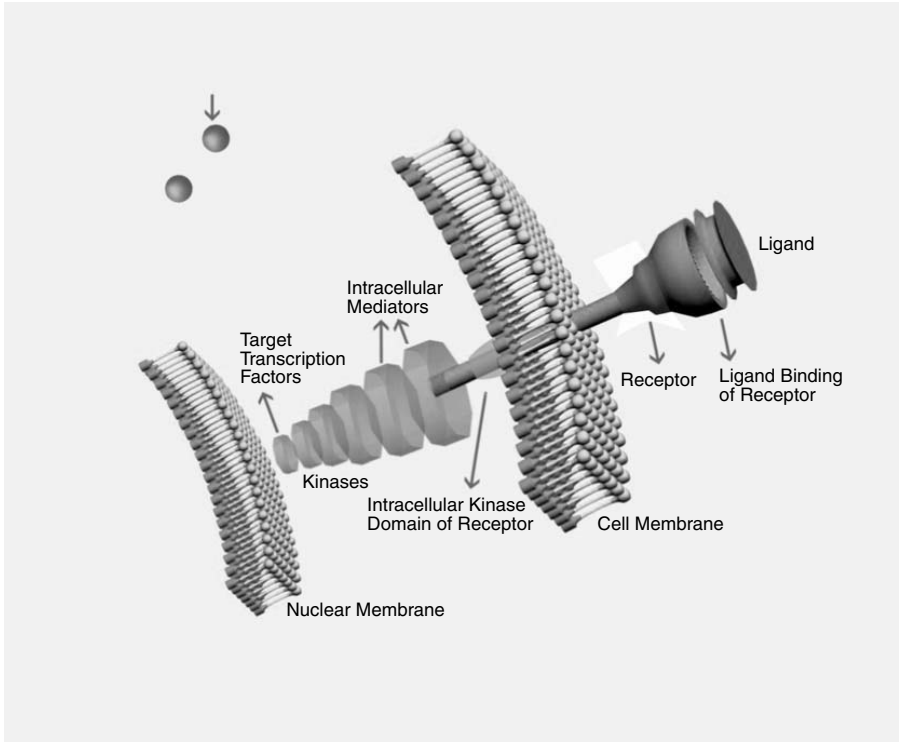

FIGURE 5.1
Signal transduction.

- Paracrine signaling, in which signaling molecules act on a neighboring target cell
- Autocrine signaling, in which a cell responds to a signaling molecule produced and secreted by itself

In most cases, steroid hormones (e.g., estradiol, testosterone) and other hydrophobic small signaling molecules act via endocrine signaling cascade. They are synthesized and secreted into the circulation by specialized endocrine cells and they bind to their corresponding intracellular receptors following direct diffusion across the plasma membrane. These intracellular receptors may be both in the cytosol or nucleus, and upon stimulation by the signaling hormone, they bind to unique hormone response elements in the promoter region of specific target genes. In addition to this classical hormone-signaling model, recent studies implicate ligand-independent and nonnuclear surface receptor-dependent models for hormone receptors. Although these untraditional actions of hormone receptors need further mechanistic investigation, these pathways seem to be involved in many physiological and pathological cellular responses.

The response to a molecular signal is mainly determined by the properties of both the ligand and the receptor as described above, but interestingly different cell types can respond differently to the same stimuli. The divergence of cellular responses in spite of the same ligand–receptor modules can be explained by the complexity of a third factor involved in cellular signaling: intracellular signaling networks.[3]

Briefly, cellular signaling pathways act on multiple intracellular targets, ranging from gene expression to protein localization. The complexity of cellular signaling arises from the large number of gene products involved, simultaneous activation of distinct signaling cascades, coexistence of different protein–protein interactions, relative abundance as well as intracellular localization of adaptor proteins, and duration/amplification of the signal. All these factors in the end determine the nature of the response to the signal — proliferation, differentiation, gene expression, cellular migration, cell cycle progression/arrest, or apoptosis.

5.3 Estrogen and Phytoestrogen Signaling

Phytoestrogens have been categorized based on their chemical structures, which resemble E2[4–6] (Figure 5.2). They come from diverse chemical classes and differ in their structure as well as in their biological activity, which affects their influence on tissues, receptors, and the genes that are transcribed. Functionally upon binding to their receptors (ERs), estrogens modulate transcription by binding to their estrogen response element (ERE) in the promoter region of target genes.[7,8] Therefore, the ER is a transcription factor that after being activated establishes a direct nuclear interaction by binding to the estrogen ERE of DNA, which confers estrogen inducibility of the gene.[8] EREs are present in the regulatory regions of estrogen target genes.

Most important features that enable estrogens/phytoestrogens to bind to an ER are the steric and hydrophobic properties of the compound as well as the hydrogen bonding between the phenolic hydroxyl group and the ER site.[9] An aromatic ring and a hydroxyl group are important for binding effectively, and the remainder of the ER will accept hydrophobic groups. Estrogenic flavonoids are similar in structure to E2. They are composed of a planar ring system that includes a hydroxy substituted aromatic ring that is approx 12 Å away from a second in coplanar hydroxyl group.[9] Two ring structures separated with two carbon atoms as well as spacing between hydrophobic and hydrogen bond interactions are also important in binding affinity to ERs. Other characteristics for ER binding affinity of a chemical are the degree and the size of branching of the alkyl group and its location on the phenolic ring and the distribution range of electron density on the A ring. The biological activity of individual phytoestrogens varies and is often reported as less active than mammal or synthetic estrogens.[10]

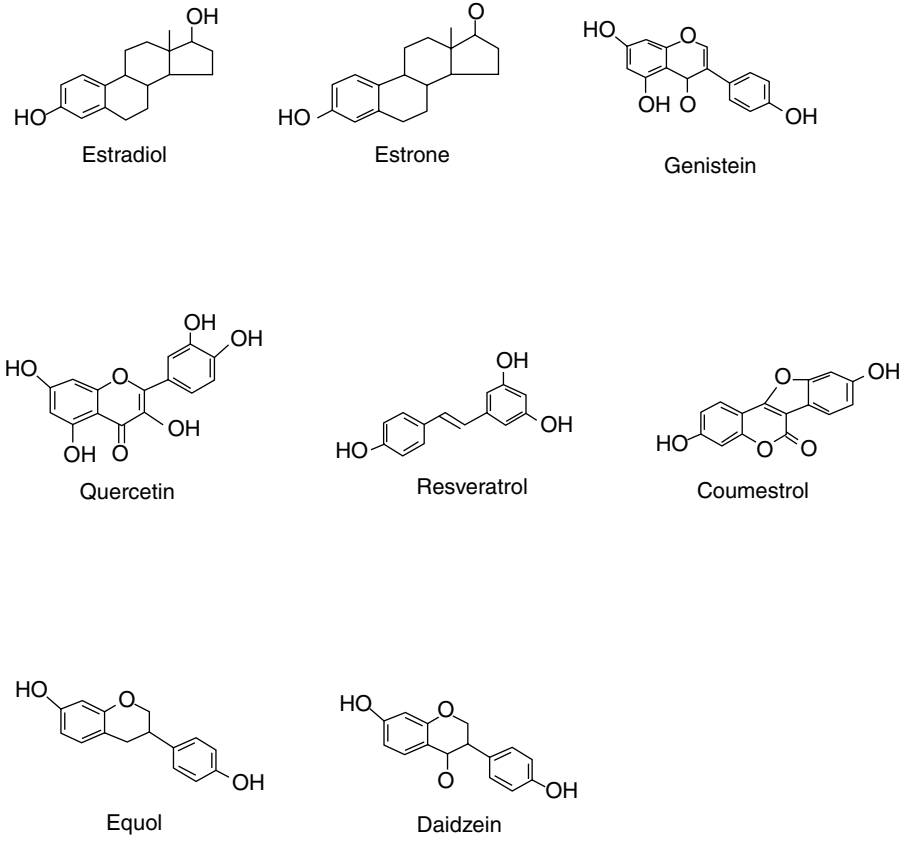

FIGURE 5.2
Structure of 17β-estradiol E2 and phytoestrogens.

5.3.1 Structure of Estrogen Receptors (ERs)

Mechanistically estrogens have been shown to bind to two types of receptors, ER alpha ERα and β. ERs belong to the steroid/retinoid receptor gene superfamily, which bind with steroidal as well as numerous nonsteroidal compounds.[11,12] Functionally upon ligand binding, ERs form dimers and modulate transcription by binding to their corresponding estrogen response element (ERE) in the promoter region of target genes. The first ER ERα was cloned in 1986, and then 10 years later ERβ was cloned in 1996.[13,14] Although the DNA binding domains of ERα and β are very similar, the overall degree of homology of the receptors is low. For the ligand-binding domain, only 55% of the amino acid sequence is shared. As a result, some ligands bind to the two receptors with different affinities. For example, E2 has a higher affinity for ERα, but genistein and coumestrol bind with higher affinity to ERβ.[11]

5.3.2 Tissue Distribution of Estrogen Receptors (ERs)

The tissue distributions of ERα and ERβ differ, although there is some overlap. Granulosa cells, kidney intestinal mucosa, lung parenchyma, bone marrow, endothelial cells, and prostate gland contain ERβ, whereas endometrium, breast cancer cells, and ovarian stroma mostly contain ERα.[14] On both ERα and ERβ the conformation of the ligand-binding domain changes in different ways when estradiol, roloxifene, or genistein binds to it. Consequently the conformation of a major transactivating domain is altered so that a different surface is exposed to the nuclear receptor coactivators or repressors. Therefore, the transcriptional effects may vary. For example, tamoxifen and raloxifen serve as transcriptional activators at activator protein (AP-1) sites when they form complexes with ERβ but suppress transcription when forming complexes with ERα.[15] Moreover, the balance of coactivators and corepressors influence the transcriptional activity of activated ERs.

Their distinct amino acid structures suggest that ERα and ERβ would recognize and bind to similar EREs, but each receptor would have a distinct spectrum of different ligands. In other words, different sets of proteins in the transcription complexes may interact with ERα and ERβ and direct them to specific targets.

5.3.3 Nuclear and Nonnuclear Actions of Estrogens and Phytoestrogens

Binding to EREs in the promoter regions of the target genes is not the only pathway through which ERs can influence gene transcription. Nonnuclear actions of estrogens have a direct effect on cell membranes and are mediated by cell surface forms of ERs and characterized by a rapid onset of their effect (Figure 5.3A, B). The examples are short-term vasodilatation of arteries and rapid activation of growth factor-related signaling pathways in neuronal cells.[16,17] A direct link has been shown by cell surface ERs and MAPK signaling pathway in osteoblasts, endothelial cells, neurons, and human breast cancer cells.[18,19] In other words, plant-derived compounds with estrogenic potency likely have effects also on other nuclear receptors; they exert antioxidative activity, are potent kinase inhibitors, show antiandrogen action including prostate carcinomas, and induce apoptosis. Both receptors can bind to other transcription factors such as activator protein AP-1 and GC box-binding protein Sp-1 sites and influence transcription of the AP-1 proteins c-jun and c-fos and the genes regulated by Sp-1.[20,21] Kushner et al. have reported that E2 mediated differentiation by binding of ER to ERE, whereas interaction of ER with AP-1 and SP-1 sites influenced pathways involved in proliferation.[22,23] Moreover, the distinct patterns of tissue distribution of these two ERs have heightened in novel estrogen targets in the body (Table 5.1).

The specific nuclear actions of estrogens are determined by the structure of the hormone, the isoform of the ER involved, the characteristics of the

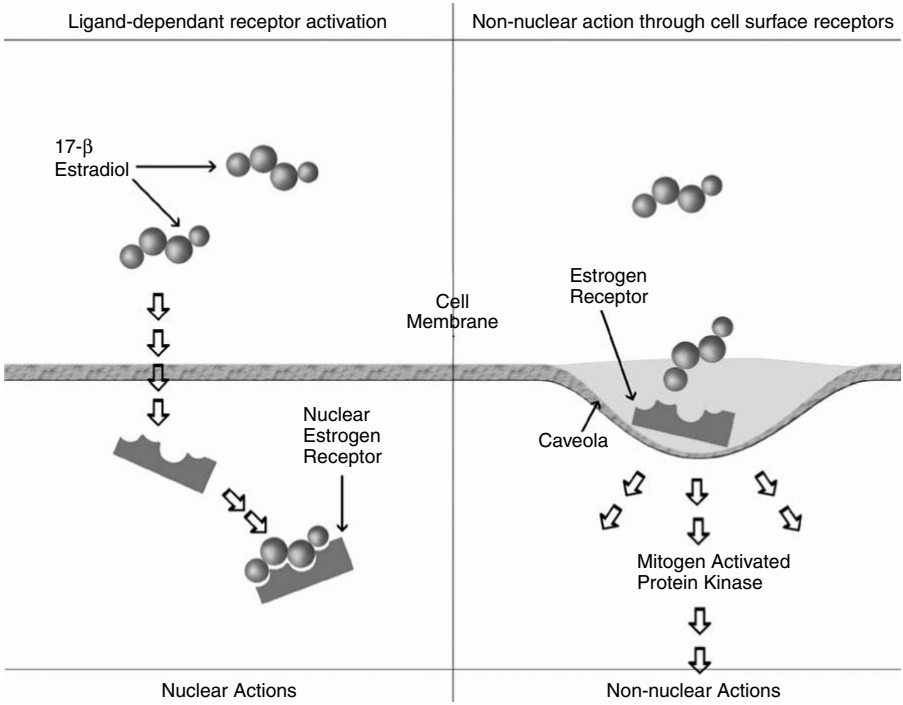

FIGURE 5.3
Nuclear (left) and nonnuclear (right) actions of phytoestrogens.

TABLE 5.1

Tissue Distribution of Estrogen Receptors and Related Pathologies

Tissue	Estrogen Receptor(s) ERα	ERβ	Pathology
Breast epithelium	+	+	Carcinogenesis
Breast stroma	–	+	
Bone	+	–	Osteoporosis
Prostate stroma	+	–	Hyperplasia
Prostate epithelium	–	+	Carcinogenesis
Brain	+	+	Stroke, dementia
Uterus	+	–	Carcinogenesis
Ovary theca cells	+	–	Polycystic ovary
Ovary granulosa cells	–	+	Polycystic ovary
Bone marrow	–	+	Leukemia, carcinogenesis
Colon	–	+	Carcinogenesis

gene promoter, and the balance of the coactivators and corepressors that modulate the final transcriptional response to the complexes of the estrogen and ERs (Figure 5.4). For example, ERβ has been shown to have ligand specificity toward phytoestrogens and is distributed in ovary, spleen, testis,

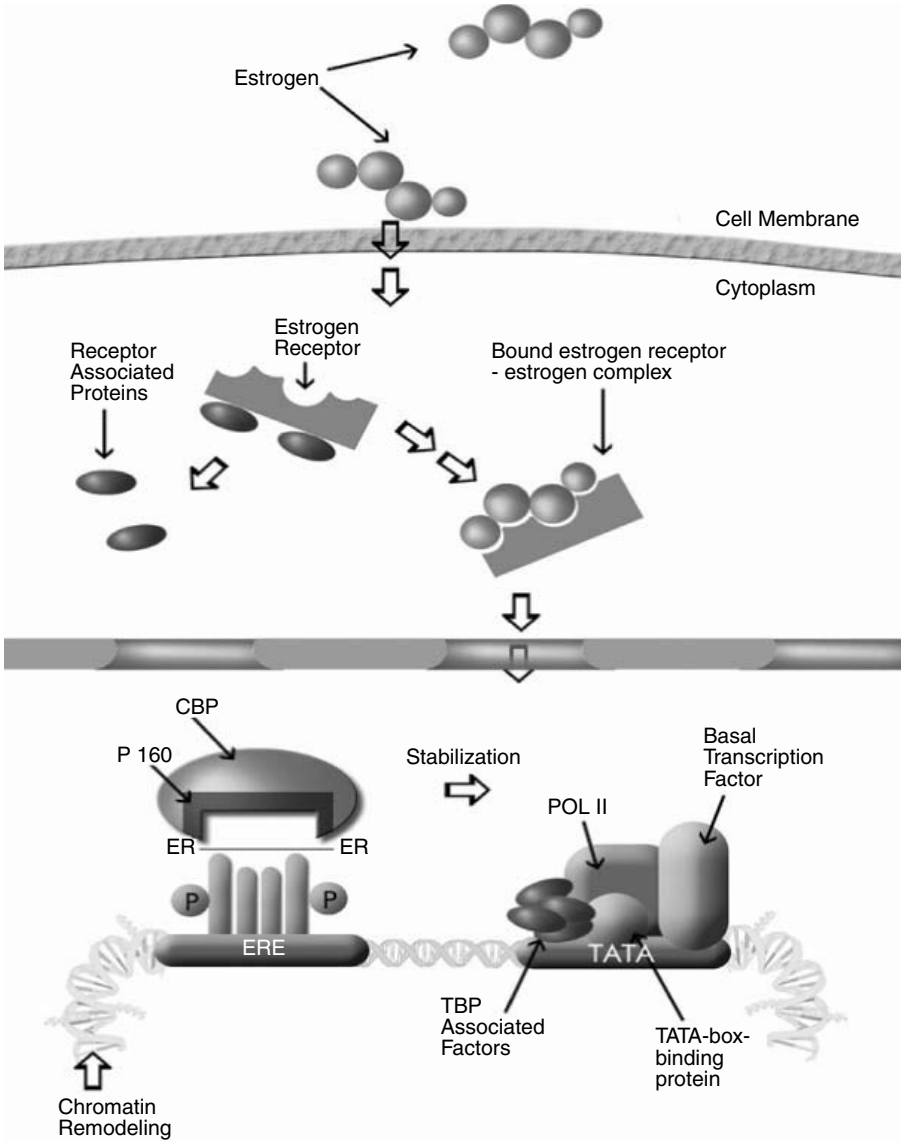

FIGURE 5.4
Transcriptional effect of estrogens and ER complex.

and thymus human tissues (Table 5.1). Phytoestrogens show a weaker binding affinity than E2, and some show a higher binding affinity for ERβ than for ERα, which may suggest different pathways for their actions and explain tissue-specific variability and phytoestrogenic action. The complexity of phytoestrogens and ER appears to be further complicated because different transcriptional activities *in vitro* are activated depending on the ligands as

well as the environment of the promoter region of specific genes for translated ERα and ERβ receptors.

The specific sequence or ERE to which ER dimer binds is a palindromic 13-base-pair inverted DNA sequence. However, nonconsensus nEREs differ from cERE in that 1-3 nucleotides predominate in endogenous ER genes, and as mentioned above, ERα and ERβ exhibit differential binding affinity to various EREs.

This clear evidence for the functions of the two receptors comes from studies involving ERβ knockout mice. ERα -/- ERβ -/- knockout mice have shown that both ERs are of physiological importance and that they have distinct and nonoverlapping functions in the body.[24–26] The uterus, breast, and pituitary tissues express predominantly ERα. The distinct patterns of tissue distribution of these receptors increased the interest in novel estrogen targets in the body and have led to awareness of new sites for pharmacological intervention in diseases such as prostate dysfunction, leukemia, and colon cancer.

In one study, it has been shown that binding of ER to ERE induced gene activation and is an important step in estrogen-induced biological effects.[27] They have investigated the effect of some dietary phytoestrogens such as the isoflavones genistein and daidzein, its metabolite equol, and coumestrol on the binding of ERα and ERβ to ERE. E2 and phytoestrogens induced an increase in ER binding to ERE in a concentration-dependent manner, E2 being more potent than the other phytoestrogens studied. The outcome of the study indicated that phytoestrogens differed not only in their binding affinities for the ER but also in their potential to increase the rate of receptor binding to the ERE at physiologically relevant concentrations, and this ability can differ from the binding affinities of these phytoestrogens to ERs.

Phytoestrogens with a stable structure and low molecular weight can pass through cell membranes, and they have been shown to interact with enzymes and receptors. These interactions allow them to bind to ERs, induce specific estrogen-responsive gene products, interfere with steroid hormone metabolism, and alter ER structure and affect transcription. Nongenomic effects that do not involve ER include cancer cell differentiation, inhibition/activation of serine-threonine and tyrosine kinases, DNA topoisomerase activation, suppression of angiogenesis, and antioxidant effects. Other effects can take place at the cellular and molecular level and potentially influence the biosynthesis and metabolism of steroids and fatty acids, the serum steroid carrier proteins, and the intracellular and transmembrane transfer of hormones to a membrane and to nuclear receptors. Phytoestrogens inhibit the enzymes needed for hormone conversion, which may reduce cancers by lowering the biological activity of sex hormones in target organs.

Animal studies have shown that potent phytoestrogens *in vitro* exert estrogenic and antiestrogenic effects *in vivo*.[28,29] Genistein and daidzein showed uterotropic action in the rat, whereas resveratrol showed no uterotropic effect consistent with their *in vitro* estrogenic activity.[30,31] In contrast, coumestrol, an ERα and ERβ agonist *in vitro*, showed similar estrogenic effects to DES

in rats but repressed ERβ mRNA expression in rats.[32] These results make it clear that results obtained in only one cell line cannot be extrapolated to effects of the tested phytoestrogen in several tissues *in vivo.*

5.4 Estrogens and Phytoestrogens in Clinical Conditions

Recently, several epidemiological and experimental studies in animals and humans have suggested that the consumption of foods rich in phytoestrogens may have protective effects on estrogen-related conditions such as menopausal symptoms and diseases such as breast and prostate cancers, osteoporosis, and cardiovascular diseases.[33,34] This phenomenon is particularly important since diet ranks second to smoking as a leading contributor to cancer incidence and mortality, and recent data suggests that almost one third of cancer deaths in the United States are avoidable by dietary change. It has been suggested that consumption of phytoestrogens (especially soy foods) may contribute to the relatively low rate of breast, prostate, and colon cancer in countries such as China and Japan.[35] The different activities and the bioavailability of phytoestrogens vary depending on such factors as the form of administration, dosage, individual metabolism, and the ingestion of other pharmacological substances. Target tissue, concentration dependency, number and type of ER, and the presence and absence of endogenous estrogens also influence the effect of phytoestrogens.

5.4.1 Carcinogenesis

High cell proliferation is a common characteristic of most cancer cells. This cellular property is well reflected in the up-regulation of signal transduction, cell cycle, and DNA synthesis in cancer cells. The proliferation rate is initially determined by the probability of switching from quiescent G0 to G1 phase of the cell cycle. In this respect, the cyclin proteins appear to play a major role in cell cycle regulation. Cyclin proteins accumulate in the G1 phase (G1-cyclins), and after they reach a peak concentration, they are rapidly degraded. It is interesting to note that most polyphenols, such as tea polyphenols genistein and silymarin, can produce cell cycle arrest at G1 phase through inhibiting cyclin-dependent kinase cdk-2 and cdk-4 activities and inducing cdk inhibitors p21 and p27.[36,37] The observation that the flavonol quercetin, flavone apigenin, isoflavone resveratrol, and tea polyphenols all arrest cell cycle progression in late G1 and G2/M stages is also interesting in this context.[38–40]

Further insight into anticarcinogenic mechanism of phytopolyphenols might be gained by looking at the individual steps in the multistage (initiation, promotion, and progression) hypothesis of cancer development.

Initiation stage either by carcinogens or after metabolic activation of procarcinogens can be prevented both by flavonoids and by other phytopolyphenols. On the other hand, mitogenic stimulation for cell proliferation is likely to be an important pivotal force in tumor promotion. However, mitogenic stimulation alone is not significant for transformation, and additional changes in gene expression are required to escape from normal growth regulation. In general, specific regulatory DNA-binding proteins or transcription factors that regulate gene expression directly by binding to specific DNA sequences in promoter regions mediate alterations in the transcription of a set of cellular genes.

Effects of various polyphenols on tumor cells suggest that different mechanisms may occur, some of them involving reactive oxygen intermediates (ROI). A great amount of evidence supports the idea that gene expression controlled by the transcription factor NF-κB is redox regulated.[41] At one level, binding of NF-κB to DNA requires a key cysteine residue that must be reduced.[42] On the other hand, ROI appear to serve as common second-messenger-like molecules in the various pathways leading to NF-κB activation.[43,44] Several mechanisms have been proposed for the activation of NF-κB. These include transformation of the cell to a more oxidized redox state either by decreasing the concentration of reduced thiols or increasing the ratio of gluthathione disulfide to reduced glutathione or by generation of the hydroxyl radical or another reactive oxidant.[41] Antioxidants such as tea polyphenols curcumin and carnasol may be expected to work by increasing the concentration of intracellular GSH or total thiols, by scavenging free radicals, or by iron chelation. Many of the compounds classified as antioxidants that have been shown to inhibit NF-κB activation are phytopolyphenols.[45] Many are effective at concentrations that may be too low to be compatible with a radical scavenging role and their effects might be better explained if they were acting on a more specific enzymatic process.

5.4.2 Specific Mechanisms for Cancer Prevention

The soy proteins genistein and daidzein are primary phytoestrogens, which have been shown to act as cancer chemopreventive agents via modulation of cellular signaling at different levels.[46,47] In contrast, recent studies have revealed tumor-promoting potential of soy isoflavones in reproductive organs, breasts, and colon.[48,49] Dietary soy proteins have been shown to stimulate epithelial cell proliferation, hyperplasia, and secretion.[50] The proposed mechanism for isoflavone-induced carcinogenesis in humans involves tumor initiation through oxidative DNA damage by pro-oxidant metabolites of isoflavones and tumor promotion through ER-mediated cellular signaling in ER (+) tissues.[48,51]

The effect of genistein, which has been shown to inhibit cancer cell proliferation *in vitro*, has been attributed to a competitive inhibition by occupying ER or to inhibition of several key enzymes, especially tyrosine kinase, which

is thought to be involved in the control of cell proliferation and carcinogenesis.[52,53] Receptor protein tyrosine kinase (RTK) activity is associated with cellular receptors for growth and differentiation and plays a role in signal transduction pathway. Binding of a polypeptide growth factor such as EGF, PDGF, and FGF to its cognate receptor triggers the RTK activity leading to a cascade of phosphorylation events.[54] Genistein is the first phytoestrogen molecule that has been shown to exert a specific tyrosine kinase inhibition. Consequently, in human MCF7 breast cancer cell line, genistein has been shown to exert antiproliferative effect at high concentrations.[55,56] Paradoxically, low concentrations of genistein stimulated cell growth.[57] In PC3-M metastatic prostate cancer cell line, high-dose genistein has been shown to induce apoptosis through inhibition of focal adhesion kinase activity independent of its estrogenic effects, but the *in vivo* correspondence of this proapoptotic activity remains to be elucidated.[58] Genistein has also been shown to decrease expression of the cell cycle phosphatase Cdc25C and induce Tyr15 phosphorylation of Cdc2 along with decreased activity.[59] p38 mitogen-activated protein kinase has also been attributed to be involved in genistein-mediated inhibition of cell proliferation. Interestingly, high-dose genistein has been reported to induce delayed and prolonged activation of p42/44 mitogen-activated protein kinase and neuronal apoptosis whereas daidzein, a structural analogue, shows no toxicity at similar concentrations, which indicates potential side effects in the nervous system with genistein used as a high-dose therapeutic agent.[60] The effects of genistein are not restricted to its tyrosine kinase inhibitory actions; it has been reported to modulate the activity of voltage-gated channels and intracellular $Ca^{(2+)}$ accumulation directly.[61] Previous studies have proposed that protein tyrosine kinase activity can directly regulate cardiac L-type $Ca^{(2+)}$ channels, and genistein can inhibit the cardiac L-type $Ca^{(2+)}$ current through modulation of protein tyrosine kinase activity.[62] A recent work by Belevych et al. reported that genistein inhibits cardiac L-type $Ca^{(2+)}$ channel activity by a tyrosine kinase-independent mechanism.[63]

Daidzein has been reported as an inactive analogue of genistein for inhibition of tyrosine kinase activity, but its cancer chemopreventive potential has been attributed to pathways other than inhibition of protein tyrosine kinases. Daidzein was shown to exert an antiproliferative effect on human estrogen-receptor-positive and -negative pancreatic cancer cells; moreover, a prominent proapoptotic mechanism was activated by daidzein in HL60 cells that has been related to up-regulation of TGF-β2.[64,65] The chemopreventive potential of daidzein has been extensively studied in prostate cancer models, as it has been postulated that soybean isoflavones act as inhibitory modulators in prostate carcinoma.[66] In a case-controlled clinical study, the serum concentrations of genistein and daidzein were found to be higher in prostate cancer patients.[67] The mechanism of action for the chemopreventive properties of daidzein needs far more investigation, and discrepancies among recent studies underline the importance of daidzein metabolizers in different tissues. Similar to genistein, daidzein at low concentrations

promotes growth of breast cancer cells in an ER-dependent manner and antagonizes the effect of tamoxifen.[68] These reports show that their combination with chemotherapeutics has to be evaluated considering dose-dependent effects of isoflavones and involvement of discrete signaling pathways in different neoplasias.

In contrast to genistein and daidzein, characterization of specific effects of coumestrol on oncogenic signaling systems is not extensively studied, and there is not enough data for estrogenic effects of coumestrol on ER (+) cells. Coumestrol was shown to be an effective aneuploidogen in mammalian cells, and coumestrol induced DNA strand breaks as well as centromere-negative micronuclei and mutations in p53 mutant Chinese hamster V-79 cells.[69] This direct mutagenic effect of coumestrol has been attributed to its inhibitory potential on topo-II function. Furthermore, as a weak estrogen-mimicking compound, coumestrol stimulates transcriptional activity of ERβ in reporter gene assays.[70]

Quercetin is another widely studied phytoestrogen in flavonoid structure, which has been reported to modulate various pathways involved in proliferation, apoptosis, and cell cycle progression involving cdc25 phosphatase, p38 MAPK, c-Jun N-terminal kinase, Erk 1/2 MAPK, PI3-Ks, and DNA topoisomerase II.[71–75] It has antiproliferative and proapoptotic activity in many human cancer cell lines, either as a single agent or in combination with other chemotherapeutic approaches. In a recent study, quercetin at 20-μ*M* concentration was shown to inhibit EGFR tyrosine kinase activity and intracellular tyrosine phosphorylation.[76] Quercetin has also been reported to prevent neoplastic invasion of melanoma cells through inhibition of PKC activation and down-regulation of matrix metalloproteinase-9 expression.[77] The effect of quercetin on PKCs is somehow controversial; in one study it has been demonstrated to sensitize lymphoblastic leukemia cells to CD95-mediated apoptosis via activating PKCα, but in another report quercetin was shown to protect against conjugated linoleic acid–mediated apoptosis via inhibiting PKC activity.[78,79] Quercetin may exert a proapoptotic immunomodulatory effect by suppressing the activation of ERK 1/2, p38 MAPK, and NF-κB signaling pathways, but it has also been reported to inhibit Taxol-induced p38 MAPK and Ask1 activation and thereby abrogates the proapoptotic effects of Taxol.[80,81] These activities of quercetin make it a promising drug candidate for treatment and prevention of various tumors, but further characterization of quercetin-modulated signaling pathways is required (Figure 5.5).

A considerable amount of effort has been ongoing with respect to resveratrol and its chemopreventive effects. Resveratrol was shown to interfere with tumor initiation, promotion, and progression in many *in vivo* and *in vitro* cancer models. The cancer chemopreventive effect of resveratrol has been initially attributed to its antioxidative properties.[82] Recent studies underline the involvement of signaling cascades, cell cycle regulators, and pro/antiapoptotic protein interactions in resveratrol-induced cancer chemoprevention.[83] MAP kinases are functionally involved in regulation of tumor

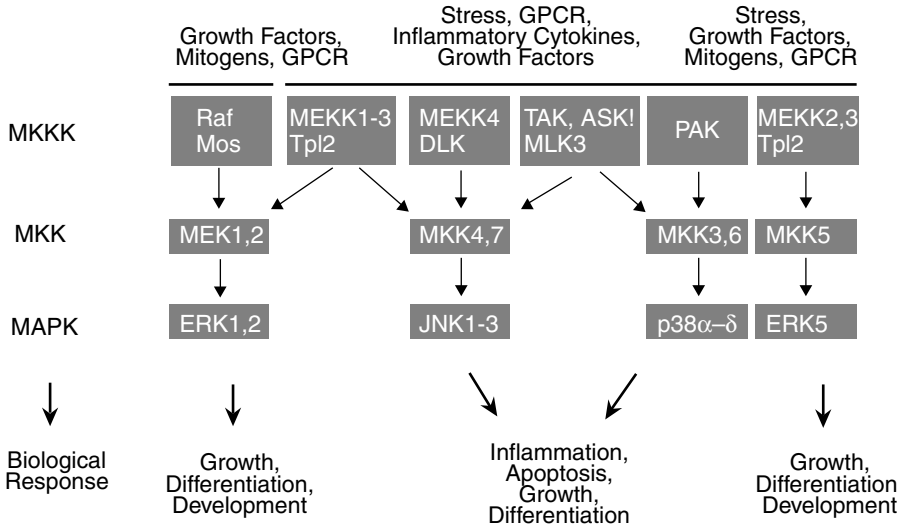

FIGURE 5.5
MAPK activation cascades.

progression and apoptosis, either through regulation of gene expression or through regulatory protein modifications. Erk 1/2 and p38 MAP kinase activation and consequent phosphorylation of Ser 15 on p53 constitutes a critical role in the stabilization, up-regulation, and functional activation of p53. Tumor-suppressive activity of resveratrol has been reported to occur through Erk 1/2- and p38-mediated p53 activation in an epidermal cell line, which is a well-established tumor progression model.[84] JNKs have also been shown to be involved in resveratrol-induced p53 activation and induction of apoptosis.[85] Resveratrol has been demonstrated to induce apoptosis in ER (+) MCF-7 human breast cancer cells.[86] ER-alpha (ERα) was shown to regulate phosphoinositide 3-kinase (PI3K) activity through interaction with p85, which indicates a nonnuclear involvement of ERα in cell proliferation and apoptosis. Resveratrol was demonstrated to modulate ERα-mediated PI3K activation either positively at low concentrations or negatively at high concentrations.[87] This selective regulation of a single intracellular signaling pathway by resveratrol defines the complexity of dose-dependent approaches for cancer chemoprevention. PKC signaling pathway is a relatively well-defined regulatory cascade in the modulation of various essential cellular processes such as apoptosis, proliferation, cell cycle progression, and differentiation. Resveratrol has been reported to abrogate PKC-catalyzed phosphorylation of different substrates *in vitro* and has been accepted as an inhibitor of various PKC isozymes.[88] It was shown to inhibit proliferation of gastric adenocarcinoma cells through utilization of a PKC-mediated signaling pathway.[89]

EGF-EGFR signaling module mediates a constitutive activation pattern of Erk 1/2 pathway, which is evidenced as an important mechanism in the

development of androgen-independent prostate cancer. Resveratrol has been demonstrated to inhibit EGFR-dependent Erk 1/2 activation through inhibition of associated PKC isozymes.[90]

Resveratrol was shown to inhibit the growth and progression of many cancer cell lines, which has been attributed to its regulatory effects on cell cycle. It has been reported to cause an accumulation of breast cancer cells in S-phase of cell cycle as well as reduced levels of Rb protein and increased expression of p53 and proapoptotic Bcl-2 protein family members.[91] Another mechanism for resveratrol-induced apoptosis in cancer cells is CD95-CD95L death receptor pathway activation in breast cancer and CD95-resistant leukemia cell lines.[92]

5.5 Menopause and Osteoporosis

Total bone mass is maintained by two mechanisms of bone remodeling: osteoclastic bone resorption and osteoblastic bone formation. Bone remodeling is under the control of parathyroid hormones, cytokines, and glucocorticoids. A deregulation in bone formation results in osteoporosis, which is characterized by both increased osteoclastic bone resorption and decreased osteoblastic bone formation resulting in increased bone fragility and fracture risk. Postmenopausal women have increased risk of developing osteoporosis as a result of decreased serum estrogen concentrations. Estrogens have been reported to preserve total bone mass, but cellular signaling mechanisms involved in this effect have been poorly characterized. Hormone replacement therapy protocols have been utilized to prevent the loss of bone mass due to estrogen deficiency in postmenopausal women. At cellular level it has been shown that estrogens enhance osteoblast differentiation and bone tissue formation, as well as inhibit bone resorption through prevention of osteoclast maturation. These effects of estrogens seem to be mediated through both ERα and ERβ receptors, but specific involvement of intracellular ERs and membrane ERs in bone turnover has not been fully identified yet.

The rate of osteoporosis differs within populations in different geographic regions with lower incidence in Asian women compared to their Western counterparts, and high content of phytoestrogens in consumed food products in the Asian region has been postulated to be involved in this protective effect.[93] Phytoestrogens such as daidzein, genistein, and coumestrol exert a potential protection against bone mass loss in ovariectomized rats and postmenopausal women.[94–97] Genistein and daidzein induces transcription of osteoblastic differentiation proteins such as BMP-2 through ERβ, but intermediate regulators of this effect remain to be elucidated.[98,99] NF-κB and AP-1 transcription factors were shown to be involved in osteoclastic differentiation in knockout mouse models, and quercetin at low doses (0.1 to 10 μ*M*) was shown to decrease osteoclastogenesis through inhibition of NF-κB and AP-1

signaling pathways.[100] Quercetin was also reported to regulate osteoblastic differentiation through activation of ERs and ERK 1/2 MAP kinases,[101] but other intermediate signaling molecules and target transcription factors involved in this signaling pathway are not identified yet.

Postmenopausal women are vulnerable to health problems other than osteoporosis such as menorrhagia, depression, hot flashes, and sleep disorders. Phytoestrogens have been proposed to alleviate these postmenopausal symptoms, but clinical studies based on patient-oriented surveys present conflicting results on this issue.[102,103]

Therapeutic strategies aiming to prevent osteoporosis and postmenopausal disorders through a phytoestrogen-rich nutritional strategy need more data available from *in vitro* and *in vivo* experimental models as well as clinical studies. Identification of cellular signaling pathways involved in osteoporosis and postmenopausal disorders would certainly provide important clues for designing novel nutritional approaches based on phytoestrogen action mechanisms.

5.6 Cardiovascular Diseases (CVD)

The advanced atherosclerotic lesion is characterized by altered vascular endothelial function, accumulation and retention of intracellular and extracellular lipids at subendothelial space, infiltration of proinflammatory monocytes and lymphocytes, advanced lipid peroxidation, and proliferation of vascular smooth muscle cells (VSMCs), which results in increased intimal thickness. Atherosclerosis has been defined as a progressive chronic inflammatory pathology, which is initiated as patchy fatty streaks in early years of life, and progression of the lesion is accelerated by high levels of circulating low-density lipoproteins and monocyte- and lymphocyte-mediated inflammation at the vessel wall.[104] In advanced stages of the lesion, lipid-laden macrophages and transmigration of proliferated smooth muscle cells to vascular intima dominate the pathological scene. Phytoestrogens might contribute to the lower incidence of CVD in Asian countries and in vegetarians. There is also sufficient evidence to support the observation that a phytoestrogen-rich diet has a beneficial effect on serum lipoproteins profile. An analysis of 38 published controlled clinical trials of soy protein consumption was associated significantly with mean reductions in total cholesterol, LDL cholesterol, and triglycerides.[105] The hypocholesterolemic effect of phytoestrogens may not be their only mechanism of cardioprotection. In most of the *in vitro* and *in vivo* atherosclerosis and ischemia-reperfusion injury experimental models, increased levels of isoflavonoids correlates with abrogation of inflammatory cell adhesion, growth factor activity, and cellular proliferation.[106]

MAPK play a crucial role in mediating proliferative response in physiological and pathophysiological conditions. Oxidized low-density lipoproteins (ox-LDLs) promote atherosclerotic lesion in both initiation and progression stages, and Ras-Raf-MEK-MAPK signaling cascade has been shown to be involved in ox-LDL-mediated VSMC proliferation.[107] Genistein has been shown to inhibit the oxidation of LDL in bovine arterial endothelial cells and protect endothelial cells against ox-LDL-induced cellular injury.[108] In addition to its antioxidant properties, genistein has also been reported to prevent VSMC proliferation through inhibition of Erk 1/2 MAP kinase activation.[109] Hypertension is also regarded as a major risk factor for cardiovascular diseases, in particular for atherosclerosis. High transmural pressure modulates gene expression and receptor expression patterns in vascular endothelial and smooth muscle cells, thereby leading to endothelial dysfunction, loss of vascular integrity, and local microvascular inflammation at the vessel wall. Genistein has been shown to suppress transmural pressure-induced proliferation of VSMCs.[110] Diabetes is one of the leading causes of mortality and morbidity in human populations, and one of the most common complications of diabetes is atherosclerosis. Plasminogen activator inhibitor type 1(PAI-1) is produced by vascular endothelial cells in response to proinflammatory cytokine stimulation, and it plays a central role in the development of atherosclerosis in diabetic patients. Genistein has been shown to completely inhibit TNFα-induced PAI-1 secretion through a potential inhibitory mechanism targeting of Erk 1/2 and NF-κB cellular signaling.[111] In addition, it has been reported to lower myocardial necrosis and ventricular arrhythmias and reduce the levels of TNFα and ICAM-1 expression in the experimental myocardial ischemia-reperfusion model.[112]

The role of enhanced tyrosine kinase activity has been established in the pathophysiology of many diseases as well as atherosclerosis. PDGF in combination with vanadate stimulates protein tyrosine phosphorylation, Erk 1/2, and PDGF receptor activation in vascular smooth muscle cells, and genistein inhibits the mitogenic effect of PDGF/vanadate efficiently.[113] Genistein in high doses significantly induces relaxation of coronary arterial ring and at low doses enhances sodium nitroprusside-induced vascular relaxation.[114] Genistein has also been shown to suppress human platelet aggregation and protein tyrosine phosphorylation induced by collagen or thromboxane A2; in addition, an increase in the intracellular $Ca^{(2+)}$ induced by thrombin was also inhibited by higher concentrations of genistein.[115,116] Although most of the studies imply the cardioprotective role of genistein, it has also been shown to promote basal ICAM-1 expression on endothelial cells and inflammatory cells transmigration into subendothelial space.[117] It has been proposed that sulfation of genistein and consequent loss of hydroxyl groups decreases its antioxidant capacity and kinase inhibitory potency and abrogates its effects on adhesion factor expression.[118]

Epidemiological surveys have revealed an inverse correlation between consumption of wine and prevalence of cardiovascular diseases. Resveratrol is abundantly present in grapes and grape products such as wine. Resveratrol

was initially characterized as a phytoalexin with potential antioxidant properties, which was assumed to play the major role in its cardioprotective function. Resveratrol has been demonstrated to suppress ROI formation and lipid peroxidation induced by LPS, phorbol esters, and proinflammatory cytokines, thereby protecting against oxidant-induced damage on proteins and DNA.[119] Resveratrol has also been shown to inhibit Cox-2 induction by LPS and phorbol esters and leads to a marked reduction of prostaglandin synthesis and arachidonic acid release.[120] Besides inhibition of oxidation of low-density lipoproteins, its antithrombotic effects through attenuation of platelet aggregation and modulation of arachidonic acid metabolism underlines another protective mechanism against cardiovascular diseases. In platelets, mitogen-activated protein kinases become activated downstream of agonist binding, and resveratrol at 10 μ*M* or higher concentrations inhibited MAPK activation induced by collagen.[121]

One of the endogenous mediators of atherosclerosis is endothelin-1 (ET-1), which is a small peptide involved in the initiation phase of lesion development. ET-1 acts as a vasopressor and a triggering signal for mitogenic responses of vascular cells. The decoys for ET-1 receptors may alleviate complications of coronary heart disease. ET-1 targets a variety of signaling molecules downstream of its receptor such as PKC and MAPKs, and resveratrol has been postulated to abrogate MAPK signaling cascades through upregulation of cAMP/PKA signaling module. Ox-LDL exerts a mitogenic effect on vascular smooth muscle cells in an ERK1/2 activation-dependent manner, and it leads to increased intracellular ROI formation. Resveratrol pretreatment alleviates ERK 1/2 activation, smooth muscle cell proliferation, and ROI generation.[122] It has been demonstrated that ROI and NO mediate signal transduction events in a number of cell lines. NO plays a major role in the vascular homeostasis controlling the enzyme guanylyl cyclase and modulating the activities of protein tyrosine kinases. Tyrosine phosphorylation is associated with mitogenesis, cell transformation, and cell death. Resveratrol induces nitric oxide synthase, the enzyme responsible for the biosynthesis of NO, in cultured pulmonary artery endothelial cells and could promote cardioprotection by modulating the expression of nitric oxide synthase and receptor tyrosine kinase activation.[123]

The cellular basis of the protective effect of resveratrol on vascular function also involves preservation of vascular endothelial integrity and function. Infiltration of monocytes into the subendothelial space is mediated by specific adhesion factors expressed on vascular endothelium, such as ICAM-1, VCAM-1, and E-selectin.[104] Endothelial adhesion factor expression is mainly under transcriptional control of NF-κB, and stimulation of vascular endothelial cells by proinflammatory cytokines and ox-LDL leads to increased expression of adhesion factors through activation of NF-κB. Resveratrol has been shown to exert an antiinflammatory action, which includes inhibition of NF-κB activation through prevention of IκB phosphorylation and degradation as well as decreased expression of ICAM-1 and VCAM-1.[119,124,125] The

upstream NF-κB-activation kinase complex, which is composed of IKKα, IKKβ, and IKKγ, has been proposed to be a target for resveratrol.[126]

The daily amount of flavonoids to be consumed has been estimated to be about 23 mg, and quercetin constitutes nearly 60% of such intake.[127] Similar to resveratrol, quercetin demonstrated a dose-dependent inhibition of both thrombin-induced and ADP-induced platelet aggregation as well as eicanosoid synthesis. In apoliprotein-deficient mice, which are prone to atherosclerosis due to deterioration of LDL transport and hyperlipidemia, quercetin has been shown to decrease the atherosclerotic lesion area without any effect on total LDL levels.[128] This effect has been proposed to be associated with reduced susceptibility of LDL to oxidation. Serum paroxanase (PON-1) can protect low-density lipoproteins from oxidation induced by metals or oxidant generators. Interestingly ox-LDL has been shown to inactivate PON-1 and exaggerates the progression of oxidative modification of lipoproteins.[129] Quercetin has antioxidant properties and may prevent LDL oxidation either by inhibiting the activity of enzymes involved in LDL oxidation (15-lipoxygenase) or by increasing serum paroxanase activity.[129,130] Heme oxygenase is the rate-limiting enzyme that is involved in oxidative degradation of heme into bilirubin, iron, and CO. Heme oxygenase-1 (HO-1) is the inducible isoform that exerts antioxidant and anti-inflammatory properties. HO-1-deficient mice have been reported to have an increased inflammatory state. AP-1 transcription factor and MRE/cMyc complex proteins control HO-1 expression. Quercetin has been shown to induce HO-1 expression in aortic smooth muscle cells, and increased mRNA and protein levels of HO-1 indicate a control at both transcriptional and translational levels.[131] The upstream signaling pathways of HO-1 expression system have not been characterized yet, but quercetin treatment was shown to up-regulate p38 MAPK activation and down-regulate Erk 1/2 MAPKs; in addition, a specific p38 inhibitor attenuates quercetin-induced HO-1 expression, indicating a p38 MAPK-dependent pathway.[131]

In addition to its antioxidant effects, quercetin protects against disturbance of the cardiovascular system by directly modulating cellular signaling pathways involved in the pathogenesis of atherosclerosis. Tissue factor (TF) is an integral membrane glycoprotein, which functions as a cellular receptor for plasma clotting factor (F)VII/(F)VIIa. The formation of ligand–receptor complex on vascular endothelial cell surface triggers a TF-dependent coagulation cascade. Although TF is expressed constitutively in cells surrounding blood vessels, it is not expressed in vascular endothelial cells, which are in direct contact with blood. In vascular endothelial cells and circulating monocytes, TF expression may be induced in response to LPS, inflammatory cytokines (TNFα and IL-1), which involves NF-κB activation, nuclear translocation, and binding to the promoter of TF gene. TF mRNA, protein, and activity levels have been detected in atherosclerotic lesions, which promotes thrombogenicity and consequent atherosclerotic plaque rupture. Quercetin as well as resveratrol was shown to inhibit LPS-, TNFα-, and IL-1-induced NF-κB activation and TF expression in HUVEC cells.[132]

As described previously, cellular adhesion molecules are mainly under the control of NF-κB transcriptional activity. Although ICAM-1 is constitutively expressed on vascular endothelial cells, it can be significantly up-regulated in response to proinflammatory cytokines and LPS. ICAM-1 mediates the formation of firm monocyte-endothelial cell adhesion via interaction with β2 integrins expressed on monocytes. The ICAM-1 gene contains numerous transcription factor-binding sites within 5′-flanking regions that are recognized by both NF-κB and AP-1. Interaction of these two transcription factors is proposed to be required for efficient transcription of ICAM-1 in response to proinflammatory cytokines and phorbol ester.[104] Kobuchi et al. have shown that quercetin down-regulated both PMA and TNFα-induced ICAM-1 expression via inhibition of JNK MAPKs and AP-1 activation in a dose-dependent manner, without any regulatory effect on NF-κB signaling pathway.[133] The upstream JNK-activating kinase potentially inhibited by quercetin remains to be elucidated.

5.7 Conclusions

Recent investigations with phytoestrogens have provided important insights as to how these compounds modulate signal transduction pathways and the role of this modulation in executing their cancer preventive actions. The pathway leading to the replication of neoplastic cells provides a mechanistic framework for discussing the role of phytoestrogens in the prevention of carcinogenesis. DNA repair mechanisms can sufficiently remove most but not all of the lesions, and in the absence of nuclear control mechanisms regulating cellular proliferation, neoplastic cells multiply. A number of compounds in fruits and vegetables are known to modulate these pathways.

The data in the literature indicates that phytoestrogens have both estrogenic and antiestrogenic effects depending on the specific tissues and the concentration of circulating endogenous estrogens. The biological activity of phytoestrogens has been demonstrated mostly in animals and in *in vitro* studies, but they appear to have physiological effects in humans. Mild estrogenic effects have been observed in postmenopausal women; however, it is too early to determine the clinical value of phytoestrogens in ameliorating the early menopausal symptoms.

In view of the current data, phytoestrogens are generally accepted as beneficial rather than deleterious, particularly when consumed in food products. The consumption of phytoestrogen-containing food products, especially soy products, may contribute to a lower risk of developing CVD and prostate cancer in healthy people. Although a large number of studies are encouraging, the field is still in its infancy.

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6

Phytoestrogens and Body Composition

V. Di Mattei, L. Scalfi, and F. Branca

CONTENTS

6.1 Body Composition and Health

The assessment of body composition is now recognized to be necessary in many areas of human nutrition, as the relative proportion of lean and fat mass, in addition to body size, are indicators of the health of an individual and of his or her physical capacity, as well as of the risk of developing several chronic diseases and the ability to recover from various illness. In recent years, considerable advances have been made in the development of new techniques, in assessing the accuracy and applicability of current techniques, and in the study of new aspects of body composition. Body composition techniques are now applicable in experimental clinical conditions, at bedside,

and in population studies, although often the former are aimed at developing prediction equations that are then applied to population studies.

Many diseases and disorders are anticipated or accompanied by abnormalities in body composition, the most common of which is obesity. Obesity is a condition of excess body fat associated with too many adipose cells (hyperplastic obesity), with too large adipose cells (hypertrophic obesity), or both. Obesity is the result of an excess of energy intake over expenditure, and it is due to genetic, environmental, and psychological factors; it is recognized to be an independent risk factor for some chronic diseases, such as coronary atherosclerosis, cardiovascular disease, diabetes, and some types of cancer.[1,2] Health care providers are concerned not only with the amount of fat but also with its distribution in the body, which is sex- and age-related. Abdominal fatness is more associated with obesity-related health problems.[3] Usually, men accumulate fat in the abdominal region (android fat distribution) and women in the hips, gluteal regions, and extremities (gynoid fat distribution). The decrease in sex steroid hormones associated with menopause may facilitate age-related increases in body fat, specifically abdominal fat.[4]

6.2 Estrogens and Body Composition

6.2.1 Premenopausal Women

Body composition changes with the onset of puberty; adipose tissue increases, suggesting that gonadal steroids can influence body fat. Premenopausal years and early pregnancy are states of efficient fat storage, possibly mediated through an estrogen-mediated reduction of lipid oxidation, with the purpose of increasing body fat for reproduction.[5] Kirchengast et al.[6] found that the absolute amount of body fat was significantly related to menarcheal age, resulting lower in women whose menarche occurred later.

There is also evidence that estrogens can modulate the osmotic regulation of vasopressin release in the brain, although the exact mechanism responsible for this effect is not known.[7,8] Vasopressin is the primary hormone involved in modulating water reabsorption in the kidney; it is synthesized in the anterior hypothalamus and stored in the posterior pituitary and is released through stimulation of central osmoreceptors. These nerve endings lie close to capillary networks, so that vasopressin release occurs rapidly after elevation in plasma osmolarity. A number of studies have demonstrated that basal plasma vasopressin concentration is elevated in the presence of high plasma concentration of unopposed estrogen. In normally menstruating women, plasma vasopressin concentration varies with the stage of the menstrual cycle and is highest at the time of ovulation and lowest at the onset of

menstruation.[9] Ross et al.[10] observed that fluid retention is at its lowest during the follicular phase and increases premenstrually.

6.2.2 Oral Contraceptives

The influence of oral contraceptives on body composition parameters is still controversial and it depends on the amount of baseline estrogens. Some studies suggest that the use of low-dose oral contraceptives does not have an impact on weight, body composition, or fat distribution. Reubinoff et al.[11] and Franchini et al.[12] have instead documented weight gain and an increase in body fat during treatment, but no changes in fat distribution. Litchfield and Grunewald[13] have indicated that women taking estrogens at high concentrations are more likely to have circumference measurements consistent with a more gynoid shape.

Oral contraceptives treatment can also affect the renin-angiotensin system. Huisveld et al.[14] have found a significantly lower plasma concentration of active renin in subjects taking oral contraceptives than in controls. Renin substrate, however, is significantly higher in the group taking oral contraceptives. This result demonstrates the well-known estrogen-induced stimulation of renin substrate synthesis by the liver and suggests a decreased secretion of renin in the kidney. Monophasic oral contraceptives users report higher levels of fluid retention and somatic symptoms than the triphasic oral contraceptives group.[10] Although hypertension and weight increase, due to increased fluid retention, are frequent in women over 35 years of age treated with oral contraceptives, De Leo et al.[15] found that new formulas have no clinical impact on such parameters in this age group.

6.2.3 Postmenopausal Women

It is well known that aging is associated to major quantitative changes in body composition: generally, fat mass increases while lean body mass decreases. However, the redistribution of fat and lean tissues is more important than the absolute quantity of tissue gained and lost. Intra-abdominal fat accumulates more rapidly than total fat, even in the absence of obesity. Aging is associated with a shifting of fat from peripheral to intra-abdominal depots. These changes are accelerated with the onset of menopause and progress linearly with time, placing middle-aged women at a higher health risk than the general population.[16,17] Menopause is defined in the gynecologic literature as the "absence of menses secondary to ovarian failure." The onset of menopause is marked by a rise in serum follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels and a fall in estrogen levels. Changes in energy expenditure, body composition, and regional body fat may be influenced and even accelerated by the menopause transition.[18] During menopause, the resting metabolic rate declines by approximately 100 kcal per day, probably due to the loss of metabolically active tissue. The

reduction in energy expenditure during rest is partly responsible for a positive energy balance, as it is not usually balanced by a corresponding decrease in daily energy intake. This phenomenon may account for the increase in fat mass.[19] Recently, it has also been shown that menopause may affect fat distribution, as more android fat and less gynoid fat has been observed in postmenopausal women compared with premenopausal women.[20] It has been suggested that estrogen depletion may be related to the increased deposition of body fat in the intra-abdominal region in postmenopausal women.[21] Premenopausal women, in contrast to postmenopausal women, have higher lipolytic activity in the abdominal adipose tissue and higher activity of lipoprotein lipase in the femoral adipose tissue.[22] Protein and carbohydrate balances are tightly regulated by mechanisms that couple intake to oxidation.[23] In contrast, changes in fat intake do not result in compensatory adjustments in oxidation. Thus, an increase in fat intake or a decrease in fat oxidation may promote an increase in total and intra-abdominal fat in postmenopausal women.[24] Menopausal transition is associated with reduced fat oxidation, and it is partially explained by the decrease in fat-free mass, because it is an important "consumer" of fatty acids.[25]

6.2.4 Hormone Replacement Therapy

Hormone replacement is the most widely prescribed drug in postmenopausal women, and one of the potential benefits of this therapy is its use as an antivisceral obesity agent, although this has not been systematically examined. Most studies have reported that postmenopausal hormone replacement therapy reduces the risk of some chronic diseases, such as cardiovascular disease[26,27] and osteoporosis.[28,29] Several studies have also investigated the effect of the hormone replacement therapy on body composition in postmenopausal women. Hassager and Christiansen[30] have shown that estrogen replacement therapy prevented total body fat accumulation, even if no measure of fat distribution was performed in this study. Other investigators have found that combined hormone replacement regimens prevented increases in central body fatness.[21,31–33] In contrast, several studies have found no effect of hormone replacement therapy on total and central fatness.[34–36]

Forsling et al.[37] have suggested an effect of hormone replacement therapy on body water. They have observed an increased vasopressin release after exogenous estrogen administration in postmenopausal women. This finding confirms the hypothesis that body water retention is a common consequence of high estrogen states (such as the ovulation time during menstrual cycle, oral contraceptives, or hormone replacement use), and it could be due in part to enhanced osmotic stimulation of vasopressin.

However, many postmenopausal women either cannot or do not want to comply with hormone replacement therapy. Consequently, the research into alternatives to estrogens for postmenopausal women has been increasing.

6.3 Soy and Obesity

Several studies have looked at the effect of soy on energy balance, body composition, and obesity.

6.3.1 Animal Studies

In a study conducted on genetically obese mice, Aoyama et al.[38] have reported that soy protein isolate and its hydrolysate were more effective in weight reduction and in lowering the body fat deposition than milk whey protein isolate and its hydrolysate. Similar findings have been observed in genetically obese yellow KK mice fed energy-restricted, low-fat, and high-protein (from casein or soy) diets. After 4 weeks, body fat content in mice fed the soy-based diet was significantly lower than in those fed the casein-based diet.[39] (See Table 6.1.)

Decreased total dissectible fat, percent body fat, and adipocyte size without significant loss of weight gain has been observed in rats when casein was substituted isoenergetically with soy protein in a starch-based diet.[40] These results indicate that soy proteins are suitable protein sources in energy-restricted diets for the treatment of obesity in mice. However, Nagasawa et al.[41] have not observed significant differences in body weight between 10-day soy and casein diets in rats. Rats fed methionine-equivalent soy or casein-based diets had similar weight gain and fat deposition,[42] suggesting that methionine supplementation may eliminate the positive effect on fat deposition previously ascribed to soy protein. Hurley et al.[43] have studied the interaction between dietary protein and carbohydrate on energy metabolism in rats. They have used casein, soy, or cod protein with either starch or sucrose. Soy protein isolate fed with starch was the most effective combination for reducing total body fat gains. This study indicates that the type of macronutrient (protein, carbohydrate, or fat) used is also important in energy metabolism and weight reduction.

6.3.2 Human Studies

Bosello et al.[44] have evaluated the short- and long-term effects of hypoenergetic diets containing proteins from different sources in 24 obese (60% above ideal body weight) adult subjects. (See Table 6.2.) Subjects were divided into two groups and were provided a very-low-energy (375 kcal/d) diet with the same amount of protein as casein or soy for 15 days followed by 60 days of a higher-energy diet (425 kcal/d). All subjects lost weight and the reduction was similar in both groups. Thus, the reduction of excess body weight seems due to a low energy intake rather than to the protein source. Similarly, in obese women Yamashita et al.[45] have not observed any differences in weight

TABLE 6.1

Effects of Soy in Obese Animals

Reference	Animals	Diet	Duration	Effects
38	Genetically obese mice	Soy protein isolate and hydrolysate compared with whey protein isolate and hydrolysate	2 weeks	Greater loss of weight and body fat in soy diet group
39	Obese yellow mice	Soy protein isolate and hydrolysate compared with casein protein isolate and hydrolysate	4 weeks	Decreased body fat content in soy diet group
40	Male Sprague-Dawley rats	Soy- or casein-based diet	7 weeks	Significantly greater total fat and adipocyte size in casein group
42	Male Sprague-Dawley rats	Soy- or casein-based diet supplemented with methionine	28 days	Similar weight gain and fat deposition in the two groups
43	Male Sprague-Dawley rats	Soy, casein, and cod diets with starch or sucrose as carbohydrate source	28 days	Lower weight gain in soy-starch diet
41	Wistar rats	Soy- or casein-based diets	10 days	No significant difference in body weight

TABLE 6.2

Effects of Soy in Humans

Reference	Subjects	Diet	Duration	Effects
44	24 obese subjects	Very low (1) and low energy diet (2) based on soy protein or casein	(1) for 15 d (2) for 60 d	Similar weight reduction in both groups
45	36 obese women	Low-energy diet based on soy protein or red meat	16 weeks	Similar decrease in body weight with both diets
46	11 obese women	Liquid formula (1000 kcal/d) based on soy or milk	4 weeks	No significant difference in weight loss between the two diets
48	100 obese women	Soy-based meal replacement formula or control diet (1200 kcal/d)	12 weeks	Treatment group lost significantly more weight than control group
49	83 obese subjects	High-soy-protein low-fat diet or control diet	6 months	Greater loss of weight and fat mass in soy diet group

loss with very low energy diets containing either lean meat or soy protein as the major protein source. Jenkins et al.[46] observed only a marginally greater weight loss in obese subjects after consumption of a low-energy diet with soy protein than after a low-energy diet with casein as a protein source. On the contrary, Fisler et al.[47] have observed that in obese men fed with low-energy diets containing either soy or collagen protein for 40 days, plasma essential amino acids were better maintained by the soy protein diet than by the collagen protein diet. These findings suggest that long-term substitution of vegetable protein for animal protein in a low-energy diet may provide an additional benefit for weight reduction in obese subjects. A recent study conducted by Allison et al.[48] on 100 obese volunteers shows that the treatment with a soy-based meal replacement formula for 12 weeks is effective in lowering weight. Moreover, Deibert et al.[49] have suggested that a high-soy-protein and low-fat diet can improve the body composition in overweight and obese people, losing fat but preserving muscle mass. However, it is not clear from all these studies whether the favorable effect of soy is related to its isoflavones content or to the protein content.

6.4 Effect of Phytoestrogens on Menopausal Body Composition

Few studies have investigated the association between body composition and isoflavones supplementation in postmenopausal women.

The DIANA randomized trial[50] was performed on 104 postmenopausal women randomized to dietary intervention (diet rich in phytoestrogens) or control group. The dietary intervention group showed a significant decrease of body weight and waist-to-hip ratio after 4.5 months of treatment.

Goodman-Gruen and Kritz-Silverstein[35] have examined the effect of typical, unsupplemented, dietary isoflavone intake on body composition in 208 healthy postmenopausal women, aged 45 to 74 years. Diet was assessed using a validated food frequency questionnaire during the past year concerning a list of foods high in isoflavone content. Women were categorized according to their genistein, daidzein, and total isoflavone intake into high (≥1 mg/day), moderate (0.001 to 0.999 mg/day), and no intake groups. Genistein and total isoflavone intake was significantly and inversely associated with weight, total body fat, and waist circumference. No association was observed between isoflavones and lean body mass and waist-to-hip ratio.

Moeller et al.[51] tested the effect of 24 weeks of treatment with soy protein isolate, isoflavone-poor soy, or whey control on regional fat gain and lean mass loss in 69 postmenopausal women. The soy protein isolate group showed a greater hip lean mass than other groups, but treatment had no effect on gain in overall body mass, fat mass, or lean mass. This result

suggests that soy protein isolate did not reduce the disease-promoting menopausal shift in regional fat mass.

Stroescu et al.[52] have evaluated the effect on body composition of 4 months of supplementation with isolate soy protein in 14 female gymnasts. This study has found a significantly greater increase in lean body mass of athletes who received daily supplementation with isolated soy protein than in the control group.

6.4.1 Mechanism of Action

Since the early 1940s, the effects of soy protein on lipid metabolism and its role in dietetic therapy for obesity have been examined. For years, the question has been whether the active component is protein itself or a combination with other component of soy protein. In addition, various kinds of soy protein (purified, ethanol extracted, isolates, hydrolysate) may contain different amounts of isoflavones, which have distinct physiological activities, particularly in lipid metabolism.

The mechanism by which phytoestrogens exert their beneficial effects on obesity is unclear. Due to their structural similarities with endogenous estrogens, phytoestrogens act as weak estrogens and compete with 17β-estradiol for binding to the intranuclear estrogen receptor protein to modulate gene transcription.[53,54] In addition to direct interaction with estrogens receptors, phytoestrogens may also act indirectly to modulate the concentration of endogenous estrogens. In postmenopausal women, overall adiposity and an unfavorable body fat distribution are associated with increased androgenicity and with a lower SHBG concentration.[55–57] Pino et al.[58] found an association between phytoestrogen action and increase in sex hormone–binding globulin (SHBG) levels, especially in subjects whose SHBG concentration are in the low end of the concentration range.

As adipose tissue is responsive to estrogen and expresses both estrogen receptor alpha and beta, Naaz et al.[59] tested the hypothesis that the estrogenic soy isoflavone genistein can have an effect on adipose tissue in ovariectomized rats. In their experiment, after 12-day diets containing 0 to 1500 parts per million (ppm) genistein, mice were killed and fat pads weighed. Mice fed 500 to 1500 ppm dietary genistein had a dose-responsive 37 to 57% decrease in fat pad compared with controls; 300 ppm genistein did not cause decreases. This result suggests that dietary genistein produces antilipogenic effects in mice at serum levels that humans are realistically exposed to. Nogowski et al.[60] have measured the effect of genistein on the liver and fat tissue of ovariectomized rats respectively by liver perfusion or incubation of isolate adipocytes with the isoflavone. When genistein acted directly on the liver during perfusion, a smaller incorporation of ^{14}C-glucose into lipids was observed, diminishing the liver triglycerides content. Acting on adipocytes, genistein strongly depressed both basal and insulin-induced lipid synthesis, when glucose was used as substrate. The effect of genistein alone

on lipolysis in the adipocytes was negligible, but it intensified the lipolysis induced by epinephrine. Genistein in food can reduce the fattening processes in ovariectomized rats, through its direct influence on lipid metabolism in the liver and adipose tissue. Another cellular study conducted on isolated rat adipocyte showed that basal lipolysis was particularly enhanced by genistein at its higher concentration.[61] It seems that the antilipogenic action of genistein may be due to an alteration on glucose transport and metabolism and to a restriction of fatty acid synthesis or their esterification.[62]

Isoflavones and lignans may also exert beneficial effects on tissue lipids through their antioxidative actions.[63,64]

6.5 Conclusion and Future Trends

Emerging evidence suggests that diets rich in phytoestrogens (soy protein and flaxseed) can have beneficial effects on obesity, both in animals and humans. The dietary components responsible for the beneficial effects of soy protein and flaxseed may be isoflavones. As maintaining daily protein intake with a low-calorie diet is important during obesity therapy, a soy protein hypocaloric diet is recommended because it has good quality, tolerability, and acceptability. However, most of the clinical trials that have been conducted on the effects of soy on obesity have been observational only, have had a relatively short duration, and have involved a small number of subjects. Long-term controlled trials on the effectiveness of soy in obesity and its complications are overdue.

Phytoestrogens could also be a useful alternative to hormone replacement therapy for preventing osteoporosis and cardiovascular disease in menopause. However, whether phytoestrogens have beneficial effects on body composition is still a matter of controversy. As estrogens are known to produce fluid retention, studies looking at body composition with isoflavone supplementation should be performed.

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7

Reproductive Hormones in Females and Hormone Replacement Therapy

Ayhan Karakoç and Metin Arslan

CONTENTS

7.1 Reproductive Hormones in the Female

The female reproductive system consists of well-regulated and integrated neuroendocrine signaling pathways between the hypothalamus and the pituitary gland and the ovaries (Figure 7.1).

The hypothalamo-pituitary-ovarian axis develops from fetal life to puberty and attains full maturity after puberty. With the first uterine bleeding episode (menarche), the ovaries begin to secrete estrogen and progesterone until menopause (the last bleeding episode). Atresia via programmed cell death (apoptosis) is the ultimate end of a follicle, which enters the growing follicular pool until the onset of puberty, after which gonadotropins orchestrate follicular development to form the preovulatory follicle in the normal menstrual cycle, which occurs about 400 times in the reproductive life of every woman from puberty to menopause.[1]

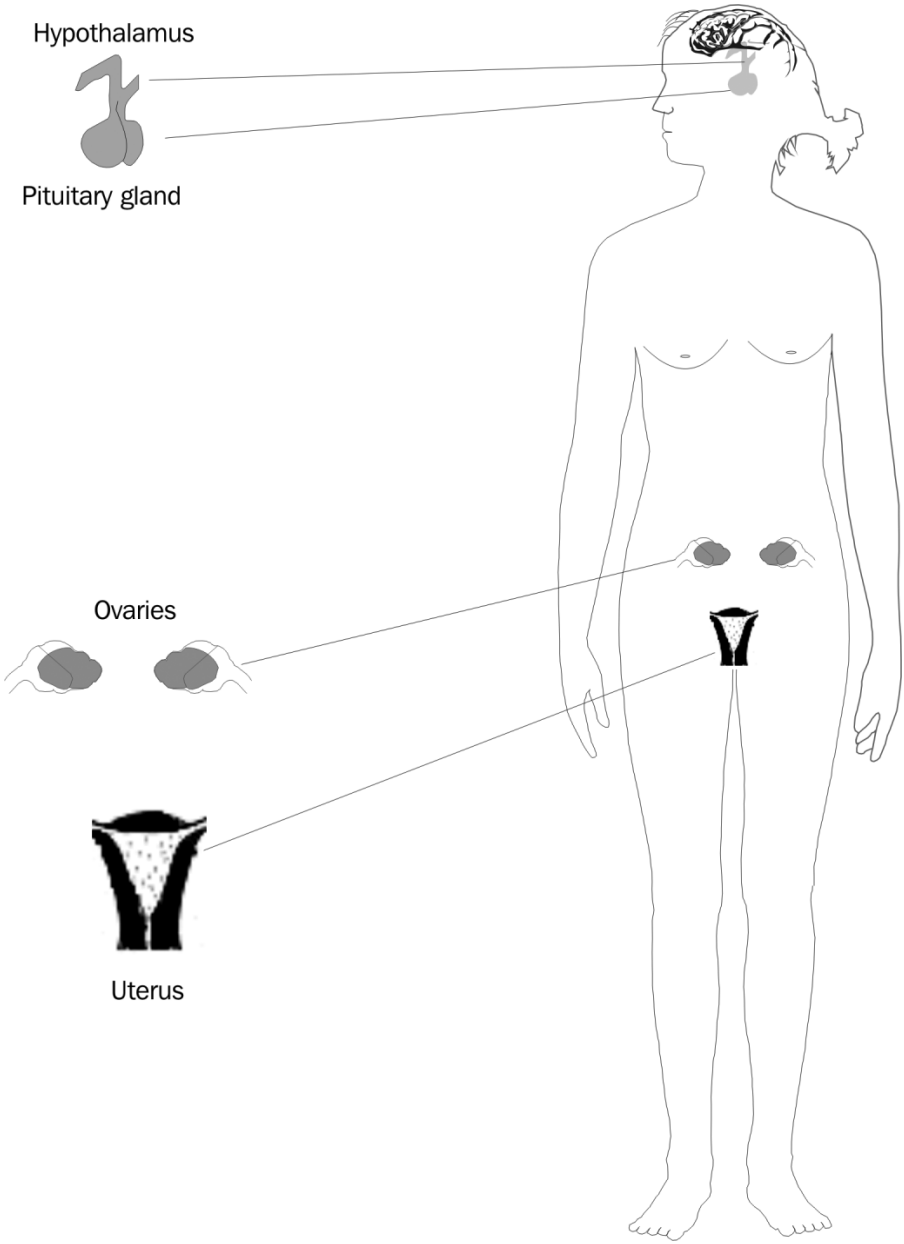

FIGURE 7.1
The reproductive system of the female.

7.1.1 Hypothalamic Hormones

GnRH (gonadotropin-releasing hormone), which is the key hormone that controls secretion of gonadotropins from the pituitary gland, is a hypothalamic hormone secreted in a pulsatile manner. GnRH is a linear decapeptide with stimulatory action only on luteinizing hormone (LH) and follicle-stimulating hormone (FSH). In humans, GnRH-secreting neurons are located mainly in the arcuate nucleus in the infundibular region of the hypothalamus and to a lesser extent in the preoptic area.

The interval between pulses and the amplitude of GnRH vary according to sex, age, and the physiological conditions. Pulsatile GnRH secretion is the major event preceding the onset of puberty. The interval between peak values of GnRH is about 90 min in adult life.

The release of LH is more sensitive to GnRH than FSH at low concentrations. Rapid GnRH pulses increase the secretion of both LH and FSH, whereas GnRH pulses at a slower rate stimulate the secretion of FSH more than LH. The continuous administration of GnRH suppresses secretion of both gonadotropins.

7.1.2 Pituitary Hormones

LH and FSH are gonadotropins secreted by the same cell from the anterior pituitary gland. Both hormones are glycoproteins with alpha and beta subunits. The beta subunits impart to them their specific biological activity. LH and FSH are also secreted in a pulsatile manner in response to the pulsatile release of GnRH.

While FSH receptors are present on only granulosa cells, LH receptors are expressed on both theca and granulosa cells of the preovulatory follicle. LH stimulates both estrogen and progesterone synthesis and secretion from the ovary, whereas FSH induces the synthesis of estrogen in the granulosa cells in the follicular phase. The development of the dominant follicle is FSH-dependent until late follicular phase where survival of this follicle becomes mainly LH-dependent.

7.1.3 Ovarian Hormones

The major function of the ovaries is the formation of mature oocytes for fertilization. The ovaries also produce and secrete hormones necessary for the development of secondary sexual characteristics and sustenance of pregnancy until 12 weeks of gestation. The follicle consists of an innermost oocyte, the surrounding granulosa cells, and outer layers of theca cells.

Estrogen and progesterone are the hormonal products of the ovary. Granulosa cells of the ovary are the main sources of estrogens in premenopausal women. The stromal cells of adipose tissue become the major site of estrogen synthesis after menopause. Estrogen produced by the syncytiotrophoblasts

of the placenta during pregnancy is also locally synthesized in the brain and breast of normal women. While the ovary synthesizes primarily 17 β-estradiol, adipose tissue synthesizes estrone and the placenta synthesizes estriol.

The synthesis of estradiol is regulated by the interaction between theca cells producing androstenedione and testosterone under the control of LH and granulosa cells of the dominant follicle converting the androgens to estradiol and estrone under the influence of FSH. This interaction between theca and granulosa cells is called the "two-cell, two-gonadotropin" theory (Figure 7.2).

Granulosa and theca cells of the ruptured follicle transform into the corpus luteum after ovulation under the control of LH. These luteinizing cells begin to secrete mainly progesterone in addition to estrogen. The progesterone level is increased by the mid-cycle LH surge, which in turn induces the expression of proteases involved in the release of oocytes.[2] Progesterone reduces primarily the GnRH pulse rate.

The ovaries also secrete glycoprotein hormones other than steroid hormones known as inhibins and activins. Inhibins and activins are members of the transforming growth factor β superfamily.[3] There are two forms of inhibins (inhibin A and inhibin B) and three activins (activin A, activin B, and activin AB). The production of these hormones is under the control of gonadotropins, and they control the production of gonadotropins (Figure 7.2). Follistatin is the activin-binding protein and is a component of the inhibin-activin-follistatin system.

7.1.4 Feedback Control of the Hypothalamo-pituitary-ovarian Axis

Feedback control of the reproductive hormones is complex in women. Both negative and positive feedback mechanisms operate depending on the phase of the menstrual cycle, age, and hormone concentrations.

Gonadal steroids have partial feedback control activity on the GnRH pulse rate. While estradiol increases the GnRH pulse rate, elevated progesterone level decreases it, especially in the late luteal phase.[4] Locally released neurotransmitters also have inhibitory or stimulatory effects on GnRH secretion. Gonadal steroids modify endogenous opioid activity while endogenous opioids mediate the negative feedback of steroids on gonadotropins and GnRH secretion.[5,6]

Gonadal steroid hormones have feedback control on both the hypothalamus and the pituitary. Estradiol and progesterone have negative feedback effects on GnRH release in general. As there is no steroid receptor in the hypothalamus, negative feedback of steroid hormones on GnRH secretion is mediated by neurotransmitters like β-endorphin.[7] In humans, estradiol has a direct negative feedback control on the pituitary, but most of the negative feedback by progesterone occurs at the level of the hypothalamus.[7]

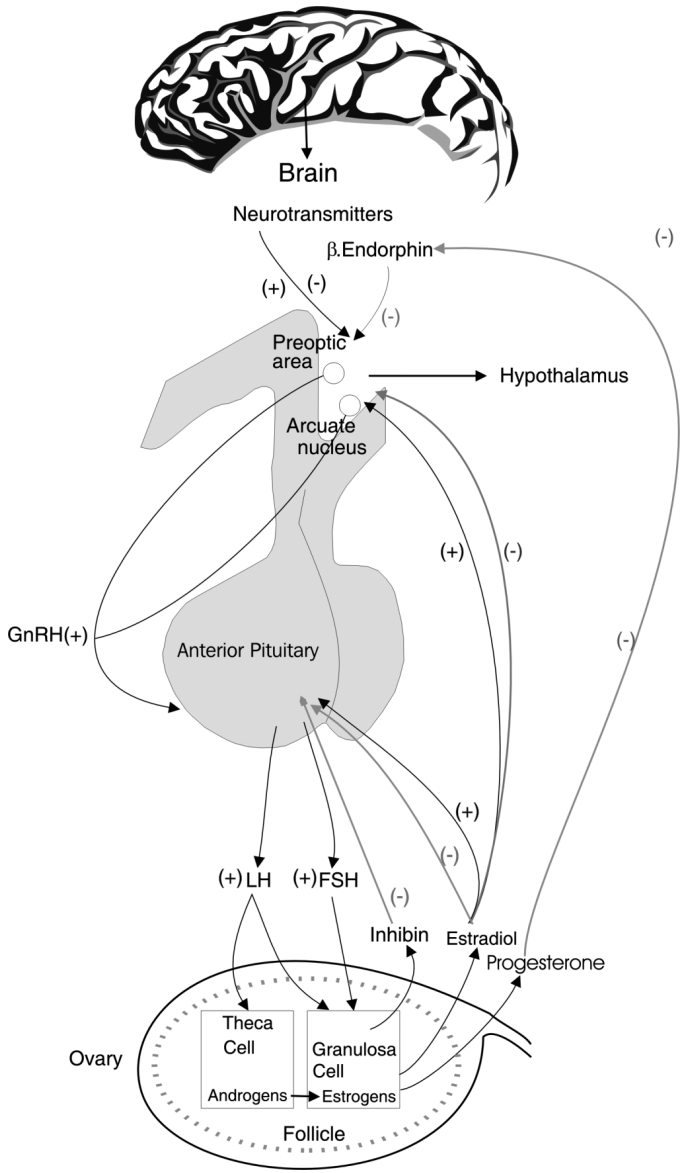

FIGURE 7.2
The feedback control of the reproductive system in the female. GnRH stimulates LH and FSH release from the anterior lobe of the pituitary gland. LH and FSH have also stimulatory effects on the functions of the ovary. The functioning of the hypothalamus and anterior pituitary gland are mainly under the negative feedback control (-) of the ovarian hormones except estradiol, which has a positive feedback effect (+) on the mid-cycle LH surge. FSH acts on the granulosa cells to produce estradiol. LH stimulates androgen production by the theca cells and estradiol and progesterone by the granulosa cells. Androgens produced by the theca cells are converted to estrogen within the granulosa cells. Progesterone has negative feedback control on GnRH secretion via β-endorphin secretion.

The hypothalamic-pituitary axis is under the negative feedback control of the ovarian steroid hormones in general. If ovarian failure as happens in menopause occurs, the LH and FSH levels increase due to the lack of negative feedback effects of the gonadal hormones.

Estradiol at high concentrations (above 200 pg/ml) has positive feedback action at the level of the hypothalamus and pituitary (Figure 7.2). High estradiol levels lead to mid-cycle LH surges by increasing GnRH secretion from the hypothalamus and the sensitivity of the pituitary to GnRH. The exact mechanisms responsible for the switch from negative to positive feedback and GnRH gene expression are not well understood. Estradiol concentration must exceed a threshold level for manifestation of the positive feedback effect on gonadotropins. Estradiol levels are generally maintained at about 500 pg/ml for 36 hours for the LH surge in women.

7.2 Reproductive Hormones Throughout Life

7.2.1 Reproductive Hormones from Fetal Life to Puberty

GnRH is present in significant amounts in the fetal hypothalamus, while LH and FSH appear in the pituitary by 10 weeks of gestation.[8] GnRH is released from the fetal hypothalamus in a pulsatile manner with LH and FSH concentrations increasing progressively from the tenth week of gestation to mid-gestation allowing for gonadal maturation and hormone production.[9] GnRH in the hypothalamus, LH and FSH in the pituitary, and serum LH and FSH concentrations remain high until late in gestation**.** Serum gonadotropins are decreased due to elevated serum estrogen levels toward term.[10]

Although fetal testosterone production is necessary for differentiation of the male genitalia, fetal estrogen, though produced in significant amounts, is not indispensable for differentiation of the female genitalia.

At birth, gonadotropins and estrogen levels are still high, but they decline during the first few days of life. Estrogen levels, however, remain low until puberty. Gonadotropin levels begin to rise at the first week of life due to weak negative feedback as a consequence of the disappearance of the sex steroids originating from the placenta.[9] During the neonatal period, LH and FSH levels are higher than that during the rest of childhood. Plasma levels of LH and FSH rise intermittently during the first 1 to 2 years of life similar to that seen in adulthood or to much higher levels.[11] After this, LH and FSH levels begin to fall with low levels of gonadotropins persisting for several years in what is known as the juvenile pause until the prepubertal years.[12]

The juvenile pause begins at 2 to 4 years of age and continues until 10 to 12 years of age.[13] At this stage in life, gonadotropins and sex steroids are all diminished, suggesting that this decrease in gonadotropin levels is independent of the ovarian steroid feedback. It is thought to be the result of the

inhibitory effect of the central nervous system, probably due to reduced GnRH pulse rate. Although gonadotropins are lowest in the juvenile pause, there are measurable levels of LH and FSH with female levels of FSH higher than male levels. However, episodic release and diurnal variation of gonadotropins also persist.[14] The estrogen levels not easily detectable with standard techniques in this period are very low, but with highly sensitive techniques measurable concentrations of estrogen have been shown in prepubertal girls.[15] However, the juvenile pause seems to be independent of the inhibitory effects of estrogen on gonadotropins, since the LH levels in Turner's syndrome are also decreased in this period of life despite an absolute estradiol deficiency in these patients.

7.2.2 Reproductive Hormones in Puberty

The age interval for the onset of puberty is 8.5 to 13 years.[13] Breast development (thelarche) is usually the first sign of puberty. Pubic hair development (pubarche) and increased velocity of growth (growth spurt) follow thelarche. The final stage of puberty is menarche with an average time from thelarche to menarche of 2.3 years (range 6 months and 5.75 years).[13]

As detectable by ultrasensitive and third-generation assays, prepubertal children at 5 years of age have diurnal rhythms of gonadotropin secretion similar to that seen in pubertal children,[16] but the amplitude of gonadotropin release per peak at this age is lower than that at puberty. At puberty, the rate of the gonadotropin pulse becomes more regular, while the amplitude of pulses rises. If a child's gonad is stimulated with gonadotropins, it can produce germinal and hormonal function. The pituitary gland in the prepubertal child can also secrete gonadotropin if it is stimulated with GnRH. Based on these observations, the onset of puberty has been suggested to depend on the level of GnRH secretion or the higher central nervous system loci; however, the precise mechanism is still not known. The stimulation of the onset of puberty is likely to be GnRH dependent.[13]

The frequency and magnitude of the GnRH pulse increases at the onset of puberty.[9] The important role of GnRH in the initiation of puberty has been confirmed by the induction of puberty in children with delayed puberty through pulsatile GnRH treatment.[17]

Prior to the appearance of the first secondary sexual characteristics during early puberty, the episodic pattern and levels of gonadotropins appear to be higher during sleep than during the day, with LH levels higher than those of FSH.[18] The LH surge is the hallmark of the maturity of the hypothalamopituitary axis. Nocturnal rise in LH activity is primarily due to an increase in the LH pulse amplitude. LH pulses during sleep are irregular at the beginning, becoming more regular with higher amplitudes as the stage of puberty advances. The nocturnal rise in LH pulse magnitude is not associated with darkness but is rather thought to be associated with rapid eye movement (REM) and non-REM sleep cycles.[19,20] The nocturnal LH pulsatile

activity persists into daytime with further increase in amplitude at night. The increase in LH throughout the day results in an increase in plasma LH concentrations, which leads to ovarian estrogen production with the subsequent induction of secondary sexual characteristics.[21] Regular daytime pulses are first seen in females at the time of the onset of thelarche. Nocturnal LH pulses disappear after menarche.[22] After puberty, LH and FSH levels are regulated by ovarian estrogen and progesterone production through negative and positive feedback mechanisms. Inhibin concentrations, which rise during puberty, also play a role in the negative feedback secretion of FSH.[23,24]

7.2.3 Reproductive Hormones in the Reproductive Years

7.2.3.1 Normal Menstrual Cycle

The normal menstrual cycle generally lasts for 21 to 35 days with a median length of 28 days. This cyclic event occurs approximately 400 times in a woman's reproductive life to support a mature ovum for fertilization. While the initial phase of follicular development is independent of gonadotropins, the selection and dominance of the preovulatory follicle is under the influence of gonadotropins. The follicular phase is the phase in which the dominant follicle is selected and matures. The dominant follicle secretes increased amounts of estradiol while the epithelial cells of the endometrium proliferate under the influence of estrogen. For this reason, this phase is also called the proliferative phase. The endometrium is prepared for implantation of the embryo in the follicular (proliferative) phase of the menstrual cycle. The follicular phase begins with menstrual bleeding and lasts until the release of the mature ovum by the ovary known as ovulation. The follicular phase varies in duration and determines the length of the menstrual cycle. Ovulation is triggered by the estrogen-dependent mid-cycle LH surge that becomes evident approximately 12 h before the ovulation[25] and also by the estrogen surge that takes place 24 to 36 h earlier.[26]

FSH stimulates the differentiation and proliferation of granulosa cells. FSH levels begin to increase at the end of the luteal phase of the previous menstrual cycle[27] and continues to increase during the early follicular phase. As the estradiol levels increase, the FSH levels fall to their lowest concentrations in the mid-luteal phase with a small increase at mid-cycle. Just before the menstrual bleeding starts, the FSH levels begin to increase again as the estradiol levels decrease to their early follicular levels by the end of the luteal phase.

LH receptors occur in the granulosa cells during the mid-follicular phase. LH also stimulates the secretion of androstenedione and testosterone by the theca cells. These androgens diffuse into the adjacent granulosa cells, where the androgens are converted to estrogen by the enzyme aromatase, whose activity is enhanced by FSH.

Estradiol levels are low during the early period of the follicular phase. Low estrogen levels form a negative feedback effect on LH. The concentration of LH shows only a minimal increase during the late follicular phase.

It is indeed under the negative feedback control of low estrogen levels during the early and mid-follicular phases. Estrogen levels rise to peak levels in the late follicular phase 24 to 36 h before ovulation. The elevated estrogen levels result in an LH peak (positive feedback).

The gonadotropins, especially LH, are secreted in a pulsatile manner during the menstrual cycle with intervals between pulses dependent on the phase of the menstrual cycle. Pulsatile secretion of GnRH accompanies the pulsatile secretion of LH.[28] The pulsatile secretion of GnRH is essential for normal reproductive function as understood from the inhibitory effect of continuous nonpulsatile GnRH administration on gonadotropin secretion.[29] During the follicular phase of the menstrual cycle, the frequency of an LH pulse is approximately once every 60 to 90 min. The increase in both LH pulse amplitude and frequency of up to one pulse every 15 to 20 minutes results in the LH surge seen during the mid-cycle.[28] The LH levels then decrease to follicular phase concentrations during the luteal phase. LH pulse frequency, however, diminishes to one pulse every 4 hours.

Progesterone levels increase slightly 12 hours prior to the LH surge.[26] The dominant follicle (Graafian follicle) ruptures under the proteolytic effect of progesterone. After release of the mature oocyte, the remaining granulosa and theca cells of the follicle reorganize to form the corpus luteum. The corpus luteum secretes estrogen and progesterone under the influence of LH.

The estrogen and LH levels decrease to early follicular phase concentrations at the end of the luteal phase. The GnRH/LH pulse rate also is reduced by the effect of progesterone. If fertilization fails, luteolysis occurs and progesterone levels decline. While FSH concentrations begin to rise, the endometrium sheds its epithelial lining, resulting in menstrual bleeding and hence the commencement of a new menstrual cycle.

7.2.3.2 Follicular Development

The primordial germ cells originate from the endoderm of the yolk sac. They migrate to the gonadal ridge by the fifth week of intrauterine life and proliferate by mitosis before differentiating into the primary oocytes. The gonads are nonfunctional in the absence of germ cells. The total germ cell number reaches its maximum of 7 million by 20 weeks of gestation. Most of the oogonia end in atresia by the process of apoptosis. Some of the oogonia enter the prophase of the first meiotic division where they remain until the time of ovulation, at which time meiosis is resumed. The formation of primordial follicles begins around mid-gestation when a single layer of pregranulosa cells surrounds each oocyte and continues until just after birth.[30]

From mid-gestation to menopause, attrition progressively decreases the number of germ cells (Figure 7.3). Formation of primordial follicles protects temporarily the germ cells from apoptosis.[31] The oogonia, which are not embedded in primordial follicles, become atretic. No oogonia are present at birth. Follicular atresia begins by the sixth month of gestation and continues throughout life; meanwhile oogonial atresia ends at 7 months of intrauterine

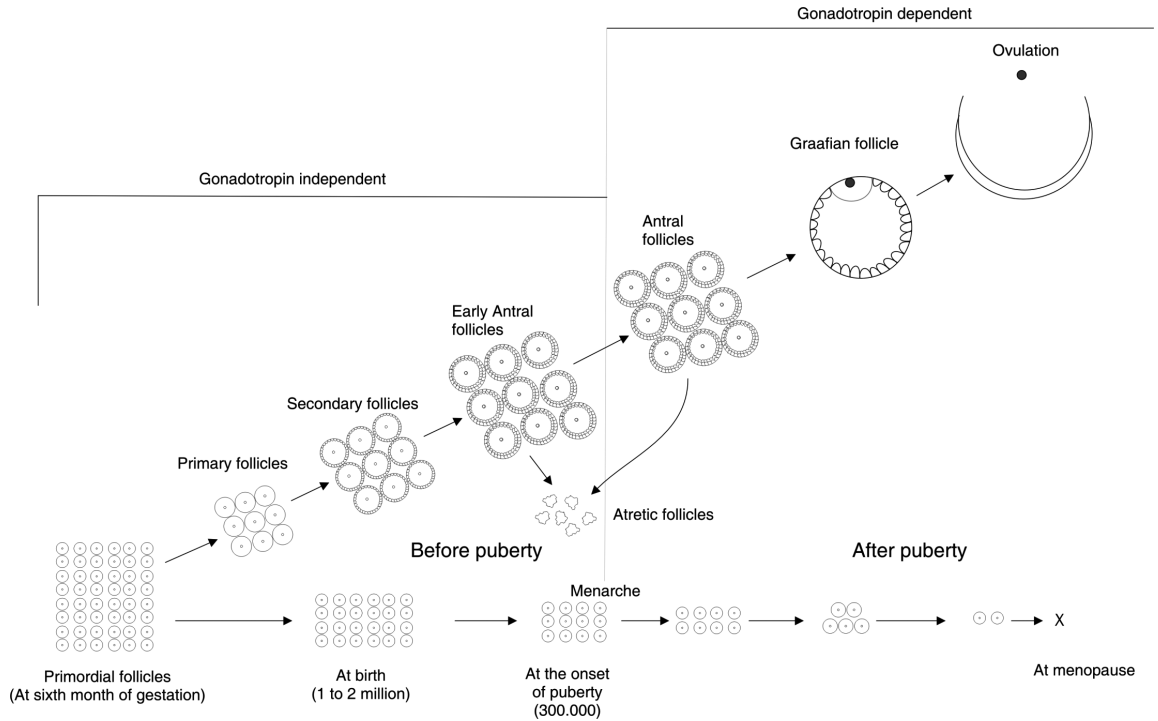

FIGURE 7.3
Follicular recruitment before and after puberty. Follicular recruitment is gonadotropin independent before puberty, and all primordial follicles develop to the antral stage once they leave the resting pool and go to atresia at this stage. After puberty, under the influence of gonadotropins, antral follicles continue their maturations and an antral follicle is selected and develops into the Graafian follicle, while the other antral follicles undergo atresia. All primordial follicles become senescent at menopause. (Adapted from McGee, E.A., Hsueh, A.J.W., *Endocrin. Rev.*, 21, 200–214, 2000. With permission.)

life. Follicular atresia profoundly depletes germ cells, so only 1 to 2 million germ cells are present at birth. This decrease continues further, and by the onset of puberty, only 300,000 germ cells remain. Of these, only 400 follicles ovulate during the entire reproductive years of the female.[32]

There are two main classes of follicles in the ovary: nonproliferating and proliferating follicles. The primordial follicles (nonproliferating) comprise 90 to 95% of the ovarian follicles and form the resting pool. Recruitment (the initiation of primordial follicular growth) begins *in utero* and continues until menopause[33] (Figure 7.3). The primordial follicular pool is depleted by recruitment from fetal life to menopause. Generally, the proliferating follicles are divided into four classes: primary, secondary, antral, and Graafian follicles. The preantral stage of follicular growth (primary, secondary, and early antral) is gonadotropin-independent (Figure 7.3) and is controlled by intraovarian factors.

Once they enter the proliferating pool, most follicles progress to the antral stage and all the growing follicles undergo atresia before puberty (Figure 7.3). After puberty, a cohort of follicles in the growing pool are rescued from atresia in the presence of FSH and so their growth continues. This stage, known as the Graafian stage, is gonadotropin-dependent, especially FSH-dependent, with normally one Graafian follicle formed in each menstrual cycle, while the remaining follicles of the cohort become atretic (Figure 7.3). Antral follicles develop into Graafian follicles in 14 days during the follicular phase of the menstrual cycle. Although the exact reason why only one follicle becomes dominant and survives is not clear, it is thought to be that this follicle is more sensitive to FSH.[34]

The continued growth of the primordial follicles into larger follicles and apoptosis of resting primordial follicles directly lead to the gradual depletion of the resting pool.[30] With increased FSH and decreased inhibin levels, more follicles are lost with the approach of menopause.[35,36]

7.2.3.3 Ovulation

Ovulation is triggered by the rise in LH levels under the positive feedback control of estradiol at mid-cycle. Increase in progesterone levels 12 hours prior to the LH surge causes expression of proteases, which lead to the release of the mature oocyte.[2] The exact mechanisms causing oocyte release from the dominant follicle are still unclear in humans. However, one candidate protein is a transcription factor, the progesterone receptor.[2] The enzyme cyclooxygenase-2 (COX-2) is another candidate for the control of ovulation. It has been shown that COX-2-deficient mice fail to undergo ovulation.[37] The physiological roles of these proteins and many others are issues still under investigation.

High doses of FSH rather than LH can also trigger ovulation.[38] The LH surge causes luteinization of follicular wall and terminates granulosa cell proliferation.[39,40]

7.2.3.4 Corpus Luteum

The remnant follicle after completion of ovulation becomes the corpus luteum. Blood vessels penetrating the follicle provide low-density lipoprotein cholesterol to the luteal cells for synthesis of progesterone and estradiol.[1] The corpus luteum also synthesizes inhibin A. The function of the corpus luteum is under the control of LH.

A prolonged LH peak is necessary for the complete formation of the corpus luteum (about 36- to 48-hour period in rhesus monkeys).[41] A small rise in the estradiol concentration occurs, but it does not cause a second LH surge due to the inhibitory effect of progesterone on GnRH/LH pulse frequency.[42] As a result, the concentration of FSH in gonadotropin cells increases. In the absence of fertilization, the corpus luteum regresses probably via apoptosis followed by a decline in the estradiol, progesterone, and inhibin A levels.

7.2.3.5 Estrogen in the Regulation of Menstrual Cycle

The follicle with the highest estrogen concentration in its microenvironment becomes dominant, whereas those with predominantly androgens become atretic. As the dominant follicle produces more estrogen into the circulation, which results in suppression of FSH, atresia of the androgen-rich follicles ensues. On the other hand, the survival of the dominant follicle continues even under suppressed FSH levels because of the availability of high FSH receptors.

The role of estrogen in the development of the follicles is controversial: follicular development occurs in the absence of estradiol.[43] Estradiol mainly regulates the secretion of gonadotropins. Estradiol plays a major role in the suppression of FSH levels during the main phase of the cycle, and decreased estradiol levels are the most important factor that initiates the secretion of FSH when the corpus luteum regresses.[44,45]

Estradiol also exerts a negative feedback effect on LH until mid-cycle, but the mid-cycle LH surge is the result of positive feedback control of estradiol.[46]

7.2.3.6 The Role of Progesterone in the Menstrual Cycle

Progesterone plays an important role in ovulation as mentioned earlier. The main neuroendocrine function of progesterone is the suppressive effect on GnRH and LH pulse frequencies, which serves as the primary mechanism of oral contraceptive treatment.[1]

7.2.3.7 Role of the Inhibin–Activin System in the Menstrual Cycle

Inhibin B is produced by granulosa cells of mature follicles, and inhibin A is secreted by luteal cells.[47] Their production is under the control of gonadotropins.

Inhibins selectively suppress FSH secretion and have no effect on LH production. The levels of inhibins increase in the menstrual cycle.[48] Inhibin

B levels rise in the follicular phase and fall in the luteal phase.[49] Inhibin A levels primarily rise to peak levels in the mid-cycle period and in the mid-luteal phase.[49]

Activins have facilitatory effects on FSH secretion.[50] Activins stimulate both the basal and GnRH-induced FSH release and enhance LH secretion. Activins have a role on granulosa cell growth, steroid hormone production, and oocyte maturation.[51]

7.2.3.8 The Endometrial Cycle

Estrogen and progesterone play a key role in the endometrial cycle and maturation. During the follicular phase, endometrial cells proliferate under the control of estradiol. The role of estradiol is limited to the follicular phase because lack of estradiol in the luteal phase does not affect endometrial maturation.[52] During the luteal phase of the menstrual cycle (the secretory phase of the endometrium), epithelial cells become secretory while stromal cells undergo predecidualization.[1] Progesterone continues the maturation of the endometrium during the luteal phase. At the end of the luteal phase, the endometrium sheds by ischemic, apoptotic, and proteolytic mechanisms, resulting in menstrual bleeding. Menstruation depends on the decreased progesterone levels. Maintenance of high progesterone levels prevents bleeding, but high estradiol levels, despite the absence of progesterone, result in bleeding.[1,52]

7.2.4 Reproductive Hormones in Perimenopause

The World Health Organization (WHO) defines the perimenopause as the period immediately before menopause (when the endocrinological, histological, and clinical features of approaching menopause commence) and the first year after menopause.[53]

Changes in the hypothalamo-pituitary-ovarian axis begin as early as the fourth decade of life.[13] The rate of loss of ovarian follicles increases and the quality of the remaining follicles decreases.[54] At this time, the menstrual cycle still occurs, but menstrual irregularities become evident in the early 1940s. It is difficult to establish the hormonal changes in the perimenopausal period, because there are high fluctuations in hormone levels from one cycle to another.[55]

The median age of the onset of hormonal and clinical signs of perimenopause is 47.5 years.[56] The most widely accepted view is that FSH levels increase first in the perimenopausal period while LH levels remain low until prior to menopause[57–59] (Figure 7.4). The elevated FSH levels are thought to be nondiagnostic of perimenopause.[60] Data from many previous reports indicate that FSH most likely first elevates during the early follicular phase.[57,61] However, many other studies have shown an increase in LH concentrations during the perimenopausal period as well.[62,63] Some

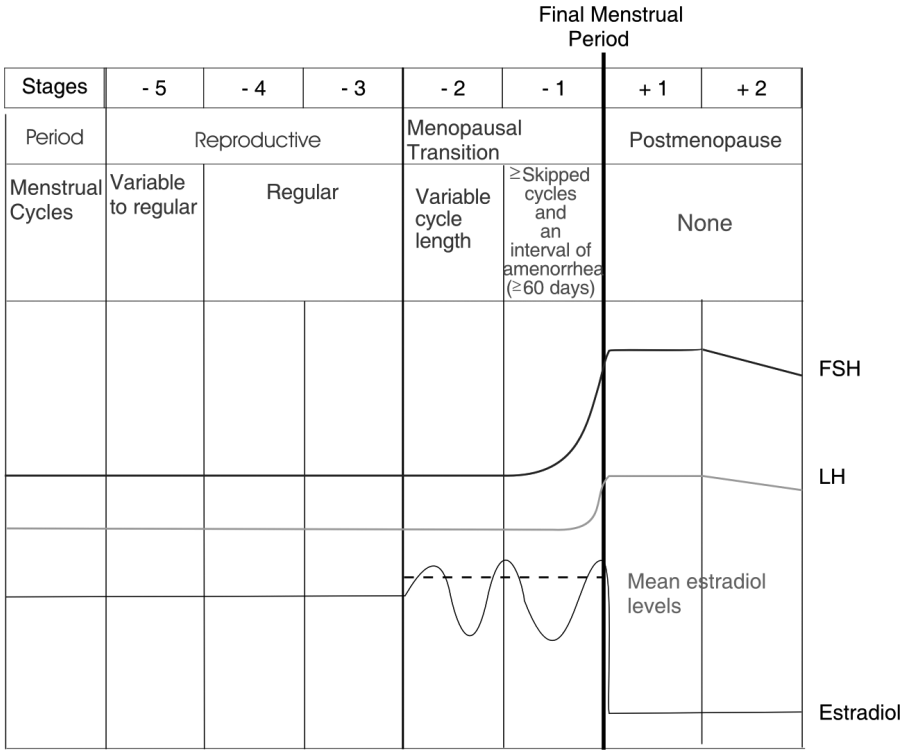

FIGURE 7.4
Stages of reproductive aging and gonadotropins and estradiol levels during reproductive aging. Menstrual cycles may be irregular in the early period of reproductive years (-5). Menstrual irregularity increases in the late menopausal transition stage (-1). Early follicular FSH levels may be high during stage –3; however, mean FSH levels are normal. FSH levels increase first in the perimenopausal period while LH levels remain low until prior to menopause. The menopausal transition is a period of fluctuations between hypo- and hyperestrogenemia, moreover with mean estradiol levels higher than reproductive years. Gonadotropins are high during postmenopausal years, but they gradually and slightly decline during the late postmenopausal period. (Adapted from Soules, M.R., Sherman, S., Parrott, E., Rebar, R., Santoro, N., Utian, W., and Woods, N., *J. Wom. Health Gender-Based Med.*, 10, 843–848, 2001. With permission.)

menstrual cycles can still be ovulatory despite elevated levels of LH in the perimenopausal period.

During early perimenopause, there is shortening of the follicular phase and therefore of the entire menstrual cycle. In the later stage of perimenopause, the ratio of anovulatory cycles increases with more luteal phase defects and prolongation of intermenstrual periods. The perimenopausal woman is still fertile due to ovulation, although it occurs rarely. For this reason, some form of contraception should be recommended until menopause or higher than 40 IU/l FSH concentration is attained to prevent unwanted pregnancies.[32]

The perimenopause is not a stage of pure hypoestrogenemia, but a status of hormonal fluctuations between hypo- and hyperestrogenemia (Figure 7.4). Indeed, serum estradiol levels do not decrease until less than a year before menopause.[32]

Estradiol levels in perimenopausal women are higher than that in younger control women[64] (Figure 7.4). Estrogen levels are elevated during both the follicular and luteal phases of the menstrual cycle.

The mechanism underlying the high estradiol levels is increased follicular response to gonadotropins. Decreased inhibin production is also thought to be the cause of elevated FSH levels.[32] Elevated estradiol levels do not have any inhibitory effects on either FSH or LH secretion, making menopause a state of hypergonadotropic hyperestrogenemia. During perimenopausal years prior to menopause, anovulatory cycles may lead to endometrial proliferation, which is not opposed by progesterone, resulting in endometrial hyperplasia or dysfunctional uterine bleeding.

Perimenopausal women have climacteric symptoms, although estrogen levels are elevated.[65] This observation can be interpreted to mean that climacteric symptoms are related to unstable estrogen levels rather than of hypoestrogenemia.

The elevated FSH and LH concentrations with low estradiol levels similar to that in the postmenopausal period are seen sometimes during the perimenopausal period, but these episodes are transient,[66] and their frequency rises toward menopause.

7.2.5 Reproductive Hormones in the Postmenopausal Period

The menopause is the final menstrual period, after which a woman does not have any menstrual bleeding for 12 months.[53]

The follicular units are completely depleted in the ovary after the menopause.[32] In the absence of estradiol and inhibin, which exert negative feedback effects on gonadotropins, both FSH and LH levels increase after menopause. Gonadotropins sharply increase for a few years after menopause and then gradually and slightly decline (Figure 7.4). Both gonadotropins may be higher than 100 IU/l; however, FSH levels are usually greater than LH levels.

Ovaries synthesize primarily androstenedione in the postmenopausal period.[67] The most important hormonal alteration seen after menopause is the very low estradiol levels. The average estradiol level is less than 20 pg/ml in the postmenopausal period (Figure 7.4).

Androstenedione is converted peripherally first to estrone and then to estradiol in adipose tissue in postmenopausal women. The mean circulating levels of estrone are slightly higher than mean estradiol levels. The change in androgen to estrogen ratio results in mild hirsutism in postmenopausal women. Serum androstenedione, testosterone, estrone, and estradiol levels do not change significantly during the postmenopausal period with aging.[68]

Because of the high content of the enzyme aromatase in adipose tissue, greater amounts of androgens are converted to estron and estradiol in obese patients than in lean subjects, and these high levels of estrogens cause an increased risk of endometrial cancer in obese postmenopausal women.[32] That is a risk, but it protects from osteoporosis at the same time.

Local estrogen synthesis from androstenedione is also important in breast cancer in postmenopausal women through aromatase and 17 β-hydroxysteroid dehydrogenase enzyme activities.[32]

7.3 Hormone Replacement Therapy

7.3.1 The Transition to Menopause and the Definition of Menopause

Menopause is defined by WHO as the final menstrual period after which a woman experiences 12 consecutive months of amenorrhea.[53] The average age of menopause is 51 years. The diagnosis of menopause is made retrospectively according to this definition. Transition to menopause includes only the period just before the final menstrual flow, and the WHO report does not recommend the use of the term "climacterium," because it is used for the perimenopausal period and the menopausal transition and to describe the postmenopausal symptoms as well.[53]

However, these definitions do not clearly define the stages of perimenopause and menopausal transition. The stages of reproductive aging workshop[69] was held in July 2001 to create a staging system for reproductive aging. This staging system took into consideration the menstrual cyclicity and endocrine changes, but not symptoms because of their variable presentations. There are seven stages: five preceding and two following the final menstrual period (Figure 7.4). The reproductive stages are –5 to –3 (early, peak, and late stages, respectively). Menstrual cyclicity is variable during stage –5 and regular in stage –4 to –3. Early follicular FSH levels may be high during stage –3. This change in FSH levels is the first sign of reproductive senescence. Stages –2 and –1 are part of the menopausal transition period, which ends with the final menstrual bleeding. Menstrual cyclicity is still normal with some variability in cycle length in the early menopausal transition period (stage –2) and becomes irregular with two or more skipped cycles and at least one episode of amenorrhea lasting ≥60 days in the late menopausal transition period (stage –1). The postmenopausal period consists of stages +1 and +2. Women are amenorrheic in this period. The early phase of the postmenstrual period (stage +1) has two subperiods, the first year after the final menstrual period and the following 4 years. Stage +2 continues to the end of life.

7.3.2 Clinical Aspects of Menopause

Some women start to experience various symptoms related to reproductive aging toward menopausal transition. In the late menopausal transition, the symptoms of reproductive aging become more prominent. Vasomotor complaints are the most frequent and important symptoms of the menopausal symptoms. However, some women may not have menopausal symptoms at all while others with menopausal symptoms may have them at varying degrees.

7.3.2.1 Vasomotor Symptoms

The most prominent menopausal symptom, the vasomotor flush (hot flash), occurs in 65% of those in the first and second years after menopause and decreases to 35% of those in the 5 to 10 years after menopause.[70] It increases during the perimenopausal and early postmenopausal years.

Women experience a sensation of heat usually involving the face and upper trunk. Reddening of the face and upper body, peripheral vasodilation accompanied by a rise in skin temperature up to 4°C, and transient increase in heart rate follow the sensation of warmth.[71] Core temperature falls because of heat loss as a consequence of peripheral vasodilation, and this episode usually ends in cold sensation and sweating. Chills, nervousness, irritability, and headache accompany the hot flushes. A hot flush episode often lasts for 4 minutes. At night, flushes are more frequent and may awaken a woman from her sleep. This may lead to fatigue, emotional lability, poor concentration, and night sweats. Hot flushes lessen in frequency and intensity with age and usually disappear in the late postmenopausal period. The association between hot flushes and hypoestrogenemia is well known, but the cause seems to be the acute estrogen withdrawal rather than the hypoestrogenemia itself. This is supported by the absence of flushes in chronic hypoestrogenemic diseases. Hypogonadal women experience hot flushes only after withdrawal of estrogen treatment. Obese women experience fewer hot flushes due to their elevated estradiol concentrations.[72]

The pathophysiology of vasomotor flushes is not fully understood. The source of hot flushes is likely the thermoregulatory center in the hypothalamus. It is thought that a change in neurotransmitter resulting from estrogen withdrawal stimulates GnRH release, which alters the temperature control center.[73] It has been shown in animal studies that a fall in endogenous opioid activity within the hypothalamus as a result of estrogen depletion may cause vasomotor symptoms similar to opiate withdrawal.[74]

7.3.2.2 Genitourinary Symptoms

Genitourinary system changes due to gonadal steroid deficiency result in many functional and psychosexual disturbances. The vulva, vagina, urethra, and uterus become atrophic. These changes result in pubic hair loss, vulvar

pruritus, vaginal dryness, dyspareunia, vaginitis, dysuria, frequency and urgency of micturition, and decline in sexual desire.

7.3.2.3 Cardiovascular System

Estrogen has a direct effect on the vascular wall as well as indirect effects such as change in total cholesterol, low-density lipoprotein (LDL) cholesterol, and high-density lipoprotein (HDL) cholesterol levels. It has been shown that menopause is associated with increases in the levels of serum cholesterol and triglycerides.

Cardiovascular tissues express estrogen receptors. Estrogen–estrogen receptor complexes induce gene expression, which has vascular effects, including regulation of vasomotor tone and response to vascular wall injury. These effects may have protective roles against atherosclerosis and coronary heart disease. Estrogen mediates rapid transient vasodilation due to the activation of the nitric oxide synthase enzyme without any change in gene expression.

Chronic effects of estrogen on the vascular system are thought to be due to its activity on vascular gene expression. The vascular effects of progesterone have not been well established.

Serum cholesterol, LDL cholesterol, and triglyceride levels increase in the postmenopausal period. This change in the lipid profile is a risk for coronary heart disease.

In the Framingham study, in which 2873 women were followed for up to 24 years after initial assessment,[75] postmenopausal women were shown to have a more than twofold risk of coronary heart disease compared with their age-matched premenopausal women. As a result, it is obvious that there is a sharp increase in the incidence of coronary heart disease and other diseases associated with atherosclerosis after menopause.

7.3.2.4 Postmenopausal Osteoporosis

More than 80% of osteoporosis cases occur among postmenopausal women and among subjects older than 65 years. Trabecular bone (spine) loss is more prominent than cortical bone (hip) loss after menopause. Bone loss in the spine does not occur until the fifth decade of life and is more dependent on the falling estrogen production.[76] Vertebral bone is especially sensitive to estrogen deficiency, because the trabecular bone of vertebral bodies is metabolically more active than the trabecular bones of other skeletal components. There is a continuous process called bone remodeling by which old bone is removed and new bone is formed. The rates of resorption of old bone and formation of new bone are equal before menopause. After menopause, an increase is seen in the number of new remodeling units, which causes a transient loss of bone mass.

Estrogen receptors have been detected on cells of osteoblast and osteoclast lineage and on many other cell types associated with bone. Estrogen

deficiency may lead to functional changes in osteoclasts, pro-osteoclasts, osteoblasts, and probably osteocytes and bone marrow cells. Consequently, estrogen actions affect cell–cell interactions, release of cytokines, and growth factors.

Osteoporosis in postmenopausal women results from both aging and estrogen deficiency. Vertebral bone loss reaches rates of 3 to 5% per year for the first 5 to 10 years, leaving no doubt that declining estrogen levels related to menopause trigger the bone loss. Fifty percent of bone mineral content in trabecular bone and 30% of cortical bone are lost in the first 20 years of the postmenopausal period.[32]

7.3.2.5 Cognitive Function Changes and Insomnia

There is a possible link between the estrogen deficiency and the increased incidence of Alzheimer's disease in postmenopausal women.[77] However, the potential benefits of estrogen replacement therapy in improving cognitive function in postmenopausal women and in the prevention and treatment of Alzheimer's disease still remain controversial.

Many cognitive functions including verbal memory, vigilance, reasoning, and motor speed may be defective. Moreover, the incidence of depression is higher among women in perimenopausal and postmenopausal periods than among premenopausal women.[78]

Insomnia is one of the clinical aspects of menopause. Estrogen therapy is thought to improve sleep by reducing the frequency and severity of hot flushes. It was shown in a prospective double-blind crossover study that the effect on quality of sleep of estrogen replacement therapy did not depend on the relief of vasomotor symptoms.[79] The effect of estrogen on insomnia was found to be the result of minimizing arousals due to nighttime arousal and not by changing the quality and quantity of sleep.[80]

7.4 Estrogen Replacement Therapy / Hormone Replacement Therapy

Synthetic estrogen, developed in the late 1930s, was marketed as estrogen replacement therapy (ERT) for the relief of menopausal symptoms. Progesterone was added to avoid the risks of ERT such as endometrial cancer after it had been recognized that estrogen alone might cause endometrial hyperplasia and endometrial cancer.[81] This combination of estrogen plus progesterone then came to be known as hormone replacement therapy (HRT). After the beneficial effects of HRT had been shown on heart disease risk factors in postmenopausal women, conjugated equine estrogen combined with medroxyprogesterone acetate became the most widely used preparation in the United States for postmenopausal women with intact uteri.[82] More than 46 million prescriptions for

premarin and 22 million prescriptions for Prempro were written in the year 2000.[83] However, the use of HRT declined more than 30% in recent years, because more and more publications report that HRT may not be as beneficial today as it was previously thought.

7.4.1 Candidates for HRT

It is difficult to make an exact list of indications for HRT in postmenopausal women. Treatment for each patient needs to be individualized. There are some benefits as well as hazards regarding the use of HRT. Hence, the balance between benefit and risk ratio must be determined for every patient. In women with premature menopause and surgical menopause, the duration of hypoestrogenemia is long, and these especially young women are good candidates for long-term HRT. Estrogen deprivation should be treated with an estrogen-progestin combination in women who have an intact uterus, and this can be replaced by estrogen alone in women without uteri. Serious vasomotor symptoms can be treated successfully with HRT in the peri- and postmenopausal periods. Short-term (6 to 12 months) HRT is a good and safe choice for women who suffer from serious hot flush episodes. The use of long-term (sometimes lifelong) HRT should be decided by both the patient and her physician. In recent years, indications of HRT are restricted to only the vasomotor symptoms and genitourinary atrophy.

7.4.2 Contraindication for HRT

Although several benefits of the female sex hormones have been described, several risks on women's health have been noted. HRT should not be recommended by the physician in patients who have an established disease and risk factors (Table 7.1).

In some situations, it will be better not to use HRT at all, but these are relative contraindications for HRT (Table 7.1). If the benefits of HRT outweigh the hazards, then HRT may be recommended in these cases.

TABLE 7.1

Absolute and Relative Contraindications for HRT

Absolute Contraindications	Relative Contraindications[a]
Breast cancer	Recent dysfunctional bleeding
Endometrial cancer	Mass in breast with unknown origin
Recurrent thromboembolic events	Family history of breast cancer
Acute phase of thromboembolism	History of thromboembolic events and hepatic diseases
Acute phase of myocardial infarction	
Active hepatic disease	

[a] There may be certain exceptions to these contraindications when the physician and patient both agree that the benefits of estrogen treatment substantially outweigh the risks.

7.4.3 Modes of HRT

Estrogen alone is given to patients without uteri. ERT may be given cyclically (3 weeks on, 1 week off) or continuously. When estrogen was used alone in postmenopausal women with uteri, it was believed that cyclic regimens had minimal risk of endometrial hyperplasia, but no evidence implicating the continuous regimen with an increased risk was available;[84] however, estrogen alone is never recommended today in postmenopausal women with intact uteri. The current preferred ERT regimen is the continuous administration of estrogen in patients without uteri, because some patients suffer recurrence of hot flushes during the period that they are off treatment.

In patients with an intact uterus, sequential or continuous combined regimens are preferred to protect against the risk of endometrial hyperplasia or cancer. The currently recommended cyclic regimen includes the use of medroxyprogesterone acetate 5 to 10 mg daily given on the last 10 to 14 calendar days and conjugated equine estrogen 0.625 mg daily given continuously. However, the occurrence of withdrawal bleeding as a result of this cyclic combined regimen is a major cause of worry for some patients considering HRT.

The continuous combined regimen is more preferred than the sequential combined regimen because it does not cause withdrawal bleeding. In this regimen, lower doses of progesterone (medroxyprogesterone acetate 2.5 mg daily) are given continuously, combined with a standard dose of estrogen. However, breakthrough bleeding in the first 3 to 4 months may occur in 40 to 50% of patients.

While the combined sequential or combined continuous regimens mentioned above are the most preferred modes of HRT, in Europe, oral micronized estradiol or estradiol valerate at doses of 1 to 2 mg daily or transdermally in gels or patches at doses of 0.05 or 0.1 mg daily are prescribed more frequently.

7.5 Estrogens

Natural estrogens are preferred to synthetic steroids in HRT. The most commonly used natural estrogen is the conjugated equine estrogen, which is composed mainly of estrone and estrone sulfate. It also contains equilin, a horse estrogen, because it is extracted from the urine of pregnant mares. The usual dose prescribed is 0.625 mg/day. Serum estradiol level 4 to 6 hours after ingestion is about 30 to 40 pg/ml with this daily dose. Conjugated equine estrogens are commonly prescribed orally but vaginal creams are also available.[85] Recently, lower doses of estrogens are available on the market.

TABLE 7.2

Types and Approximately Equivalent Doses of Estrogens

Natural	Synthetic
17–β estradiol (micronized) 1–2 mg/d	Ethinyl estradiol 10–20 μg/d
Estrone sulfate 1.25–2.5 mg/d	Qinestrol 0.1–0.2 mg/week
Estradiol valerate 1–2 mg/d	Diethylstilbestrol 0.1–0.5 mg/d
Conjugated equine estrogen 0.625–1.25 mg/d	Dienestrol
	Mestranol

17-β-estradiol has been successfully micronized, and it is effective over 24 hours when given orally. Estradiol valerate is another estrogen prescribed orally.[85] There are many other natural and synthetic estrogens (Table 7.2). Ethinyl estradiol is not used for postmenopausal HRT; it is usually prescribed for contraception. Their potencies differ with different doses of estrogen needed to suppress FSH to the same degree (Table 7.2). Oral estrogens, which result in higher serum levels of estrone, are metabolized to estradiol in the intestinal mucosa and in the liver. They are found in higher doses in the liver and intestinal mucosa and are rapidly excreted in the urine and bile. Many parenteral administration routes have been developed to prevent this intensive first-pass metabolism. The application sites for parenteral estrogens are mainly the skin, vagina, and subcutaneous tissue.

The first-pass metabolism is avoided when parenteral routes are used, and therefore the plasma levels measured reflect the delivered and absorbed dose. The epithelial lining of the vagina rapidly absorbs estrogen and leads to improvement of vaginal atrophy and urinary symptoms.

The pharmacokinetics of intranasal estradiol application differ from other parenteral forms. The time necessary for the maximal plasma level to be attained is shorter (10 to 30 min) than the other forms. The peak values decrease by 10% after 2 hours. It has a pulse-like plasma profile. The plasma levels are low between pulses, but the effect on symptom relief is equivalent to oral estradiol treatment.[86]

7.6 Progestins

Progestins are synthetic derivatives of progesterone used in HRT. They act primarily on intracellular progesterone receptors and may act weakly on other steroid receptors like androgen and glucocorticoid receptors. They are derived from testosterone (19-nortestosterone derivatives) or from progesterone (17-OH progesterone derivatives and 19-norprogesterone derivatives).[85]

19-nortestosterone derivatives include the estrone group (norethisterone and its derivatives) and the gonane group (levonorgestrel and its derivatives). Desogestrel, ethanogestrel, gestodene, and norgestimate are in the

gonane group, whose members are collectively referred to as the third-generation progestins. The 19-nor derivatives of progesterone include trimegestone, nomegestral acetate, and nestorone. They bind only to progesterone receptors. Medroxyprogesterone acetate, cyproterone acetate, megestrol acetate, and the much newer compound drospirenone are 17-OH progesterone derivatives. The most prescribed molecule in this group, medroxyprogesterone acetate, has little androgenic activity. Natural progestins and micronized progesterones are also used.

7.7 Benefits and Risks of HRT

There are many benefits and risks of HRT, although some are considered controversial (Figure 7.5). Controversies arise from the discrepancies between observational studies and recent randomized controlled trials.

7.7.1 Benefits of HRT

7.7.1.1 Effects on Menopausal Symptoms

The effectiveness of HRT with standard regimens for relief of vasomotor and urogenital symptoms is well established.[87,88] However, lower doses are also

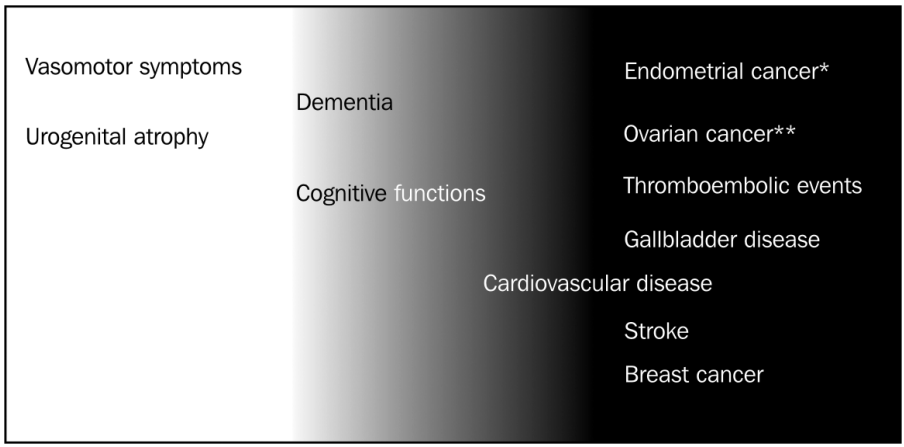

FIGURE 7.5
Schematic view of the effects of hormone replacement therapy (HRT). The white area contains beneficial effects of HRT. The gray area represents controversial effects of HRT. Light gray = probably beneficial, dark gray = probably harmful. The black area contains harmful effects of HRT. *Endometrial cancer is not seen with the continuous combined regimen, which is the most widely used HRT application method today. However, the risk of endometrial cancer is present with the long-term cyclic combined regimen. **It is thought that continuous combined regimen may cause ovarian cancer (especially endometrioid type).

as effective as the commonly prescribed higher doses for the relief of menopausal symptoms.[89] Hot flushes and night sweats are the only symptoms universally reported to respond to estrogen treatment and are the primary indications for HRT. Estrogen treatment reduces the incidence and severity of hot flushes.[90,91] The most important component of HRT effective on hot flushes is estrogen. The type or dose of progesterone does not seem to affect the effectiveness of estrogen at standard HRT doses. Although low-dose HRT is as effective as standard dose in treating menopausal symptoms, more time is required for the relief of symptoms.

Vaginal dryness, dyspareunia, and pruritus are alleviated by any of the locally applied estrogen preparations.[92] HRT also relieves some urinary complaints. HRT seems to improve urgency, frequency, and dysuria, but its effect on urinary incontinence is controversial. Although earlier studies show improvement by up to 50%,[93,94] a recent study reported worsening of incontinence with HRT when compared with placebo.[95] HRT also reduces the risk of recurrent genitourinary tract infections, probably by modifying the vaginal flora.[96]

The loss of energy, sleep problems, and mood changes are also alleviated by HRT.[97,98] HRT improves the quality of life in the short term.[99] The effects of HRT on sleep disturbances and mood disorders are thought to be due to its relieving effects on hot flushes, although some studies have noted the opposite results.[79,80]

7.7.1.2 Benefits on Osteoporosis

Accelerated bone loss related to estrogen deficiency begins at the menopausal transition period before the final menstrual period.

The beneficial effects of estrogen on bone loss have been well documented in many studies measuring bone mineral density in comparison with placebo. Data from many observational studies indicate that estrogen replacement reduces the risk of osteoporotic hip fracture by 30% and that of the spine fracture by 50%.[100] However, only a few randomized controlled trials evaluated the effects on fracture risk. While a meta-analysis reviewing the randomized controlled trials between 1977 and 1999 concluded that the literature failed to show whether estrogen therapy reduces fracture rates or not, another meta-analysis reported that estrogen treatment reduced hip and wrist fractures by 40%.[101,102]

The Women's Health Initiative (WHI) study, a randomized, placebo-controlled, double-blind, multicenter trial, comparing conjugated equine estrogen plus medroxyprogesterone acetate given continuously or conjugated equine estrogen alone with placebo, reported that the estrogen-plus-progestin arm resulted in a decreased risk of hip fracture with five fewer cases of hip fracture per 10,000 person-years.[103] This combined continuous estrogen-plus-progestin arm of the WHI study was stopped by the National Institutes of Health (NIH) on July 9, 2002, due to an increased risk of breast cancer and a lack of overall benefit.

In a recent publication reporting the results of the estrogen-alone arm of the WHI study, a significant risk reduction in osteoporotic fractures was shown.[104] Six fewer hip fractures per 10,000 person-years ($p = 0.01$), six fewer clinical vertebral fractures per 10,000 person-years ($p = 0.02$), and 56 fewer total osteoporotic fractures per 10,000 person-years were reported in this trial.

7.7.1.3 Benefits on Cognitive Function and Dementia

Several epidemiological studies indicate that HRT decreases the incidence of Alzheimer's disease by 20 to 60%.[105] Also, a meta-analysis of observational studies showed that HRT was associated with a decrease in the incidence of dementia.[106] However, HRT did not seem to be effective on every cognitive dysfunction in postmenopausal women free of Alzheimer's disease.[106] This meta-analysis highlights the fact that 66 to 95% of women in the studies included in the analysis used conjugated equine estrogen. There is not sufficient data to determine the independent efficacy of progestins or other estrogens, different doses, or duration of treatment. In another earlier meta-analysis, it was concluded that estrogen users have 29% reduction in the risk of developing dementia.[107]

However, the results of the Women's Health Initiative Memory Study (WHIMS), which were published previously and recently, were conflicting and contrary to the other previous studies.[108–111] WHIMS is a randomized, double-blind, placebo-controlled ancillary study of WHI. The estrogen-plus-progestin trial of the WHI was stopped in July 2002 due to significantly more noncognitive adverse events as mentioned above.[103] The WHI estrogen-alone trial was terminated on February 29, 2004, because NIH considered the excess risk of stroke in the active hormone group to be unacceptable in healthy women in the absence of any benefit for coronary heart disease.[104] WHIMS examined whether HRT (estrogen alone or estrogen plus progestin) reduced the risk of dementia and whether HRT improved global cognitive function in healthy women aged 65 years or older. The results of the stopped arm of WHIMS published in 2003 reported that the estrogen-plus-progestin treatment increased the risk of dementia and provided no benefit for mild cognitive impairment for women aged 65 years or older.[108] In addition, the estrogen-plus-progestin treatment did not improve global cognitive function when compared with placebo in the WHIMS report.[109]

The recent results of the estrogen-alone arm of the WHIMS report published in 2004 were also against the benefits of estrogen on dementia and global cognitive function.[110,111] The estrogen-alone arm of WHIMS did not only fail to show any reduction in the incidence of dementia and mild cognitive impairment but also increased the risk when analyzed together.[110] Pooling the data for estrogen alone and estrogen plus progestin showed an increase in the risk of both end points. In addition, estrogen treatment had an adverse effect on global cognitive functions especially in women with lower cognitive function initially.[111] It was concluded that neither estrogen

alone nor estrogen-plus-progestin combination therapy should be initiated in old women for the purpose of protecting cognitive function. We absolutely agree with this conclusion. The advanced age of the patients in the WHIMS seems to be its weak point. The ideal time for initiating HRT is the menopausal transition and early postmenopausal periods. Irreversible neurodegenerations that estrogen therapy cannot improve may be evident many years before the age of 65 years.

7.7.1.4 Benefits on Colorectal Cancer

HRT reduces the risk of colorectal cancer in observational studies.[112] The WHI study also demonstrated a decrease in the incidence rate of colorectal cancer in the estrogen-plus-progestin group when compared with placebo.[103] However, in the recent report of WHI estrogen-alone trial, estrogen treatment did not seem to be effective on the development of colorectal cancer.[104]

7.7.2 Risks of HRT

7.7.2.1 Endometrial Cancer

Endometrial hyperplasia and endometrial cancer are the historical harmful effects of unopposed HRT (estrogen alone) in postmenopausal women with an intact uterus. Progesterone decreases the risk of endometrial cancer and has been added to estrogen treatment since the 1980s. However, the protective effect of progesterone decreases beyond 5 years of use of sequential regimens.[113] The combined continuous regimens suppress the endometrium for much longer durations.[114]

7.7.2.2 Ovarian Cancer

Although the development of ovarian cancer under HRT is thought to be a risk, the results of studies are conflicting. While a prospective study reported an increased risk in ovarian cancer mortality among women using estrogen for 10 years and beyond,[115] continuous combined HRT did not increase the risk of ovarian cancer.[116]

7.7.2.3 Thromboembolic Disease

It is widely accepted that an increased risk of deep vein thrombosis and pulmonary emboli is present in women taking HRT. The increase in risk seems to be greater in the first year of use.[103,117,118] The estrogen component of HRT is the likely cause, because estrogen in the absence of progestin is associated with venous thromboembolism.[119,120]

Many prothrombotic factors increase while fibrinolytic factors decrease during HRT. The risk of thromboembolic events is greater in women with predisposing factors such as a family history of thromboembolic disease,

prolonged bed rest, advanced age, lower extremity fracture, and cancer. Use of aspirin and statins appears to be protective.[118]

7.7.2.4 Stroke

The evidence of increased risk of stroke with HRT comes from the WHI study. Both the estrogen-plus-progestin arm and the estrogen-alone arm of the WHI study showed significant increase in the risk of stroke.[104,121]

7.7.2.5 Gallbladder Disease

HRT increases gallbladder disease and the need for biliary tract surgery. Estrogen enhances hepatic lipoprotein uptake and inhibits bile acid synthesis; as a result, biliary cholesterol increases with its attendant increase in the rate of cholelithiasis.[122]

7.7.2.6 Breast Cancer

The risk of breast cancer is the prime issue in most of the epidemiological, observational, and recently randomized clinical trials. Several epidemiological studies have reported an increased risk of breast cancer,[99] with the risk being higher with estrogen-plus-progestin combined treatments than with estrogen alone.[123] A 2.3% increase in the risk of breast cancer for every year of hormone use was reported in a large meta-analysis from observational studies.[124] It is generally thought that the risk of breast cancer is minimal unless HRT is taken for more than 5 years. In addition, it has been reported that estrogen users are less likely to die from breast cancer than nonusers[125] due to earlier diagnosis and better prognosis. Available data also indicate that the increase in breast cancer risk is more likely to affect lean subjects.[124] Increased risk of breast cancer falls after cessation of HRT with return to baseline levels occurring within 5 years.

The WHI study also showed an increased risk of breast cancer with the estrogen-plus-progestin regimen.[103] The study concluded that for every 10,000 women taking HRT, there would be eight more cases of breast cancer. However, mortality rate was not different between the HRT group and placebo group, although an increase in tumor size and nodal involvement was observed. The estrogen-alone arm of the WHI study showed that there was a 23% lower rate of breast cancer in the treatment group compared with the placebo group.[104]

7.7.2.7 Cardiovascular Disease (CVD)

Observational studies indicate a reduction in CVD of 50% in postmenopausal women receiving HRT.[126] This CVD-protective effect of HRT has been attributed to the beneficial lowering effects of estrogen on serum lipid levels. Indeed, the randomized controlled trial "Heart and Estrogen/Progestin Replacement Study (HERS)" showed that the mean LDL cholesterol levels

decreased and the HDL cholesterol levels increased in the HRT group when compared with placebo group.[117] Also, the mean triglyceride levels increased in the HRT group. However, the direct effects of estrogen on blood vessels contribute to the cardiovascular protective effects of estrogen. Estrogen enhances endothelial-dependent vasodilation via the nitric oxide synthase enzyme as a short-term effect. Long-term effects of estrogen develop by alterations in vascular gene expression. The role of progestins in the development of CVD is not well understood, but progestins seem to attenuate the beneficial effects of estrogens.

One of the controversies about the effects of HRT on CVD is whether any beneficial effect of HRT in secondary prevention (beneficial effects in women with established coronary artery disease) exists or not. A large cohort study in which the clinical outcomes of 114,724 women aged 55 years or older with documented myocardial infarction had been evaluated was published recently. A 35% improvement in the survival rate was found in women receiving HRT. The beneficial effects of HRT on cardiovascular events were found in all age groups.[127] However, this study was not a randomized, controlled study. The Estrogen Replacement and Atherosclerosis (ERA) Trial is the first randomized angiographic end-point study to test the effect of ERT and HRT on the progression of atherosclerotic coronary stenosis.[128] In this study, conjugated equine estrogen 0.625 mg/d with or without medroxyprogesterone acetate 2.5 mg/d was compared with placebo. No significant difference was found between the three groups on the progression of coronary atherosclerotic stenosis. The results showed that both the combined regimens and ERT are ineffective in the prevention of CVD.

The HERS is a randomized, double-blind, placebo-controlled trial to determine whether conjugated equine estrogen (0.625 mg/d) plus continuous medroxyprogesterone acetate (2.5 mg/d) was superior to placebo in preventing recurrent cardiovascular events in 2763 women with established coronary disease.[117] The mean age of the participants was 66.7 years at baseline. The patients were followed up for 4.1 years on the average. The primary outcomes were nonfatal myocardial infarction or coronary heart disease death. Primary coronary heart disease events occurred in 172 women in the hormone group (33.1/1000 women per year) and in 176 women in the placebo group (33.6/1000 women per year) in HERS. There was no significant difference between the groups. A post hoc time-trend analysis revealed a significant 52% increase in cardiovascular events in the first year in the HRT group compared with placebo. In the later years, there was a nonsignificant trend toward fewer events in the HRT group than in the placebo group. Despite a nonbeneficial effect on cardiovascular events, a decrease in LDL and an increase in HDL cholesterol levels in the HRT group were observed.

The discrepancy between the findings of randomized, controlled trials and observational studies may reflect the differences between study populations and treatments. Most of the observational studies of HRT enrolled postmenopausal women who were younger (than the women in the randomized, controlled trials) and healthier. It is not known whether the results of

secondary prevention trials may be applicable to younger women, different HRT regimens, or different routes of delivery.

The study populations of the HERS and ERA trials had higher mean ages (66.7 years and 65.8 years, respectively) than the usual age at which HRT is started. It can be hypothesized that if women are given HRT early enough after menopause, it might be possible to prevent the development of CVD more easily than to prevent its progression once it is established. However, based on the HERS and ERA trial, the American Heart Association has advised against the use of HRT in women with established coronary heart disease.[129]

Another issue that awaits an answer is the effect of HRT on primary prevention. Most of the observational studies in postmenopausal women without coronary heart disease demonstrated that ERT/HRT users have a 30 to 50% reduction in cardiovascular events, although this benefit is reduced by the addition of progestins.

However, the results of WHI do not support the beneficial effects of HRT seen in those observational studies.[103,104] The WHI trials of hormone therapy were designed in 1991–1992 and planned to last for 8.5 years. A total of 16,608 postmenopausal women aged between 50 and 79 years (mean 63.3 years) with an intact uterus were recruited in 1993–1998.[103] The trial was stopped early based on the observation of health risks exceeding benefits over an average follow-up of 5.2 years. Coronary heart disease events increased by 29% (37 vs. 30 per 10,000 person-years) in the women taking HRT over those placed on placebo with statistical significance of 0.05. Nonfatal myocardial infarctions were more common than other coronary heart disease events. Although the estrogen-plus-progestin arm of the WHI study was stopped earlier, a sufficient number of coronary heart disease events had occurred to suggest that continuation of treatment to the end of the planned time would not change the unfavorable results in CVD.

The estrogen-alone arm of the WHI study was also stopped earlier than the planned date by NIH after an average of 6.8 years follow-up period because of ineffectiveness of estrogen on heart disease (primary outcome) and an increased risk of stroke.[104] No significant effect of ERT was observed on coronary heart disease events. There were 177 patients with coronary heart disease events in the estrogen group and 199 patients in the placebo group (49 per 10,000 person-years in the ERT group vs. 54 per 10,000 person-years in the placebo group, 9% reduction, which is not statistically significant). LDL cholesterol levels decreased while HDL cholesterol and triglyceride levels increased significantly in the ERT group. However, the results of the estrogen-alone arm of the WHI study showed a possible modest, cumulative beneficial effect of estrogen on coronary heart disease with the long-term treatment, although a statistically nonsignificant increase was observed in the first year.

The study population of the estrogen-alone arm of the WHI study had advanced age like the studies in the secondary prevention of coronary heart disease. It is not known whether the results of the estrogen-alone trial of

WHI can be applied to younger women in perimenopausal period or not. This controversy was also noted in the original paper of WHI: "The current study suggests that younger women who use conjugated equine estrogen may be at reduced risk of coronary heart disease but this possible association may be just a matter of chance."[104] It is also unknown if the other estrogens and different routes of administration will show the same effects on coronary heart disease.

7.8 Brief Recommendations

1. Current use of HRT is restricted for alleviation of vasomotor and urogenital symptoms. It is therefore recommended in the menopausal transition and early postmenopausal years (especially in the first year after the final menstrual cycle) because these symptoms are more prominent in these periods. We think that prescribing HRT for a short time period (6 to 12 months) will be better.
2. All women experiencing premature menopause before the age of 40 should be encouraged to use HRT. There is no evidence that the use of HRT by women under the age of 50 years increases the risk of breast cancer.
3. HRT should never be prescribed for the prevention of CVD or any other age-related chronic diseases present or expected in the future in a postmenopausal woman.
4. Alternative treatments are available today for the treatment of chronic diseases seen in postmenopausal years, such as bisphosphonates for osteoporosis and statins for hyperlipidemia.
5. If a woman has been on the HRT for more than 5 years without any side effects, the decision to continue the therapy should be dictated with consideration for the patient's own desire.

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8

Benefits and Risks of Phytoestrogens

Bensu Karahalil

CONTENTS

8.1 Introduction

Plants have been producing chemicals for millions of years. Through countless generations they have been perfecting a miscellany of chemicals, some benign and some deadly. Many of the plants that produce phytoestrogens and other endocrine disrupters are edible. More than 300 foods have been shown to contain phytoestrogens.[1] They are naturally occurring phenolic plant compounds, present in foods such as beans, cabbage, soya beans, grains, and hops, and are part of a wider class of polyphenols found in all plants.[2–5] Structurally, they are similar to endogenous estrogens. However, their activity is weaker than that of endogenous estrogens. They are estrogenic and antiandrogenic and share a similar mechanism of action through their affinity for and binding to estrogenic receptors (ER-α and β-).[6]

Phytoestrogens were first noted in 1926 to have estrogenic effect and activity, but it was not known whether they would affect living organisms. In the 1940s, estrogen toxicity was associated with plant exposures; phytoestrogens were shown to induce infertility and developmental toxicity in certain animals, and coumesterol caused "sheep clover disease," inhibiting fertility.[2,6]

There are many studies on the toxicity and beneficial effects of phytoestrogens. Both the possible beneficial and adverse effects of phytoestrogens are still being investigated, taking into account that there may be specific groups of the population who are more susceptible to the effects of phytoestrogens. For example, consumption of soy products or phytoestrogens is traditional in the diet of people who live in Eastern countries. Asians, Japanese, Koreans, and Chinese consume more phytoestrogens than the people of Western countries, including Americans.[7–10] Dietary intake of phytoestrogens is greater in vegetarians than in nonvegetarians. Epidemiologic and experimental studies have shown that there is an inverse association between intake of dietary phytoestrogens and hormonally related diseases such as breast cancer, prostate cancer, and menopause.[11] Population studies are used to evaluate the risk and benefit of phytoestrogens. In this chapter, the possible beneficial and adverse effects of phytoestrogens will be discussed and comparable evaluations made.

8.2 What Are Phytoestrogens?

8.2.1 Definition

Phytoestrogens (hormonally active substances, sometimes called "dietary estrogens"): *phyto* = plant; *estrogen* comes from *estrus* (period of fertility for female mammals; derived from the name of an ancient Anglo-Saxon fertility

goddess, oestro) + *gen*, to initiate, generate.[12] Phytoestrogens are a diverse group of nonsteroidal plant compounds.[2,13] They are naturally occurring chemical constituents of certain plants that have estrogenic, and in some cases, antiestrogenic or antiandrogenic activity in animals and humans. They were first noted in 1926 to have estrogenic activity.[2,14] Besides phytoestrogens, estrogenic activity has been reported among compounds produced by animals and microorganisms, as well as in industrially manufactured chemicals and their breakdown products.[2] Phytoestrogens cannot be classified as nutrients as their absence from a diet does not induce a characteristic deficiency syndrome, nor do they participate in any essential biological functions.[11]

8.2.2 Sources and Types of Phytoestrogens

Phytoestrogens having subgroups are naturally occurring estrogens and synthetic contaminants. The majority of phytoestrogens found in typical human diets can be categorized into two primary classes: (1) isoflavonoids (flavonoids) and (2) lignans or resorcylicacid lactones (non-flavonoids). Isoflavonoids are compounds of isoflavones and coumestans[2,15] (Figure 8.1).

Multiple phytoestrogens can originate from a single plant. The content of phytoestrogens in food varies greatly.[2,16,17] Isoflavonoids include the isoflavones genistein and daidzein, which occur mainly as the glycosides genistin and daidzin, respectively, in soya beans and consequently in a wide range of soya-derived foods and to a lesser extent in other legumes. These are also abundant in grains, seeds, vegetables, berries, fruits, tea, coffee, and wine.[3]

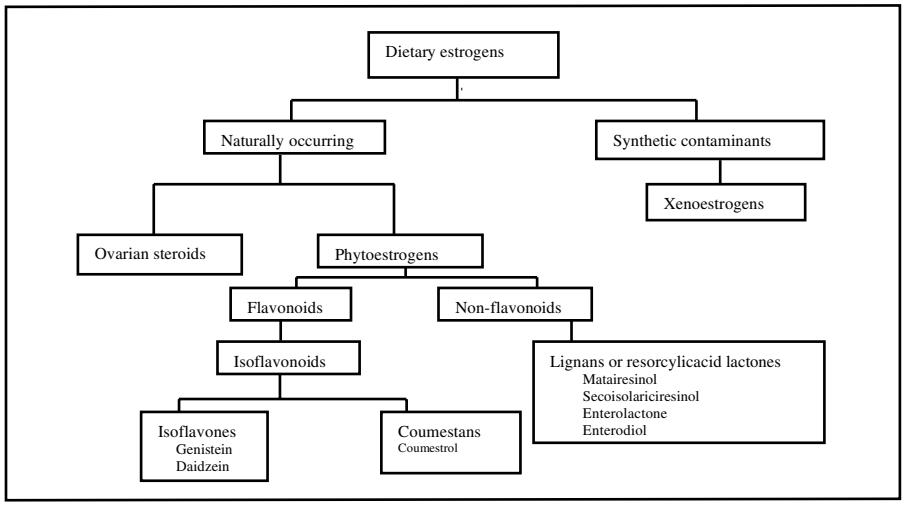

FIGURE 8.1
Classification of dietary estrogens.

Inside the bright-green flesh of the soybean lie the isoflavones genistein and daidzein, which are the phytoestrogens responsible for either increasing or decreasing estrogen levels in the body. Genistein and daidzein may be metabolized from other isoflavonoid plant precursors, biochanin A and formononetin, respectively.[2,15]

There are more than a thousand types of isoflavones, but the most commonly investigated are genistein and daidzein, which are also thought to have the highest estrogenic properties. The amount of phytoestrogen in plants depends on the processing techniques used and its relative abundance in the specific soy product of interest.[18]

Traditional soya foods are made from soya beans and include both fermented and nonfermented soya foods. They contain isoflavones mostly present as β-glucosides, some of which are esterified with malonic acid or acetic acid. Fermented soya foods such as miso or tempeh contain mostly unconjugated isoflavones.[7]

Coumestans are found in various beans such as split peas, pinto beans, and lima beans; alfalfa and clover sprouts are the foods with the highest amounts of coumestans. These estrogen-like compounds were identified in the 1940s, when an epidemic of infertility among sheep was investigated.[19]

Lignans form the building blocks for lignin, which is a main component of the plant cell wall. They are found in high-fiber foods such as cereal brans and beans; flaxseeds contain large amounts of lignans.[20] Mammalian lignans are not present in our diets, but their precursors are present in fiber-rich foods such as flaxseed, unrefined grain products, particularly rye, and some berries.[21] The main mammalian lignans, enterolactone or enterodiol, are found in flaxseed (in huge quantities), lentils, whole grains, beans, fruits, and vegetables.[18] They are formed from matairesinol and secoisolariciresinol by gut microflora.[20,22]

8.2.3 Chemical Structure of Phytoestrogens

All phytoestrogens are nonsteroidal heterocyclic phenols with a structure similar to β-estradiol.[13] Figure 8.2 shows the similarity of the most common phytoestrogens (genistein and daidzein) to estrogens.[4,23]

Isoflavones make up the most common form of phytoestrogens. They have a common diphenolic structure that resembles the structure of the potent synthetic estrogens diethylstilbesterol (DES) and hexestrol[4] (Figure 8.3). The phenolic ring is a key structural element of most compounds that bind to estrogen receptor.[6]

Lignans are compounds possessing a 2,3-dibenzylbutane structure and exist as minor constituents of many plants.[20] The chemical structure of plant lignans differs somewhat from that of mammalian lignans in that they have phenolic hydroxyl groups in the meta position only in their aromatic rings.[3,24,25]

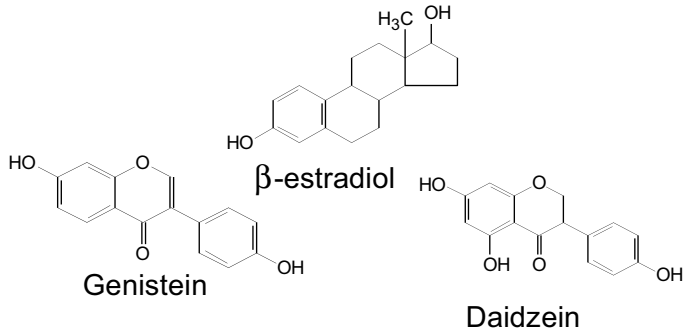

FIGURE 8.2
Similarity of isoflavones to estrogens.

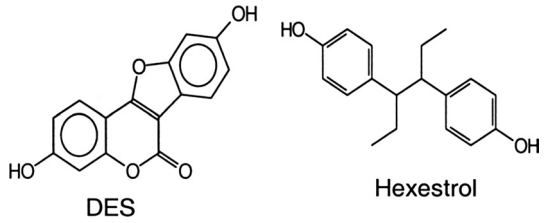

FIGURE 8.3
The structure of synthetic estrogens diethylstilbestrol and hexestrol.

Structure–activity relationships of phytoestrogens may provide clues to the molecular basis for estrogenic agonism and antagonism, and the absence of a specific lipophilic region in phytoestrogens may affect binding to the estrogen receptor and thus affect biological activity.[4,26]

8.2.4 Dietary Intake of Phytoestrogens

There is limited current information about the amount of phytoestrogens in human diets. Variations in phytoestrogen content occur because of genetic differences in plants (for example, soy varieties), location, season, infection with fungal diseases, processing, and as part of the plant's defense response.[27]

The isoflavones are the best-researched phytoestrogens and are found in soy products, namely, soybeans, tofu, and other soy derivatives as mentioned above.[28] One of the common and major examples is soy-based infant milk or food. Babies fed with soy formula milk (SFM) gain weight normally, but there is concern that this feeding may expose the baby to abnormally high levels of estrogens. This is because soya beans, from which SFM is made, contain appreciable amounts of isoflavones, genistein and daidzein, and

their related glycones, genistin and daidzin, which have weakly estrogenic activity, and these are not a natural part of our diet. Concerns are also raised due to long-term health effects of exposure during development.[27,29,30] There is also the other side of the coin. The infant formula industry is an $8 billion per year business. Huge advertising budgets are spent to convince women that it is better and more convenient to bottle-feed their babies. The rationale is that infant formula contains less dioxin, polychlorinatedbiphenyls (PCB), and organochlorine pesticides than breast milk. It is very well known that these chemicals, endocrine disrupters (to be discussed soon), are lipophilic and they bioaccumulate in adipose tissues including breast milk. However, infant formula may have contaminants introduced in the manufacturing process including contaminated water (when the formula is over- or under-diluted) and potential contaminants in bottles and nipples.[31]

Lalles and Peltre showed the average isoflavone concentrations of some soy-based infant formulas and showed that an infant regularly consumes 4 to 6 times more soy foods than an adult. Aglycone forms (genistein and daidzein) present in formula are more biologically active than glycones.[32]

The concentration of phytoestrogens found in soy-based infant formulas is several magnitudes higher than that found in human breast milk. It has been estimated that intake by infants of isoflavones from soy-based formula is approximately 4 mg/kg bw/day. Infants are able to absorb significant levels of isoflavones such as genistein and daidzein. The Working Group concluded that infants fed soy-based formula are the population subgroup exposed to the highest concentration of isoflavones and that exposure via breast milk is low by comparison. On the other hand, no data on the transfer of lignans from the maternal diet to breast milk have been published.[33]

If a baby develops a true intolerance to cow's formula milk, which is very rare, then SFM can be used in the knowledge that millions of children worldwide have been reared on it without any major problems as far as we are aware. Mothers who have to choose SFM by force of such circumstances should therefore do so without worrying that they will harm their baby. But where there is a choice, it should not be recommended to feed babies with SFM, not because it is proven to cause harm (for it is not) but because it is dabbling with the unknown and therefore taking an unknown risk on behalf of the baby.[34]

There are many published data on dietary intake of phytoestrogens. There are a number of population subgroups that may be expected to have a higher risk than those with an average dietary intake of phytoestrogens, and these subgroups are used to evaluate the risk-benefit ratio of phytoestrogens through comparable subgroup studies. These population subgroups can be categorized into four classes: (1) vegetarians and vegans, (2) particular ethnic groups (e.g., Japanese and Chinese), (3) consumers of soy-based foods, and (4) consumers of phytoestrogen-containing dietary supplements.[3,5,11,35–41]

Epidemiological data indicate that Asiatic societies widely consume a phytoestrogen-rich diet, predominantly in the form of soy, and thus they have a lower risk of so-called Western diseases such as breast and prostate cancer,

osteoporosis, and cardiovascular disease. The typical concentration of genistein in soy foods is 1 to 2 mg per g of protein, and Asians consume 20 to 80 mg of genistein per day in the usual diet. By contrast, the average American ingests only 1 to 3 mg per day.[35] It was calculated that, in the 1980s, the maximum intake of phytoestrogens that an average person in the U.K. consumed was less than 1 mg/day.[11] The amount of phytoestrogens in plants depends on processing techniques; for example, the cooking of foodstuffs containing phytoestrogens reduces phytoestrogen concentrations and their chemical form changes, due to loss in the water, and during roasting of soybeans. On the other hand, boiling or frying does not affect the total isoflavone content of foodstuffs.[18,33]

Nagata et al. carried out the best survey of what types/quantities of soy are eaten in Asia. One thousand two hundred forty-two men and 3596 women participated in an annual health checkup program in Takayama City, Japan. It was clearly observed that these people consumed mostly soy products including tofu (plain, fried, deep-fried, or dried), miso, fermented soybeans, soymilk, and boiled soybeans. The estimated amount of soy protein was 8.00 ± 4.95 g/day for men and 6.88 ± 4.06 g/day for women.[42]

The majority of fresh bakery items, in particular the traditional family bread loaf, now contain soya flour, which is added to ensure lasting freshness. In the U.K., the main intake of phytoestrogens is as the isoflavones genistein, daidzein, and glyciten, which are contained in plants primarily as glycoside conjugates. Soya usage has increased substantially in recent times in the U.K.[11] Vegetarians are a significant minority of the U.K. population and more than 5% of the population does not eat fish or meat. Vegetarians may have lower dietary exposure to substances that are more prevalent in meat or fish. Conversely, they may have higher dietary exposure to substances that are present in vegetables.[11]

TABLE 8.1

Comparison of Estimated Dietary Isoflavone Intakes in Different Groups

Groups	Isoflavone	References
Japan	25–45	44
U.K.	1	5
Asia	150–200	44
Western population	<1	4
Vegetarian	~ 3	33
Infants fed soy-based formula	40	33
Consumers of dietary supplements or soy-rich diet	> average consumer	33

TABLE 8.2

Daily Intake Values and Contents of Phytoestrogens in Baby-Feeding Materials

Phytoestrogen Source	Content of Phytoestrogens	Daily Intake	References
Soy-based infant formula	18–41 mg/l	4 mg/kg bw	33
Soya milk	–	100 mg daidzein/daidzin 100 mg genistein/genistin (when consumed 36 oz/day)	5
Soya protein isolate	–	38 mg genistein (when consumed 38 oz/day)	5
Vegetarian mothers` breast milk	2 – 32 µg/l	> nonvegetarian mothers' breast milk	33
Breast milk	Varies with maternal diet		33
Cow's milk	ND		33

Note: ND = not detectable.

8.3 Pharmacokinetics of Phytoestrogens

The absorption, distribution, metabolism, and excretion (ADME) of phytoestrogens have not been fully clarified in human adults or infants. Most of the information concerns the isoflavones daidzein and genistein, and there is less knowledge about the lignans enterolactone and enterodiol.

8.3.1 Absorption

Isoflavones are present in food mainly as glycosides. Isoflavones are absorbed as aglucones, which are more readily absorbed than the parent glycosides due to their higher hydrophobicity and lower molecular weight.[44] Absorption of aglucones occurs mainly in the intestine. Acid hydrolysis of glycosides takes place in the stomach and saliva.[4,33,44] The liver and the enterocytes of the human small intestine contain β-glycosidase enzymes capable of efficiently hydrolyzing naturally occurring flavone and isoflavone glycosides. β-glycosidase enzymes in the gut microflora also play a role in glycoside hydrolysis.[15] Uptake requires hydrolysis of the isoflavone glycosides to their aglucone form. In the human, before absorption, the isoflavones may be further metabolized by the gut microflora and are converted to a hormonally inert form and reduced to estrogenically active and nonestrogenic metabolites.[7] There is little information about processes concerning lignans and coumestans prior to absorption. Lignans undergo bacterial hydrolysis and metabolism. This causes removal of glucose residues, and diphenolic compounds (enterolactone and enterodiol) occur by demethylation and dehydroxylation. These phenolic compounds are absorbed and

TABLE 8.3

Concentration of Phytoestrogens in Some Foods, mean (μg/100 g)

Sources of Food	Isoflavones				Lignans		Coumestans
	G	D	Bio A	F	SEC	M	Coumestrol
Soy							
Soy-flower	93,900	67,400	70	30	130	—	—
Kikkoman firm tofu	21,300	7600			—	—	
Hatcho miso	14,500	7300			—	—	
Soy drink	2100	700			—	—	
Soymilk	310	30			—	—	
Linseeds	—	—	—	—	369,900	1027	—
Clover seeds	323	178	381	1270	13	3,8	5,3
Vegetables and legumes*							
Alfalfa sprouts	117.6	151.7	66.9	3898.9	TR	ND	105.3
Asparagus	TR	57.8	ND	ND	68.4	TR	TR
Broccoli sprouts	ND	43.7	ND	ND	TR	ND	ND
Carrots	ND	ND	ND	TR	38.3	TR	ND
Cauliflower	ND	TR	ND	ND	30.2	ND	ND
Brussels sprouts							
Clover sprouts	70.9	71.3	751.2	4019.9	ND	ND	97.7
Garlic	TR	ND	TR	ND	26.6	37.4	ND
Mung bean sprouts	424.1	387.2	TR	26.1	TR	TR	2000.4
Sweet potatoes	TR	ND	ND	ND	ND	40.6	ND
Oat							
Bran	6,9	3,5	—	—	110	0	—
Meal	—	0	—	—	13,4	0,3	—
Rye							
Meal	—	—	—	—	47	65	—
Bran	—	—	—	—	132	167	—
Mung							
Bean	365	9,7	14	7,5	172	0,25	1,8
Sprouts	1902	745	—	—	468	0,87	1032
Pumpkin seeds	1,53	0,56	—	—	21370	—	—
Chick peas	76,3	11,4	838	215	8,4	—	5

Note: G = Genistein, D = Daidzein, Bio A = Biochanin A, F = Formononetin, M = Matairesinol, ND = None detected, TR = Trace as 25 μg/100 g.

Source: Modified from References 8, 42, and 43.

enterodiol may be further metabolized to enterolactone in the gut[4,7,15,33,44] (Figure 8.4).

Isoflavones in aglycone form (as in fermented soy) are more bioavailable in food than isoflavones in glycoside form (as in unprocessed soy). This suggests that conversion to aglycones increases the bioavailability of isoflavones (Figure 8.4). It has also been shown that processing techniques are a major factor influencing the bioavailability of phytoestrogens and for the phytoestrogen content of plants.

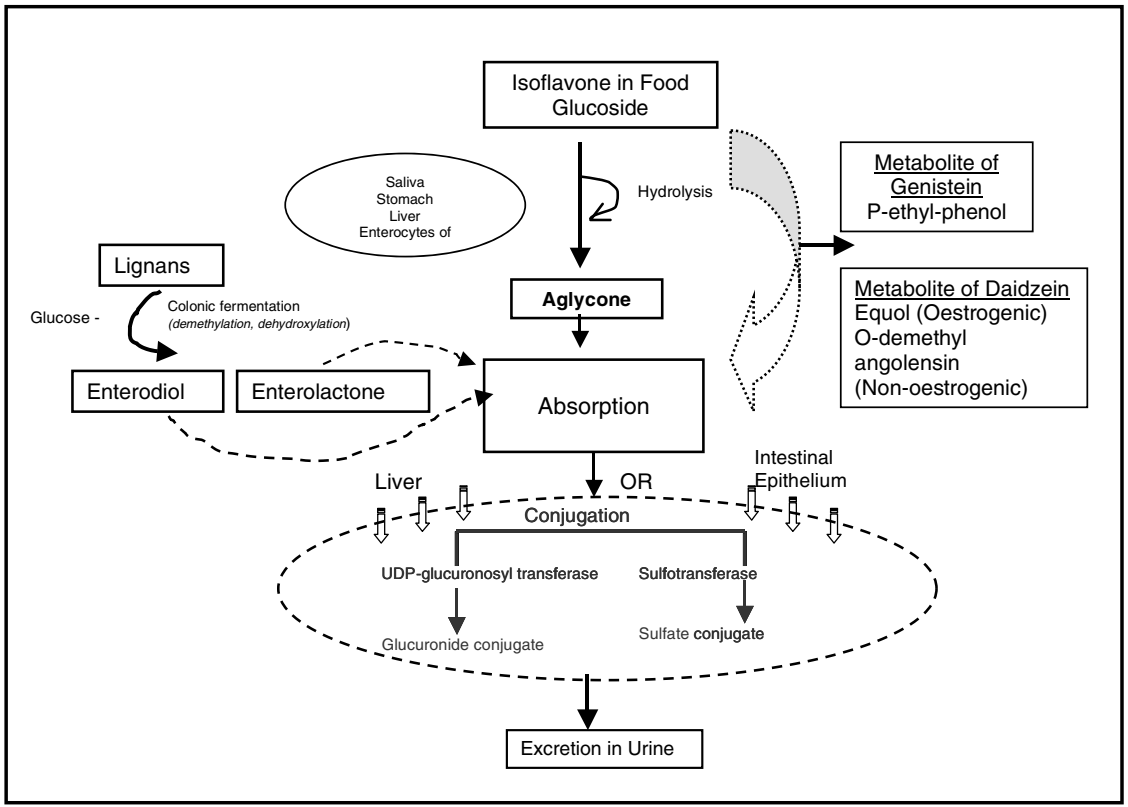

FIGURE 8.4
The pathway of absorption, metabolism, and excretion of isoflavonoids and lignans.

8.3.2 Distribution

Isoflavone and lignan phytoestrogens have been detected in a number of body fluids such as urine, plasma, feces, prostatic fluid, semen, bile, saliva, breast milk, breast aspirate, and cyst fluid.[6]

8.3.3 Metabolism

The importance of the gut microflora in the metabolism of phytoestrogens has been clearly established. Some bacteria (*Lactobacilli, bacteriodes,* and *bifodiobacteria*), possess β-glucuronidase and arylsulfatase activity. Daidzein, genistein, and lignans are converted to their metabolites via microfloral metabolism. Once absorbed, isoflavones and lignans are efficiently reconjugated with glucuronic acid or sulfate. Conjugation occurs in the liver or within the intestinal epithelium.[7]

Isoflavones have been identified in human biological fluids within plant tissues. Daidzein and genistein are hydrolyzed by the action of bacteria in the large intestine.[7,20] Human biological fluids such as plasma, urine, feces, etc., are used to estimate concentrations of phytoestrogens and assess the extent of exposure to dietary intake. Daidzein can be metabolized in the large intestine by bacteria to form the isoflavon equol (estrogenic) or *O*-desmethylangolensin (nonestrogenic), whereas genistein is metabolized to the nonestrogenic *p*-ethyl phenol.[7,20,45]

Isoflavones exist as sugar derivatives called glycosides, and concentrations vary widely depending on stressors such as viral, bacterial, fungal, or herbivore attack. These compounds undergo hydrolysis in the human gut, yielding aglycones. Like the lignans, these aglycones meet one of three fates: they may be excreted or absorbed from the gut, or they may undergo further metabolism. The four most common isoflavones are formononetin, daidzein, genistein, and biochanin A.[23] If it is not absorbed into the body, it may be further metabolized to *p*-ethylphenol, a hormonally inert compound. Formononetin may be metabolized mostly to equol, a more potent phytoestrogen, and to *O*-desmethylangolensin. The lignan phytoestrogen precursors (matairesinol and secisoloiciresinol) are present in foods as glycoside and are converted by gut bacteria to the two main mammalian lignans enterolactone and enterodiol. Matairesinol undergoes dehydroxylation and demethylation directly to enterolactone, whereas secisolariciresinol is converted to enterodiol, which can then be oxidized to enterolactone.[45]

8.3.4 Excretion

The major excretion routes of phytoestrogens are urine and bile. Several bacterial metabolites of phytoestrogens have been detected in urine and feces. Conjugated phytoestrogens excrete in bile and then deconjugate by gut bacteria and undergo enterohepatic circulation. Excretion via the feces

is thus largely determined by the degree of enterohepatic circulation. After absorption, enterolactone and enterodiol are converted to their β-glucuronides and eventually excreted in urine.[7,33]

Products of both lignan and isoflavone metabolism may be excreted or absorbed. If absorbed, the phytoestrogens undergo conjugation in the liver with glucuronic acid — or to a lesser extent, sulfate — and then they are excreted in the urine or in the bile.[6]

8.4 Mechanism of Action and Relevant Actions of Phytoestrogens

The endocrine system is actually many systems, having complex interactions and interdependencies. Normal endocrine function is often dependent on cyclical events, rather than steady state.[46]

Phytoestrogens are structurally and functionally similar to 17-β-estradiol (Figure 8.2). Phytoestrogens are much weaker than human estrogens, with 100 to 10,000 times less activity.[13,17,18,36,47] They act primarily as an estrogen or as an antiestrogen according to the amount of endogenous estrogens and the types and number of receptors. Although phytoestrogens have generally weaker activity than endogenous steroidal estrogens, their amount in the body is greater than that of endogenous estrogens and they act as antiestrogens by competing with the more potent endogenous estrogens for binding to ERs[13] due to weaker activity.[1] Estrogenic and antiandrogenic activity depend on the abundance of endogenous estrogens. There is an inverse association between activity of phytoestrogens and abundance of endogenous estrogens.

Two ER subtypes (ER α and ER β) bind structurally diverse endogenous steroids, phytoestrogens, and synthetic chemicals.[48] Although phytoestrogens prefer ER β for binding, most endogenous steroidal estrogens show no such difference, so phytoestrogens show their activity preferentially.[28] Isoflavones are weak estrogen agonists, binding the estrogen receptor with an affinity that is orders of magnitude lower than that of estradiol.[49] The binding affinity of genistein to ER β is 5 to 20 times greater than to ER α. This suggests that isoflavones may be tissue selective in their effects.[50]

In vitro experiments have also shown that the soy isoflavones can bind to estrogen receptors and act as competitive agonists or antagonists to endogenous estrogens depending on relative concentrations and affinities.[30]

Phytoestrogens show their action via multiple mechanisms: (1) Binding estrogen receptors and acting estrogenically or antiestrogenically, (2) interacting with some pivotal enzymes and affecting the production of sex steroid-binding proteins through hormone or antihormone action, and (3) other, nonhormonal actions.[13] The estrogen receptor is the only steroid receptor able to additionally interact with a large number of nonsteroidal

compounds, including phytoestrogens and environmental and drug xenoestrogens (and their metabolites), which frequently show a structural similarity to the steroid nucleus of estrogen.[7]

Most phytoestrogens have low potency, and people who consume diets rich in these substances may have a reduced risk of developing some hormone-related diseases. However, the actual health risk or benefit of a diet rich in plant hormones is largely unknown. Diet influences estrogen production in various ways. A diet of proportionally higher total calories relative to body mass can also alter the metabolism of estrogen in the gut and stimulate earlier onset of menses.[51]

8.5 Biological Effects of Phytoestrogens

The structure of phytoestrogens resembles that of steroidal hormones 17β-estradiol, and phytoestrogens can bind to the estrogen receptors to show their biological effects.[2]

Phytoestrogens may also be able to function as antioxidant species. They inhibit enzymes involved in the synthesis of thyroid hormones, as well as signal transduction, apoptosis, cell cycle, and differentiation pathways.[52]

Epidemiological and experimental studies suggest that consumption of a phytoestrogen-rich diet may have beneficial effects on health. This type of diet may prevent or alleviate hormonally related diseases such as cardiovascular disease, menopausal symptoms, and postmenopausal osteoporosis. Epidemiological data also indicate that Asiatic societies, which consume a phytoestrogen-rich diet, have a lower risk of so-called Western diseases such as breast and prostate cancer, osteoporosis, and cardiovascular disease. On the other hand, phytoestrogens exhibit harmful impacts on the endocrine system and may have adverse effects like endocrine disrupters. There are many studies on animals, wildlife species, and humans that support the existence of these adverse effects.[24]

Milligan (1998) showed that daidzein had minimal acute estrogenic activity and genistein was around 1/1000th to 1/10,000th that of naturally occurring estrogens. None of them were shown to have estrogen-blocking effects. However, genistein and daidzein in soy infant formula are capable of competitively inhibiting the action of thyroid peroxidase (TPO) through competitive inhibition, and they stimulate thyroid dysfunction and provide goitrogenic principles.[53]

Isoflavones such as genistin and daidzin glycosides are biologically inactive. After aglycones of genistein and daidzein form via gastrointestinal enzymes, further metabolism of genistein and daidzein leads to the end products (equol, *o*-desmethyl-angolensin, *p*-ethylphenol).[15] These end products may be responsible for many clinical benefits, and furthermore

biological activity depends on preferential binding and activity at estrogen receptors α- and β.[44,54]

Studies in experimental animals suggest that genistein (i.e., a soy phytoestrogen) has antitumor activity. There is also evidence that genistein can act as an antioxidant. The antioxidant effects were predominantly directed against oxidative damage to membrane lipids and lipoprotein particles and also against oxidative DNA damage. These properties are particularly strong for equol. It is an effective antioxidant and shows structural similarity to the tocopherols.[7,14]

Preparation of isoflavone extracts from soya could well result in the loss of important components that act synergistically with the isoflavones. This approach could also result in daily phytoestrogen intake being increased so far above normal dietary levels that toxicity occurs.[7]

8.6 Toxicity of Phytoestrogens

Identifying chemicals that can cause harmful health effects is not a simple matter. Because the endocrine system is so complex, there are currently no generally accepted, validated methods to screen chemicals for possible hormonal activity that might lead to adverse health effects, but rapid progress is being made.

8.6.1 Xenoestrogens and Endogenous Estrogens

Thousands of man-made chemicals have been released into the environment in vast quantities since the chemical industry began to boom in the 1950s. This has created many problems for the environment. These chemicals disrupt and change the balance of hormone systems in wildlife and humans. Endocrine disruption is a complex, controversial field, and it could be the cause of declines in the populations of many wildlife species over the past 50 years. Researchers believe that the trend toward lower male fertility may be due to environmental estrogens, including the soy phytoestrogens.[55–57]

Hormone disrupters or endocrine disrupters are xenoestrogens (industrial chemicals and pesticides), natural estrogens (ovarian steroids; endogenous hormone), and phytoestrogens (dietary estrogens). They represent a broad array of compounds of varied origins and could include natural hormones, various pharmaceuticals including birth control pills, and estrogen replacement products and other steroids. These compounds excrete and release with wastewater effluent when taken by humans.

The term *estrogen* refers both to natural estrogen hormones in the body and to estrogen products used in medications. The main forms of estrogen found in women's bodies — endogenous estrogen — are the following:

- Estradiol, the main estrogen made by women's ovaries before menopause (also described as 17-beta estradiol and E2)
- Estrone, a weaker estrogen produced both in the ovaries and in fat tissue from other hormones, and the main estrogen found in women after menopause (E1)
- Estriol, the weakest of the three main forms of estrogen, made in the body from other estrogens (E3)

Endogenous estrogens regularly release in the body, and there is no toxicity in normal body conditions. However, when the hormone balance is changed in some conditions such as menopause, the weaker estrogen, estron, is increased and causes problems depending on the balance of hormones. The use of hormone therapy such as hormone replacement therapy to alleviate or prevent menopausal symptoms may cause other hormonally related diseases such as breast cancer. Because of the link between natural hormones and breast cancer, the possibility that oral contraceptives ("the pill") and hormone replacement therapy could cause breast cancer has been studied. It has also been suggested that estrogenic chemicals in the environment could add to lifetime estrogen exposure and thereby increase the risk of breast cancer.

The chemical industry is still manufacturing and using these chemicals called endocrine disrupters or hormonally active substances. These chemicals, including phthalates, are used as plasticizers in plastics (PVC), alkyl phenols and their derivatives (industrial and domestic detergents), bisphenol A (an ingredient of lacquers that are used in dental treatment and to coat metal containers [food cans]), a number of chlorinated organics, such as polychlorinated biphenyls (PCBs) that were used in transformers but were banned some years ago (however, a large quantity of PCBs is still present), dioxins, brominated flame retardants, parabens, which are used as preservatives in cosmetics and antibacterial toothpastes, and butylated hydroxyanisole (BHA), which is a food antioxidant. Xenoestrogenic compounds may work through several estrogen-related mechanisms or through other mechanisms unrelated to estrogen. Various studies have shown that many xenoestrogens have a dramatic effect on the reproductive systems in both wildlife and laboratory animal populations. There is also some research that suggests that there may be a relationship between environmental estrogens and human reproductive disorders[60] (Table 8.4).

Estrogens were first found in plants in the early 1930s, but it was not known whether they would affect living organisms. Exposure to these estrogenic chemicals causes adverse effects. It is a well-known example that when women used DES, which was taken to prevent miscarriages during pregnancy in the 1960s, it caused vaginal tumor in some daughters of these women.[61] Shortly after, it was discovered that Australian sheep experienced a loss of fertility and a number of reproductive lesions. Animal husbandry experts linked the problem to the sheep's grazing on *Trifolium subterraneum*

TABLE 8.4

Known and Suspected Xenoestrogens and Toxic Effects

Synthetic and Natural Xenoestrogens	Chemical (Example)	Toxic Effect	References
Organochlorine	DDT and its metabolites (DDD, DDE) cloro-DDT, chlordane, dicofol	Reduced male fertility; premature puberty; suppression of the	55, 61, 62
Organophosphate	Parathion	immune system; toxic to humans, mammals, birds; breast cancer; testicular cancer	
Organohalogens	Polychlorinated biphenyls (PCBs), polychlorinated dibenzofurans (PCDFs), percloroethylene (PERC), dibromochloropropane (DBCP)	Effect on male fertility, small penis, decline in sperm quality, decreased reproductive capacity	58, 59,62
	TCDD, dioxins	Overall reduction in male fertility	47, 55, 59
Halogenated phenols	Pentachlorophenol (PCP), polybrominated bisphenol-A,	Low testosterone/ gonadotropin	58,63
	Octylphenols (OPs), nonylphenol (NP) (neonatal exposure, rat)	Decreased reproductive organ weight, increased cryptorchidism	47
Synthetic estrogen	Diethylstilbestrol (DES)	Vaginal adenocarcinoma	15, 61

a species of clover that contains high levels of coumestrol.[56] Animals eating the plants receive larger-than-normal doses of estrogen, decreasing their fertility and thereby reducing the population that feeds on the plant. The infertility syndrome was called clover disease.[2,6]

Xenoestrogens are lipophilic and resistant to biodegradation, and they bioaccumulate in adipose tissues.[63] Once they have entered the environment or body, they persist in the body for years due to their long half-life. In contrast, phytoestrogens are readily metabolized and spend relatively little time in the body, and they are the only environmental estrogens that are suspected of having positive effects on humans.

Since the late 1950s, many dramatic declines in wildlife populations, caused by reproductive failure and problems with the development of young, have been associated with exposure to man-made chemicals. In 1962, Rachel Carson warned of the dangers of man-made chemicals to the environment and to humans in her book, *Silent Spring*, concluding, "Our fate is connected with the animals." *Our Stolen Future*, written by Theo Colborni, Dianne Dumanoski, and John Peterson Myers, warned of an impending crisis, reporting that synthetic chemicals are now in the environment.

Although exposure to endocrine disrupters often has little effect on the exposed organism, the offspring of that organism may suffer drastic repercussions, lower sperm counts, undescended testicles, early puberty, and

thyroid dysfunction. If these chemicals are present in the body at the right concentration and at the right time, they can adversely affect hormone balance or disrupt normal function in the organs that hormones regulate.[62]

There are many studies to show toxicity of environmental estrogens on humans and animals. Boys in Taiwan exposed to PCBs while in their mother's womb when their mothers unwittingly consumed PCB-contaminated rice oil during a 10-month period in 1979 have smaller penises compared to normal boys in Taiwan.[63] Alligator eggs exposed to pesticides produce male alligators with abnormal sex hormones.[64] One study carried out in Belgium showed that children who had emigrated from countries such as India and Colombia (DDT is still used in these countries) are 80 times more likely to start puberty unusually young. High levels of a chemical derivative of DDT were found in their blood.[65] Tyrone Hayes found hermaphrodites in frogs at exposure levels as low as 0.1 ppb atrazine.[66]

A number of xenoestrogenic compounds induce or promote breast cancer experimentally. Epidemiological studies have found that breast fat serum lipids of women with breast cancer contain significantly elevated levels of some chlorinated organics compared with noncancer controls.[67]

Since phytoestrogens are natural plant products, their interactions with the body are very different from other synthetic environmental estrogens. They are the only environmental estrogens that are suspected of having positive effects on humans. They do not bioaccumulate in adipose tissues, unlike xenoestrogens.[68]

They can also cause infertility, endometriosis, sexual dysfunction, and masculinization of females, as well as feminization of males, in a variety of animal and bird species. After the discovery of "clover disease" depending on coumestrol consumption, toxicity in SBF for infants was noticed depending on phytoestrogens. SBP have been used for >60 y.[69] Initially developed for infants unable to tolerate the proteins in cow's milk-based formulas (CMF), SBF are now used more widely and account for ~25% of infant formulas sold in the United States.[70] Concerns about the use of soy-based infant foods have been raised due to long-term effects of exposure during development. The greatest phytoestrogen risks by far to consumers are the estrogenic toxins genistein and daidzein present in all modern soy products and food ingredients.[30]

SBFs are manufactured from soy protein isolates and contain significant amounts of phytoestrogens of the isoflavone class. As determined by HPLC, the isoflavone compositions of commercially available formulas are qualitatively and quantitatively similar and are consistent with the isoflavone composition of soy protein isolates. Genistein, found predominantly in the form of glycosidic conjugates, accounts for >65% of the isoflavones in soy-based formulas. Isoflavones circulate at concentrations that are 13,000- to 22,000-fold higher than plasma estradiol concentrations in early life.[71]

Setchell et al. found that the daily exposure of infants to isoflavones in soy infant formulas is 6- to 11-fold higher on a body-weight basis than the dose that has hormonal effects in adults consuming soy foods.[72] There is strong

evidence from both *in vitro* and *in vivo* studies that persistent TPO inhibition will occur in infants subjected to soy formulas long term (for more than 3 months).[73]

Soy phytoestrogens such as genistein can inhibit 17--hydroxysteroidoxido reductase, an enzyme that is required for the synthesis of testosterone and the development of the central nervous system (CNS)-gonadal axis. Therefore, it is quite possible that phytoestrogens, along with other endocrine-disrupting compounds such as DDT, may contribute to the worldwide decrease in male fertility, including that in male insects.[74]

Phytoestrogens are now strongly implicated in thyroid disorders, behavioral and developmental disorders, and cancer. Thyroid problems are now of epidemic proportions. Theodore Kay of the Kyoto University Faculty of Medicine noted in 1988 that "thyroid enlargement in rats and humans, especially children and women, fed with soybeans has been known for half a century." Recent research leaves little doubt that dietary isoflavones in soy have a profound effect on thyroid function in humans.[75]

Isoflavones also encourage early sexual development in girls but delay or feminize development in boys. The soy isoflavones, genistein and daidzein, inhibit an enzyme that plays a critical role in the development of testosterone.[76]

Preliminary studies indicate that children given soy formula go through puberty much earlier than children who were not fed soy products, because the phytoestrogens/isoflavones in soy act like a hormone in the body, causing the infant to have hormones like those in the adult body. A 1994 study done in New Zealand revealed that, depending on age, potency of the product, and feeding methods, infants on soy formula might be consuming the equivalent of up to ten contraceptive pills a day. By exposing your baby to such large amounts of hormone-like substance, you are risking permanent endocrine system damage (pituitary gland, pineal gland, hypothalamus, thyroid, thymus gland, pancreas, ovary, testis, adrenal glands).[77]

8.6.2 Male Health and Infertility

Phytoestrogens such as isoflavones and coumestrol, if given in high enough doses or during critical stages of development, cause several reproductive tract disorders, including impaired fertility.[13]

Congenital abnormalities of the male genital tract are increasing. Soy phytoestrogens may be implicated, according to a study that found a higher incidence of birth defects in male offspring of vegetarian, soy-containing mothers. There are also links between high soy diets during pregnancy and nursing and eventual developmental changes in children.[78]

Developmental exposure to phytoestrogens results in toxicities similar or identical to those of other estrogens.[70] Phytoestrogens are prototypical environmental estrogens known to induce infertility in livestock fed phytoestrogen-containing forage. The human sperm count decrease over the past five decades might relate to the introduction of soya to the West. Although it has

been known for many years that estrogen administration has deleterious effects on male fertility, data from transgenic mice deficient in estrogen receptors or aromatase point to an essential physiological role for estrogen in male fertility.[79]

In experimental animals, some phytoestrogens cause abnormal development of female reproductive tract tissues, including the uterus (inhibition of uterine gland differentiation) and ovary (polyovular follicles, altered follicle development), as well as having actions on the developing brain (causing infertility due to neural failure to regulate estrus cycles).[80]

There is a wealth of evidence that shows that mammals exposed to estrogens during critical periods of sexual development can suffer a drastic reduction in fertility. Human exposure during development occurs primarily through phytoestrogen-rich soy infant formula. High consumption of soy-rich diets has been associated with altered reproductive function in women, decreased serum estrogen levels, and increased sex hormone-binding globulin levels. Genistein is a major ingredient in tofu found in significant levels in the blood of Asians.[81,82]

Weber et al. studied the short-term effects of dietary phytoestrogens on the levels of testosterone and prostate weight in adult Sprague-Dawley rats. Plasma testosterone and androstenedione levels were significantly lower in the animals fed the phytoestrogen-rich diet compared with animals fed the phytoestrogen-free diet, and prostate weights were also significantly decreased.[83,84]

8.6.3 Phytoestrogens and Thyroid

Goiter (thyroid dysplasia) can occur as a result of hypothyroidism caused by iodine deficiency. Soybean and goiter have long been associated in animals and humans. Rodents are useful risk assessment models for thyroid toxicants, despite significant differences between rodent and human thyroid physiology. There is a negative interaction of low dietary iodine and soy, and thyroid carcinoma was observed in rats fed an iodine-deficient diet consisting of 30% defatted soy. In humans, goiter has been seen in infants fed soy formula. This is usually reversed by changing to cow's milk or iodine-supplemented diets.[33]

Ishizuki et al. carried out a study to elucidate whether soybeans would suppress the thyroid function in healthy adults. They selected 37 subjects who had never had goiters or serum antithyroid antibodies. They were given 30 g of soybeans every day and were divided into three groups subject to age and duration of soybean administration. Their findings suggested that excessive soybean ingestion for a certain duration might suppress thyroid function and cause goiters in healthy people, especially elderly subjects.[85]

8.7 Possible Benefits of Phytoestrogens for Human Health

Soy has clearly been a functional food in the spotlight during the 1990s. In addition to being a high-quality protein, soy is now known to play a preventive and therapeutic role in a number of chronic diseases, including heart disease, osteoporosis, and cancer.[86]

8.7.1 Breast and Prostate Cancer

The agents responsible for the initiation of human mammary and prostatic cancers remain unidentified. The role of endogenous estrogens in breast cancer risk is widely recognized. Phytoestrogens have biphasic effects on the proliferation of breast cancer cells in culture.[7] Both tumorigenic and antitumorigenic effects of phytoestrogens have been reported. Although estrogens stimulate the growth of many breast tumors, there is a negative correlation between the incidence of breast cancer and the phytoestrogen-rich diet of certain Asian populations.[87] The phytoestrogens and breast cancer hypothesis stems in part from the low breast cancer mortality rates in Asian countries, where soyfoods, which are high in isoflavones, are commonly consumed.[88] Protective effects for women were observed primarily in Asian immigrants, not in U.S.-born Asians.

Based on animal studies, there is a possible beneficial role of phytoestrogens in reducing the risk of prostate cancer. On the other hand, there are no significant positive findings from human clinical trials. As for breast cancer, there are promising results obtained from dietary studies in addition to the epidemiological evidence.[5] The possible role of phytoestrogens in breast cancer is far from being conclusive. Animal studies have demonstrated that in mice and rats, transplacental exposure to genistein, a major component in phytoestrogens, acts as an estrogen and may actually increase the incidence of mammary tumors in female offspring.[89]

The results of the case-control human studies of the connection between eating soy products and breast cancer risk are conflicting.[15,44] Animals that were given soy phytoestrogens developed fewer mammary (breast) tumors in many,[15,44,90] but not all,[91] studies. Some studies have reported no link and others have reported a decrease in the risk of breast cancer among women eating soy compared to women who did not eat soy. No studies have reliably demonstrated an increase in the risk of breast cancer among women eating soy. Studies of breast cells have shown that soy phytoestrogens can either encourage or discourage growth within the breast.[92] It is unclear if these effects on cells in the laboratory are the same or different from those on breast cells in the body. Both lignan (from brans, beans, and seeds) and coumestan phytoestrogens (from beans and sprouts) have been studied for a possible effect on breast cancer risk. Two studies have found higher levels of lignan phytoestrogens in the urine of women who may be at lower risk

for breast cancer, such as Japanese women.[93] Other studies compared women without cancer to women with breast cancer; the women with breast cancer had significantly lower levels of lignan phytoestrogens in their urine.[94]

Breast cancer is still a major cause of death for women in Western countries.[15,44] Prostate cancer is now the second leading cause of cancer death in men, exceeded only by lung cancer. Breast and prostate cancer are much less prevalent in Far Eastern countries, where there is an abundance of soya phytoestrogens in the diet, than in Western countries.[54] In Eastern countries such as Japan, Korea, China, and Taiwan, the mean daily intake of soya products is about 10 to 15 times higher than in the United States and Western countries. The incidence of breast cancer in Japan is much lower than in the United States. It was shown that the large amount of soy protein significantly lowers the cancer risk of women in the former country.[54] More research on the mechanism of the body's response to naturally occurring estrogenic substances from plants and other sources, including ethnic differences, is needed to understand the differences in cancer rates between Japan and the United States.

Studies have shown that actions of phytoestrogen in prevention of breast cancer risk do not use only estrogenic but also nonhormonal actions. Based on animal studies, there is a possible beneficial role of phytoestrogens in reducing the risk of prostate cancer. Ozasa et al. carried out a nested case-control study as part of the Japan Collaborative Cohort (JACC) Study. They examined whether a high serum concentration of phytoestrogens reduces the risk of prostate cancer among Japanese men. They found that serum genistein, daidzein, and equol dose dependently reduced prostate cancer risk.[95]

It has been observed that the prevalence of breast cancer among women in Japan is one of the lowest in the world and that Asian men have even lower rates of prostate cancer. These rates are generally lower in the homeland, but when Asian women migrate to America, breast cancer incidence significantly increases.[96] Hence it has been suggested that environmental factors, especially the diet, play an important role. Migration of people from countries such as Japan, Korea, China, and Taiwan to the United States has been shown to increase their risk of breast and prostate cancer. It was observed that prostate cancer risk increased in the same generation in men, whereas breast cancer risk was observed in the next generation for women. These changes in breast and prostate cancer risk may depend on changes in diet.[7]

Ingram et al. conducted a case-control study of 288 subjects to find an association between the urinary excretion of phytoestrogens and breast cancer. They found that the increased urinary excretion of some phytoestrogens was associated with a substantial decrease in the risk of breast cancer.[94]

Vegetarians, who have a low risk of breast cancer relative to that of omnivores, have a significantly higher dietary intake and urinary excretion of lignans.[97]

8.7.2 Menopause

Women are reluctant to receive postmenopausal estrogen replacement therapy since they consider prescription estrogens as being "unnatural" and consider that plant-derived and herbal therapies are safer, although there are no government standards controlling their quality. For this reason, the use of phytoestrogens is increasing from day to day. *In vitro* and animal studies show that phytoestrogens may play a pivotal role in inhibition of the growth of cancer cell lines, the reduction of cholesterol levels, prevention of osteoporosis, and alleviation of menopausal symptoms.[15,44]

There has been tremendous interest in the possibility that dietary phytoestrogens may be an alternative to postmenopausal hormone therapy because of concerns about side effects and long-term health consequences that prevent many women from using hormone therapy for problems associated with the menopausal transition.[30]

Given that phytoestrogens are weak estrogen agonists, they will exhibit their most potent estrogenic effects in a low-estrogen environment, when there are few competitors available. Thus, they may be predicted to exhibit more estrogenic properties in postmenopausal women. Phytoestrogens reduce the number and intensity of hot flashes, although the reduction is a modest 10 to 20%.[98]

Nagata et al. conducted a prospective study in premenopausal Japanese women who received either a soymilk-supplemented diet or a normal diet. The women were followed up during three consecutive menstrual cycles. They found an inverse association between hot flashes and soy food or isoflavone consumption. These data suggest that consumption of soy products has a protective effect against hot flashes.[99]

8.7.3 Osteoporosis

Osteoporosis is related to multiple factors including aging, hormone deficiency, and diet.[2] There have been several *in vitro* and animal studies that have shown that phytoestrogens prevent postmenopausal bone loss. However, only three human studies have been reported. These studies suggested that phytoestrogens, especially ipriflavone (synthetic isoflavone), increased bone mineral density (~ 1.28 to 4%) compared with the baseline in pre- and postmenopausal women.[44,98] Population studies also showed that osteoporosis-related fractures are lower in Asia than in most Western communities, possibly due to the phytoestrogen-rich soybeans and vegetables consumed in large quantities in the Asian diet.[15]

8.7.4 Cardiovascular Disease

Mortality due to cardiovascular disorders (CDs) is similar in men and women. The lipid profile (serum total, LDL, and VLDL cholesterol) plays a

pivotal role in cardiovascular disorders. The lipid profile, estrogen, and CD form an interacting triangle. When estrogen levels are decreased, the levels of cholesterol are increased, and this state may cause CDs. In this context, phytoestrogens may be protective in CDs. In point of fact, the mechanism of cardioprotective effects of phytoestrogens is uncertain.[2,7]

Epidemiological and numerous animal studies support the notion that soy consumption is cardioprotective.[2,7] For example, men in Japan, who consume relatively large amounts of soy, have approximately one-sixth the risk of CDs as men in the United States.[54]

Serum cholesterol levels are low in women compared with men; however, after menopause these levels increase due to loss of endogenous estrogen secretion and become high compared with men.[98]

Recent reports have linked the dietary intake of soy-based foods with a reduction of coronary heart disease (CHD). Intact soy protein appears to be effective in both animals and humans in lowering plasma total cholesterol, LDH cholesterol, and triglyceride.[7,35,98]

8.7.5 Endometrial Cancer

An antiestrogenic effect of phytoestrogens may be relevant to the inhibition of endometrial carcinogenesis by lowering endometrial cell proliferation or reducing levels of ovarian steroids through down-regulation of the hypothalamus and pituitary.[100]

8.8 Risk Evaluation of Phytoestrogens

How do people evaluate whether the effect of phytoestrogens on humans is serious? Risk evaluation makes use of four distinctive steps: hazard identification, exposure assessment, dose-response relation, and risk characterization. With phytoestrogens, the hazard can be defined as "serious effect." These adverse effects can be either detrimental or beneficial. The assessment of exposure time and the amount of phytoestrogens are very important tools for evaluation. Most plant estrogens and synthetic estrogens that have been identified are much less potent than endogenous estrogen, and for activity to take place, a large amount of phytoestrogens must be consumed. As for the exposure period, Asian people, for example, consume more phytoestrogens than American people do, and the total amount of phytoestrogens ingested via diet in their lifetime is consequently greater. Epidemiological studies show that Asian people have lower risk of hormonally dependent diseases such as menopause and prostate and breast cancer.[5,6] This suggests that long exposure and high consumption are required to have biological effect. Understanding a substance's potency and its behavior in the body

allows assessment of whether the amount that reaches a sensitive organ is sufficient to cause an adverse health effect.

Hazard and risk assessment of chemicals is usually focused on individual compounds. However, risk assessment of substances in the environment can be especially complicated, and it is more difficult to assess risks, hazards, and benefits for chemical mixtures, because people and wildlife are exposed to many chemicals at the same time. When several very similar chemicals are combined in a mixture, compound interactions may result in additive, synergistic, or antagonistic effects. Furthermore, many studies have used phytoestrogen-containing foods and assumed that they are responsible for the biological effects observed. However, there are other active components used in these foods as a test material, which could also contribute to these effects. Experimental conditions are not equivalent to the level of dietary exposure in humans. Many studies also use the subcutaneous route of administration, which excludes the critical influence of gastrointestinal and hepatic metabolism. Consumption of nonfermented and fermented soy may have biological effects in opposite directions.

Risk assessment of phytoestrogens is complex due to all the circumstances mentioned above. Phytoestrogens can elicit agonist and antagonist actions and nonestrogenic effects, which are age, tissue, and gender dependent. There are also interspecies differences in ADME (absorption, distribution, metabolism, and excretion) and extrapolation data from animals to humans. An evaluation of the risk and benefit of dietary phytoestrogens is critically dependent on the nature, timing, conditions, and extent of exposure.

Consumption of large amounts of soy involves a risk of mega-dosing per isoflavones. According to Protein Technologies International (manufacturers of isolated soy protein), soy consumers consume 100 g of soy protein per day, and their daily genistein intake could easily exceed 200 mg per day, definitely to be avoided. When compared to the study of Fukutake et al., Japanese males consume less than 10 mg of genistein per day.[101]

Generally, phytoestrogens are much less potent than the endogenously produced estrogens, but phytoestrogens can be present in much greater quantities. Animal studies enable one to put forward an idea about phytoestrogens. However, this is not of great use due to the reasons mentioned above (interspecies differences in ADME, extrapolation data from animals to humans, etc.). Population studies provide much more realistic data than *in vitro* experiments and animal studies, because they reflect more fully the facts related to phytoestrogens.

It has been hypothesized that phytoestrogens reduce the risk of a number of cancers and hormonally related diseases. However, epidemiological studies addressing these issues are hampered by the lack of a comprehensive phytoestrogen database for quantifying exposure. Horn-Ross et al. suggested developing a database on phytoestrogens for use with food-frequency questionnaires in large epidemiological studies. One hundred eighteen female volunteers (African-American, Latin, and White women residing in California) participated in this study. The content of seven specific phytoestrogens

was determined by high-performance liquid chromatography–mass spectrometry (HPLC-MS). A database was developed, which is important in assessing the relationship between phytoestrogen exposure and cancer risk in epidemiological studies.[8] Another study on intake of dietary phytoestrogens was conducted, and the intake levels of phytoestrogens in the study were low. However, these levels can be compared to other Western cohorts.[20]

There is a great deal of research on different population groups[11,101] showing that there is an association between phytoestrogen exposure and hormone-related conditions. Consumption of soy containing isoflavones reduces lipid peroxidation *in vivo* and increases the resistance of LDL to oxidation. This oxidant effect may serve to prevent the risk of arteriosclerosis, cardiovascular disease, and cancer.[14]

8.9 The Future of Phytoestrogen Research

Epidemiological studies suggest that phytoestrogen-containing foods may have beneficial roles in hormonally related diseases and detrimental effects due to their estrogenic properties in sufficient doses. However, there is almost no evidence to link the effect of phytoestrogen-containing foods to phytoestrogens alone. Many other components such as soya and linseed are biologically active and may be responsible for all these effects in humans.

Many studies using phytoestrogens including soya and their products have demonstrated conflicting results. In the future, more research is needed, and it must be determined whether or not phytoestrogens are the main active components, what mechanisms are involved, and how they work.

Phytoestrogens could be used as an effective, safe, and reliable means to alleviate menopausal symptoms and may offer additional beneficial effects compared to the pharmaceutical properties already available, but this needs to be confirmed with well-controlled clinical trials.

In order to understand how phytoestrogens may act, we need more information on the cell-, tissue-, or organ-specific actions of the individual compounds. Long-term clinical trials with well-characterized and standardized phytoestrogen preparations are necessary to confirm the beneficial health effects in humans.

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9

Soy Isoflavones and Health

Xing-Gang Zhuo, Melissa K. Melby, and Shaw Watanabe

CONTENTS

9.1 Introduction

There are many studies reporting the human health effects of soy-based foods on the prevention of hormone-related cancers, cardiovascular diseases, and prevention of osteoporosis.[1–5] Soybeans are consumed in many Asian countries such as Japan[6] and are a major source of isoflavones (IFs), one group of phytoestrogens.[7–11] In this chapter we review recent research on soy-based foods and the biological activities and health benefits of their constitutive IFs.

9.2 Isoflavone Content, Intakes, Serum Concentration, and Urine Excretion

Soybeans are well known to be rich in the isoflavones genistein and daidzein.[9–11] Intake of total isoflavones (sum of daidzein and genistein) calculated from the 3-d dietary records (DR) of 115 Japanese women using the isoflavone composition table of Japanese soy-based foods (Table 9.1) was 47.2 ± 23.6 mg/d. Genistein composed most of the isoflavone intake (30.5 ± 15.6 mg/d), and daidzein intake (16.6 ± 8.0 mg/d) was about half that of genistein (Table 9.2). Isoflavone intake was attributable to tofu (bean curds, sum of various types, 37.0%, 49.4 g/d), natto (fermented soybeans) (31.0%, 14.8 g/d), and miso (fermented soybean paste) (15.7%, 17.0 g/d), which accounted for 84.3% of pulse consumption by weight in the dietary records.[12]

Yamamoto et al.[13] collected food frequency questionnaire (FFQ) data, DR, and blood and urine samples from 215 subjects among Japanese Public Health Committee (JPHC) Study participants, estimated isoflavone intakes from FFQ and DR, and measured serum isoflavone concentration and urine excretion. For daidzein, mean intakes estimated from FFQ and DR, serum concentration, and urine excretion were 18.3 mg/d, 14.5 mg/d, 119.9 nmol/L, and 17.0 μmol/d and for genistein, 31.4 mg/d, 23.4 mg/d, 475 nmol/L, and 14.2 μmol/d, respectively; results were similar when analyzed separately by sex. Spearman correlation coefficients for FFQ energy-adjusted daidzein intakes and those from DR, serum concentration, and creatinine-adjusted urinary excretion were 0.64, 0.31, and 0.43, respectively. Similar results were observed for genistein.

TABLE 9.1

Concentrations of Daidzein and Genistein in Japanese Foods

Food	(μg/g wet food) Daidzein	Genistein
Soybean		
(Raw)	1006.3	1437.7
(Boiled)	135.8	472.5
Green soybean, immature		
(Raw)	33.3	63.1
(Boiled)	48.3	66.0
Soybean powder (kinako)	823.3	1301.6
Soybean sprout	49.6	79.3
Mung bean sprout	3.4	2.4
Soymilk	78.2	156.6
Okara[a]	33.1	57.1
Yuba (soymilk sheet)		
(Dried type)	518.2	1106.5
(Raw type)	167.4	395.0
Tofu (soybean curd)		
(Momen type)	166.2	269.2
(Silken type)	130.1	206.4
(Packed type)	168.6	280.7
(Backed type)	166.8	291.2
(Okinawea type)[b]	87.2	184.0
(Dried type)	168.2	556.7
Deep-fried tofu		
(Thick type)	148.7	257.4
(Thin type)	84.2	179.1
Ganmodoki[c]	132.5	338.4
Soy sauce		
(Common type)	8.4	4.7
(Light color type)	4.9	2.7
Miso (soybean paste)		
(Light yellow type)	176.5	300.0
(Dark yellow type)	212.6	293.2
(White type)	104.2	257.6
Natto (fermented soybean)		
(Common type)	366.2	607.4
(Tera type)[d]	685.6	1186.5
Tempeh[e]	525.9	1326.2

[a] Bean curd residue.

[b] Okinawa, southern part of Japan, domestic type.

[c] Deep-fried tofu with mixed vegetables and seaweeds.

[d] Fermented with *Aspergillus oryzae.*

[e] Fermented soybean from Indonesia.

Source: Arai, Y., Watanabe, S., Kimira, M., Shimoi, K., Mochizuki, R., and Kinae, N., *J. Nutr.*, 130, 2243–2250, 2000.

TABLE 9.2

Intakes and Contributions of Various Foods to Isoflavone Intake by Japanese Women

Daidzein		Genistein	
mg/(d, person)			
16.6 ± 8.0[a]		30.5 ± 15.6	
Food	**% of Intake**	**Food**	**% of Intake**
Tofu[b]	35.9	Tofu	37.3
Natto[b]	30.1	Natto	32.5
Miso[b]	20.3	Miso	17.6
Deep-fried tofu (Thin type)	3.1	Deep-fried tofu (Thin type)	3.8
Soy sauce[b]	1.9	Ganmodoki	2.0
Ganmodoki	1.7	Boiled soybean	1.8
Kinako	1.6	Kinako	1.5
Boiled soybean	1.1	Deep-fried tofu (Thick type)	0.9
Dried-type tofu	1.0	Soymilk	0.7
Deep-fried tofu (Thick type)	0.9	Green soybean, immature	0.6

[a] Values are mean ± SD, n = 115.
[b] Value are sums of various types.

Source: Arai, Y., Watanabe, S., Kimira, M., Shimoi, K., Mochizuki, R., and Kinae, N., *J. Nutr.*, 130, 2243–2250, 2000.

9.3 Measurement of Isoflavones

The isoflavone contents of Japanese soy-based foods were analyzed using the hydrolysis and extraction method of Mazur et al.[14] combined with the HPLC method described by Gamache et al.[15] Freeze-dried samples (0.01 g) were put in glass tubes and 0.5 ml of distilled water was added to each. After the samples stood for 10 min, they were hydrolyzed overnight at 37°C with 0.5 ml of an enzyme solution. The hydrolyzed samples were extracted twice with 5 ml diethyl ether, and the ether fraction was dried by evaporation under nitrogen gas flow. The residue was dissolved in 1 ml methanol, and 20 µl of that solution was analyzed by HPLC with diode-array UV detection scanning from 250 to 400 nm. Isoflavone peaks were detected at 254 nm. Peak areas and a calibration curve of standards were used to quantify each isoflavone.[12]

Measurement of plasma isoflavones is difficult, because of the low levels present in the blood and the necessity of sample pretreatment to obtain aglycone forms.[16] The commonly used HPLC ultraviolet (UV) detector has inadequate sensitivity (usually 100–1000 ng/ml) for most plasma samples. A high-performance liquid chromatography electrochemical detector (ECD)

that detects 10 to 100 ng/ml has recently become available. Gas chromatography-mass spectrometry (GC-MS) is so far the most sensitive method, able to detect 1 ng/mL, but it requires a skillful technique. A recently developed time-resolved fluoroimmunoassay provides an alternative sensitive method with a detection limit of less than 5 nmol/L, and is especially useful for analysis of large numbers of samples from epidemiological studies.[17]

9.4 Metabolism of Isoflavones

Absorption of IFs in the alimentary tract mostly involves IFs in their aglycone forms.[18–21] It has been suggested that bacterial hydrolysis of the glycosyl bond of glycosidic forms of isoflavones must precede absorption from the intestinal tract. In humans, absorption of the unconjugated form of IFs occurs primarily in the small intestine and large intestine. Fecal excretion of intact isoflavones varied from 0.4 to 8%. This variation results from different compositions of intestinal bacterial flora. Isoflavones that possess a 5-OH group, such as genistein, are much more susceptible to C-ring cleavage by rat intestinal bacteria.[22] Certain strains of *Clostridium* spp. in the human colon can cleave the C-ring and produce monophenolic compounds anaerobically.[23] O-desmethylangolensin (O-DMA) and equol, the main metabolites of daidzein, are probably produced in the colon.[20,21] Hutchins et al.[24] compared urinary excretion after consumption of fermented and unfermented soy foods and found that fermented soy foods such as soy sauce and miso (fermented soybean paste) resulted in faster absorption of isoflavones

Altered kinetics of urinary daidzein and genistein excretion following prolonged intake of IF were reported by Lu et al.[25,26] Watanabe et al.[18] conducted a pharmacokinetic study of soybean isoflavones after a single ingestion of baked soy powder (*kinako*, containing 103.23 μmol daidzein and 111.89 μmol genistein) and further analyzed the kinetic properties of IFs in seven healthy male volunteers. The plasma concentration of genistein increased after 2 h and reached its highest value of 2.44 ± 0.65 μmol/L 6 h later. The plasma concentration of daidzein peaked at 1.56 ± 0.34 μmol/L at the same time, but it was always lower than that of genistein. Peak plasma concentrations of O-desmethylangolensin (O-DMA) and equol appeared after the daidzein peak. In contrast with plasma, daidzein was the main component in urine. Urinary daidzein excretion started to increase shortly after the rise in its plasma concentration and reached a peak of 2.4 μmol/h 8 h after ingestion of kinako. Genistein excretion in the urine paralleled that of daidzein, but the value at 6 h was approximately half (1.1 mmol/h). Total recovery of daidzein, O-DMA, and equol from urine and feces was 54.7%, calculated from daidzein intake; 20.1% of administered genistein was recovered as genistein. The individual plasma and urinary concentrations of equol and O-DMA were quite variable and bimodal: Subjects were classified as

high and low producers. Recovery of daidzein and genistein from urine was 35.8% and 17.6%, respectively, and 4.4% of ingested daidzein and 4% of genistein were recovered in feces. The half-lives of plasma genistein and daidzein were 8.36 and 5.79 h, respectively. King and Bursil[27] reported similar values. Genistein seems to be absorbed more efficiently from the intestinal tract and remains in circulation longer.

9.5 Health Benefits of Isoflavones

9.5.1 Hormone-Related Cancer Prevention

9.5.1.1 Breast, Endometrial, and Ovarian Cancer

Hirayama[28] reported that breast cancer incidence was lower among women with higher frequency of miso soup consumption. An inverse correlation between breast cancer risk and consumption of soy protein as well as total soy products was also observed by Lee et al.[29] Adlercreutz et al.[2] and Murkies et al.[30] found that urinary excretion of phytoestrogens was significantly lower in breast cancer patients compared to controls. One case-control study[31] clearly showed a significantly reduced odds ratio of 0.27 (95% CI: 0.10 to 0.69) for breast cancer patients with >185 nmol/day excretion of equol, compared to <70 nmol/day. Duncan et al.[32] reported that equol producers generally had lower concentrations of estrone, estrone sulfate, testosterone, androstenedione, dehydroepiandrosterone (DHEA), DHEA sulfate, and cortisol and higher concentrations of sex hormone-binding globulin and mid-luteal progesterone, a hormonal pattern generally consistent with lowered breast cancer risk. In the case of breast cancer, transfer of IF from mother to fetus could have a preventive effect on the mammary gland after birth.[33] Early exposure to IF may suppress the development of mammary buds, leading to a decreased risk of breast cancer in adulthood. In human immigrant studies in the United States and Brazil, breast cancer incidence was increased from the second generation, while that of prostate cancer increased from the first generation.[34] These results suggested that early life events may modify the breast cancer risk. Results from animal experiments suggest that the high intake of soy-based foods among Japanese should result in a lower incidence of breast cancer.

Xu et al.[35] reported that regular consumption of soy foods, measured as amount of either soy protein or soy isoflavones, was inversely associated with the risk of endometrial cancer among Chinese women in Shanghai. Compared to women with the lowest quarter of intake, the adjusted odds ratio of endometrial cancer was reduced from 0.93 to 0.85 and 0.67 with increasing quartile of soy protein intake (P for trend 0.01). A similar inverse association was observed for soy isoflavones and soy fiber intake.

Chen and Anderson[36] found that genistein and daidzein independently modify cytokine production and reduce ovarian cancer cell proliferation via, at least in part, an estrogen receptor-dependent pathway.

9.5.1.2 Prostatic Cancer

Sonoda et al.[37] reported that consumption of all soybean products, such as tofu and natto, was associated with decreased prostate cancer risk in a case-control study in Japan. Consumption of natto showed significantly decreasing linear trends for risk. A case-control study in China[38] showed a reduced risk of prostate cancer associated with consumption of soy foods and isoflavones. Kumar et al.[39] found that supplementing early stage prostate cancer patients with soy isoflavones, even in a study of short duration, altered surrogate markers of proliferation such as serum PSA and free testosterone in a larger number of subjects in the isoflavone supplemented group than the group receiving placebo.

9.5.1.3 Colon Cancer

Spector et al.[40] reported an inverse association between higher soy consumption and colon cancer onset in their analysis of 13 epidemiological studies. Dietary soy isoflavones protect ovariectomized ERalphaKO and wild-type mice from carcinogen-induced colon cancer.[41]

9.5.2 Cardiovascular Disease

In October 1999, the U.S. Food and Drug Administration approved a health claim for the relationship between consumption of soy protein and reduced risk of coronary heart disease. Dietary intake of whole soy foods containing 60 mg/d of isoflavones results in significant serum levels of phytoestrogens and reductions in several key clinical risk factors for cardiovascular disease (CVD) in postmenopausal women.[42]

9.5.2.1 Hyperlipidemia

A meta-analysis[43] published in 1995 of 38 controlled clinical studies in 29 scientific articles reported that ingestion of 47 g/d of soy protein was associated with the following net changes in serum lipid concentrations compared to the control diet: decrease in total cholesterol of 0.60 mmol/L (23.2 mg/dl; 95% CI, 0.35 to 0.85 mmol/L [13.5 to 32.9 mg/dl]), or 9.3%; decrease in LDL-C of 0.56 mmol/L (21.7 mg/dl; 95% CI, 0.30 to 0.82 mmol/L [11.2 to 31.7 mg/dl]), or 12.9%; and a decrease in triglycerides of 0.15 mmol/L (13.3 mg/dl; 95% CI, 0.003 to 0.29 mmol/L [0.3 to 25.7 mg/dl]), or 10.5%. The changes in serum total cholesterol and LDL-C concentrations were directly related to the initial serum total cholesterol concentration ($P < 0.001$). Since that time, a number of studies have re-examined the effect of soy

protein and/or IF on blood lipid levels in humans and observed substantially weaker effects. Changes in LDL or non-HDL cholesterol levels attributable to the substitution of 25 to 50 g of soy protein for animal protein range from zero to a 5% decline in individuals with moderately elevated total cholesterol levels.[44–49] A similar meta-analysis of 10 clinical trials published in 2003 reported that a daily intake of 36 g soy protein with 52 mg soy-associated IF on average decreased low-density lipoprotein (LDL) cholesterol by 0.17 ± 0.04 mmol/L (mean ± SE), and increased HDL cholesterol by 0.03 ± 0.01 mmol/L.[50]

9.5.2.2 Hypertension

An intervention using soy protein isolate (40 g soy protein, 118 mg isoflavones) resulted in a significant decrease in blood pressure compared with casein placebo.[47] After 3 m of soymilk consumption, systolic blood pressure decreased by 18.4 ± 10.7 mmHg compared with 1.4 ± 7.2 mmHg in the cow's milk group ($P < 0.0001$), and diastolic blood pressure decreased by 15.9 ± 9.8 mmHg vs. 3.7 ± 5.0 mmHg in the cow's milk group ($P < 0.0001$). Urinary genistein was strongly ($r = -0.588$) and significantly ($P = 0.002$) correlated with the decrease in blood pressure, particularly for diastolic values.[51] When compared to the casein-based diet, the soy-based diet attenuated the development of hypertension and decreased serum total cholesterol level in spontaneously hypertensive rats (SHRs).[52]

9.5.2.3 Atherosclerosis

Adams et al.[53] reported that the inhibitory effect of soy protein isolate on atherosclerosis in mice did not require the presence of LDL receptors or alteration of plasma lipoprotein. Isoflavone aglycone-rich extract without soy protein attenuated atherosclerosis development in cholesterol-fed rabbits.[54]

9.5.3 Osteoporosis

Higher intake of isoflavone (calculated from dietary intake) was associated with higher bone mineral density of femoral neck in mid-life Australian-born women.[55] Postmenopausal women with the highest level of isoflavone consumption had greater bone density at the spine.[56] A soy-rich diet stimulates bone osteoblastic activity, as evidenced by significant increase in osteocalcin concentrations. Bone mineral density (BMD) decreased significantly only in the control group but not in the intervention groups. These data suggest that soy products could be effective in reducing the risk of osteoporosis in asymptomatic postmenopausal women.[57]

9.6 Mechanisms of Isoflavones in Health Effects

9.6.1 Cancer Prevention

9.6.1.1 Hormone-Related Effects

Historically, isoflavone competition with endogenous estrogen was hypothesized to mediate estrogen-related cancer prevention.[58] Estrogenic effects of isoflavones have been extensively investigated[58–60] due to the chemical and biological similarity to mammalian estrogens (Figure 9.1). Kuiper et al.[61] found that genistein and daidzein bind more strongly to ER-beta than to ER-alpha (Table 9.3). Breinholt et al.[62] reported weak estrogenic activities of isoflavones, and equol showed much stronger activity than the other isoflavones (Table 9.4). One intervention study[63] showed significantly increased sex hormone-binding globulin (SHBG), which may lower the activity of estradiol (E2) in the circulating blood. Inhibitory effects of IF on aromatase CYP19 also result in low E2. Endometrial cells predominantly have ER alpha, so the binding capacity of IF is much lower compared to E2, which is used by replacement therapy. Therefore, IF may reduce the risk of endometrial and ovarian cancer by reducing plasma E2 levels and binding competitively to ER in the target organs. Lu et al.[64] reported that a soy diet with low levels

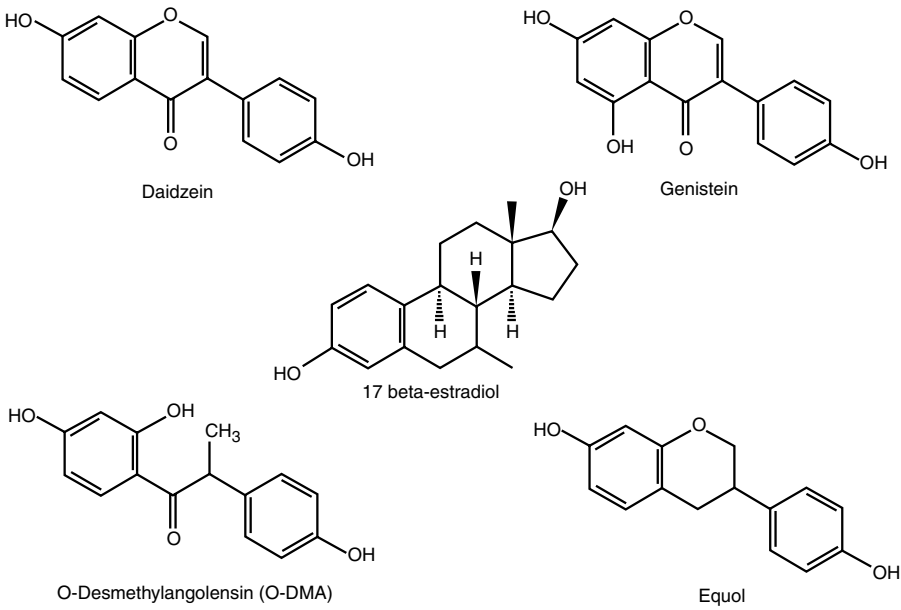

FIGURE 9.1
Structural similarities of estrogen and isoflavones.

TABLE 9.3

Relative Binding Affinities of Isoflavones for Estrogen Receptors α and β

Compound	ER α	ER β
17β-estradiol	100	100
Tamoxifen	4	3
4-OH-tamoxifen	257	232
Genistein	4	87
Daidzein	0.1	0.5
Ipriflavone	< 0.01	< 0.01

Source: Kuiper, G.G., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., van der Saag, P.T., van der Burg, B., and Gustafsson, J.A., *Endocrinology,* 139, 4252–4263, 1998.

TABLE 9.4

Estrogen Activities of Isoflavones

Compound	Yeast Screen	MCF-7 Cells
17β-estradiol[a]	100%	100%
Equol	2.3×10^{-2}	1.3×10^{-2}
Genistein	4.5×10^{-3}	2.6×10^{-2}
Daidzein	2.8×10^{-4}	1.1×10^{-2}

Source: Breinholt, V. and Larsen, J.C., *Chem. Res. Toxicol.,* 11, 622–629, 1998.

of isoflavones significantly reduced daily circulating levels of 17β-estradiol over the entire menstrual cycle by 20% and progesterone by 33% compared with levels during the home diet period in premenopausal women, but had no effect on LH, FSH, or sex hormone-binding globulin (SHBG), which may have implications for breast cancer prevention. Soy diets containing varying amounts of genistein stimulate growth of estrogen-dependent (MCF-7) tumors in a dose-dependent manner.[65] Patisaul et al.[66] reported that soy isoflavone supplements antagonize reproductive behavior and estrogen receptor alpha- and beta-dependent gene expression in rat brain.

As dietary soy-phytoestrogens decrease testosterone levels and prostate weight, low prostatic cancer incidence among Japanese has also been considered to be due to high IF levels.[67] The incidence of occult cancer among Japanese and U.S. whites was almost the same (25%), but the clinical cancer incidence differed.[68] Soy intake appears to suppress the progression to the clinical cancer.[69] Hikosaka et al.[70] found that isoflavone supplements can inhibit PhiP-induced rat prostate carcinogenesis without any adverse effects, possibly by inhibiting progression of prostatic intraepithelial neoplasia (PIN)

to carcinoma, and that down-regulation of ornithine decarboxylase (ODC) and prothymosin alpha (PTA) could be related to the underlying mechanisms. Thus, intake of dietary isoflavones can be promising for prevention of human prostate cancer.

Genetic predisposition may be also related to cancer susceptibility.[71,72] An inhibitory effect of IF on 5alpha-reductase was considered relevant to prevention of prostatic cancer, but a recent study using a 5alpha-reductase inhibitor did not yield different cancer detection rates.[73] Inhibitory effect of IF on NF-kappaB activity may also be effective in suppressing cancer growth.[74,75] It may reduce oxidative stress to form DNA adducts. Estrogenic effects of IF in the low estrogen environment of males may also help inhibit the growth of prostatic cells.[76] Chemoprevention trials of soy isoflavones are now being carried out in Phase 2/3 Cancer Prevention Trials.[77,78]

An inverse correlation of soy product consumption with colon tumor incidence may be related to enhanced colonic synthesis of the antimitotic hormone 1,25-dihydroxyvitamin D3 in the mouse colon.[79]

9.6.1.2 Antioxidant Effect

Free radicals are considered to be among the most potent carcinogens in the body. Suppressed production of peroxides, such as PCOOH and PEOOH, is thought to decrease the mutations leading to cancer. In one animal experiment,[80] treatment of Sprague-Dawley rats that drank soy hypocotyls tea in place of ordinary water showed decreased blood PCOOH and PEOOH levels, as well as decreased oh8dG in the liver and kidneys. oh8dG is also considered an indicator of DNA damage.[81] The excision repair of DNA followed by excretion in urine suggests dose-dependent oxidative damage to DNA; therefore, decreased urinary excretion of oh8dG reflect decreased DNA damage in the body.[82] We also found that human equol excretors showed a stronger antioxidant effect.[83] Soy isoflavone supplementation decreased levels of 5-hydroxymethyl-2-deoxyuridine (5-OhmdU), a marker for oxidative DNA damage in humans.[84] Davis et al.[74] reported that soy isoflavone supplementation inhibited TNF-alpha-induced NF-kappa B activation in blood lymphocytes in healthy men and resulted in a reduction of 5-OhmdU. This may be a possible mechanism of the cancer-preventive effects of soy isoflavones.

9.6.1.3 Cytological Activities

Genistein reversed the transformed phenotypes of v-H-ras NIH3T3 cells through attenuating the content of phosphotyrosine and also significantly inhibited the proliferation of ras-3T3 cells in a dose-dependent manner.[85] Genistein inhibited in a dose-dependent manner the growth of HGC-27 cells derived from human gastric cancer. Flow-cytometric analysis showed that genistein almost completely arrested the cell cycle progression at G2-M. This effect was reversible when genistein was removed from the culture medium.

In contrast, daidzein arrested the cell cycle at G1. These results suggest that the G2-M arrest by genistein is unique.[86] Tyrosine-specific protein kinase activity of the epidermal growth factor (EGF) receptor, pp60v-src, and pp110gag-fes was inhibited *in vitro* by genistein.[87]

Numerous laboratory studies indicate that IFs exhibit many diverse biological activities, both in vivo and in vitro, which may be related to anticancer functions.

9.6.2 Cardiovascular Disease

A significant increase (9.3%, $p < 0.05$) in the mean lag-time of low-density lipoprotein cholesterol oxidation was seen and was positively correlated with serum phytoestrogens ($p < 0.05$). Significant increases were found in mean levels of high-density lipoprotein cholesterol (HDLc) (3.7%, $p < 0.05$). Significant decreases were observed in total cholesterol: HDLc ratios (5.5%, $p < 0.006$). Urinary excretion of total isoflavones was negatively correlated with very-low-density lipoprotein cholesterol, triglycerides, and total cholesterol: HDLc ratios ($p < 0.04$) in normal postmenopausal women.[42] Substitution of soy foods for animal products, regardless of isoflavone concentration, reduces the CAD risk because of both modest reductions in blood lipids and reductions in oxidized LDL, homocysteine, and blood pressure in hyperlipidemic men and women.[88]

9.6.2.1 Hyperlipidemia

The proposed mechanisms for the soy–cholesterol effect include the stimulation of bile acid excretion and an increase in liver LDL cholesterol receptor activities that increased bile acid production and excretion.[89] Which components of soy produce the beneficial effects on plasma lipids remains unclear, but studies have suggested that 60 to 70% of the effect is probably related to soy isoflavones.[43]

9.6.2.2 Hypertension

Dietary soy exerts an antihypertensive effect in ovariectomized (OVX) SHRs. This effect does not involve the nitric oxide system but may be related to an as yet undefined interaction with the autonomic nervous system.[90]

9.6.2.3 Atherosclerosis

Increasing dietary isoflavone intake was associated with decreased aortic stiffness[91] in postmenopausal women, suggesting a protective effect on the risk of atherosclerosis and arterial degeneration through an effect on arterial walls, especially among older women. Yamakoshi et al.[54] reported that antioxidative action of isoflavones and their antioxidative metabolites inhibit the oxidation of LDL, thereby exerting an antiatherosclerotic effect in

cholesterol-fed rabbits. Soy protein isolate, in comparison with casein, promoted a decrease of lipid peroxides, cholesterol, and triglyceride content of atherogenic lipoproteins (beta-VLDL and LDL), which had beneficial effects over atherosclerosis progression in cholesterol-fed rabbits.[92]

9.6.3 Osteoporosis

Women with the highest daily intake of dietary genistein had significantly lower urinary type I collagen cross-linked N-telopeptides (N-Tx) concentrations, a marker of bone resorption, than those of women who reported no daily genistein consumption,[56] suggesting that usual, unsupplemented dietary isoflavone consumption may be protective against bone loss in postmenopausal women through a reduction in bone resorption. Dietary inclusion of whole soy foods resulted in significant increases in serum osteocalcin (10.2%, $p < 0.025$) and significant decreases in mean urinary N-telopeptide excretion (13.9%, $p < 0.02$) in normal postmenopausal women.[42] Daily IF consumption prevented ovariectomy-induced bone loss, both by depressing bone resorption and stimulating osteoblast activity in the ovariectomized rat.[93] Soybean isoflavones dose-dependently reduce bone turnover but do not reverse established osteopenia in adult ovariectomized rats.[94]

9.7 Discussion

Further studies are needed to delineate more precisely the nature of estrogenic and/or antiestrogenic effects of isoflavones in humans. Despite the well-documented effects of estrogens on leptin production, even high levels of isoflavone consumption did not alter leptin concentrations in either premenopausal or postmenopausal participants.[95] Estrogenic and antiestrogenic effects of IF should be studied further with respect to nuclear proteins, and the interaction of IF metabolites with macromolecules in the body should also be clarified.

In contrast to epidemiological studies, Sorensen et al.[96] reported that high amounts of soy isoflavones present in a Western-type high-risk diet did not protect against intestinal tumor development in a relevant animal model such as the Min mice.

Six weeks of isoflavone supplementation (Novasoy, 50 mg daily of isoflavone equivalents) had no measurable effect on bone turnover markers (bone specific alkaline phosphatase [BAP] and pyridinoline creatinine ratio [PYR]) in adolescent boys.[97] Thus, longer-term studies of bone density may be desirable.

We found that a decreased level of FSH during the preovulatory phase was prolonged by IF tablet intake, resulting in a prolongation of the LH

surge. This suggested that isoflavones affect the hypothalamo-pituitary level by a feedback-like mechanism. Changes in androstenedione, testosterone, and thyroxin, caused by IF tablet intake, may also be caused by an altered function of the hypothalamus and hypophysis. Further study of the influences of IF on the hypothalamo-pituitary-gonadal axis are necessary.

Recently, IF supplements have become increasingly popular and are frequently advertised as natural alternatives to estrogen replacement therapy. Setchell et al.[98] reported that the IF content in commercial supplements was highly variable and emphasized the need for standardization of IFs. There are over 20 kinds of isoflavone supplements available on the Japanese food market. Recommended daily intake of soy isoflavones and isoflavone aglycones is 9 to 125 mg and 14 to 121 mg, respectively. The multiple effects of soy phytoestrogens should be clarified before supplements are used extensively for heath promotion and disease prevention.

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10

Wine Antioxidants/Phytoestrogens

Kallithraka Stamatina

CONTENTS

10.1 Wine Phenolic Compounds

Currently, more than 8000 phenolic compounds are known to occur widely in plants. In the past most interest in phenols focused on their antinutritional effects, which arise because some polyphenols (e.g., tannins) bind and precipitate dietary carbohydrates, proteins and digestive enzymes and thereby reduce food digestibility.[1] It is this ability to bind proteins that accounts for their astringency as precipitation of proline-rich proteins and mucopolysaccharides in saliva leads to a loss of lubrication of the oral mucosa and the sensation of astringency that characterizes some foods and beverages.[2]

However, recent interest in plant phenols has focused on their potential benefits to human health, with particular reference to those phenols in fruits

and vegetables, green tea, red wine, and cocoa. Wine phenols occur in greatest abundance in red wines (1000 to 4000 mg/l) compared with white wines (200 to 300 mg/l). Chemically, phenols are cyclic aromatic compounds possessing one or more hydroxyl groups associated directly with the ring structure. Two distinct phenol groups occur in wines, the flavonoids and the nonflavonoids. Flavonoids are characterized as molecules possessing two phenols joined by a pyran (oxygen containing) carbon ring structure. Flavonoids (Figure 10.1) include flavolols (e.g., quercetin, myricetin) with the 3-hydroxy pyran-4-one C ring, flavonols (catechins) lacking the 2,3-double bond and the 4-ketone structure, as well as polymers of the latter, defined as procyanidins, and anthocyanidins.[1] These flavonoids often occur as glycosides, glycosilation rendering the molecule less reactive toward free radicals and more water soluble, thus permitting storage in the vacuole. Common glycosilation positions are the 7-hydroxyl in flavones, the 3- and 7-hydroxyl in flavonols, and the 3- and 5-hydroxyl in anthocyanidins.[3] The sugar most usually involved in the glycoside formation is glucose.

Nonflavonoids are structurally simpler, but their origin in wine is more diverse. In wines, not aged in oak, the primary nonflavonoids are phenolic acids, which are derivatives of hydroxycinnamic and hydroxybenzoic acids.[4] Flavonoids are derived primarily from the skins, seeds, and stems of the grape, and characterize red wines more than white wines. In red wines, they commonly constitute more than 85% of the phenol content. The factors that affect the amount of total phenol found in a wine are skin and seed contact time during fermentation, agitation of juice, intensity of pressing, ethanol concentration, fermentation temperature, and grape variety.[5]

Young red wines have relatively high levels of procyanidins (tannins) and anthocyanidins, giving the wine a purple hue; these decline as the wine matures with the formation of stable new oligomeric and polymeric red-orange pigments with molecular weights of 2000 to 4000 Da. Aggregation of procyanidins, possible by acetaldehyde-mediated cross-linking, results in precipitation of these components and less astringency in the matured wines.[1]

All of these phenolic compounds, including resveratrol, a trihydroxystilbene (Figure 10.2), have been shown to have antioxidant properties *in vitro*.[6] Not surprisingly, red wines exhibit strong antioxidant capacity, which tends to be several orders of magnitude more potent than that of white wine and is directly related to the total phenolic content.[7]

10.2 Health Benefits of Moderate Wine Consumption

The potential health benefits of red wines arose from the proposed "French Paradox," which drew attention to the relatively low incidence of coronary heart disease mortality in French people who habitually eat a diet high in

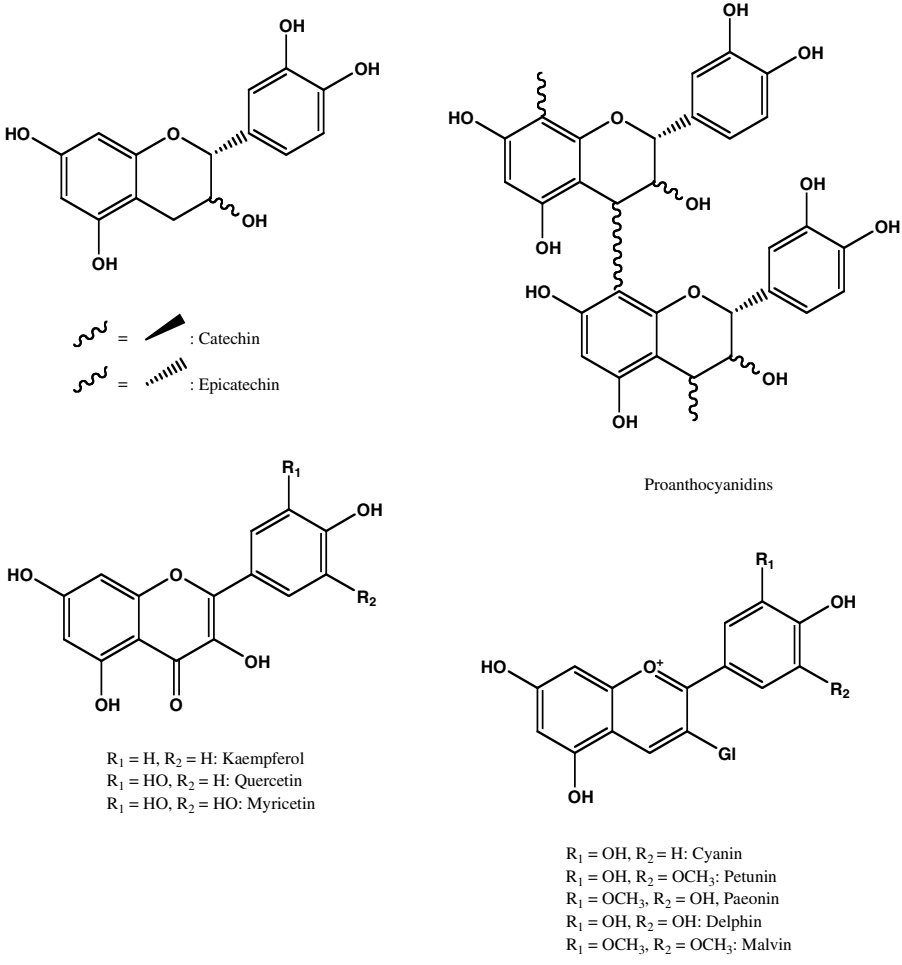

FIGURE 10.1
Principal flavonoid polyphenolic compounds encountered in wines.

saturated fat but who also regularly consume red wine.[8] Possibly some of the cardioprotective effect enjoyed by the French may arise from their moderate consumption of fresh fruits and vegetables and some from the effect alcohol per se has on raising high-density lipoprotein (HDL) levels and lowering platelet aggregation.

The most universal property of wine polyphenols relates to their function as antioxidants, manifested by their ability to trap free radicals and inhibit their enzymatic generation and to block the oxidation of membrane lipids and low-density lipoprotein (LDL), demonstrated *in vitro* as well as *in vivo*, thereby reducing inflammatory and atherogenetic processes triggered by the oxidized components.[9]

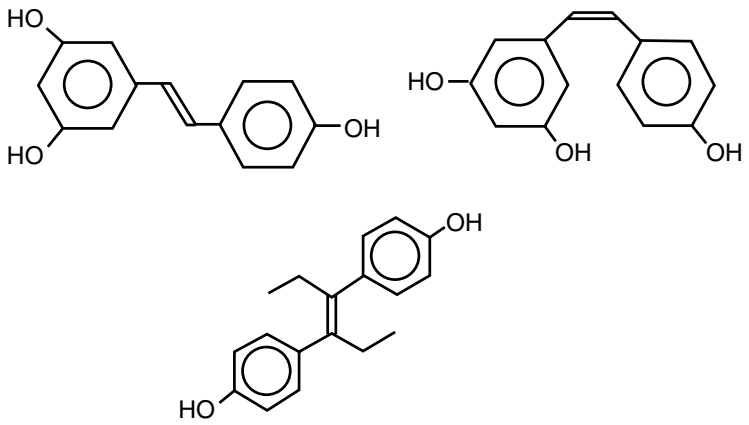

FIGURE 10.2
Cis- and *trans*-resveratrol.

More selectively, wine phenols can, at least *ex vivo*, modulate the synthesis and the release of nitric oxide by vascular endothelium. If the vasorelaxant effect of phenolic compounds also occurs *in vivo* in man, they could conceivably help maintain coronary artery patency and contribute to reinforce antithrombotic defenses strictly related to antiaggregatory activity of nitric oxide.[10]

Wine phenols have been shown to behave as strong inhibitors of platelet activation *in vitro* and in experimental models. In humans, it is possible to speculate that the long-term inhibition of platelet activity by regular and moderate consumption of wine may retard atherogenesis and prevent thrombosis and related arterial ischemic disease.[9]

In addition, a number of studies indicate that wine phenolic compounds may also lower the risk of certain cancer types. *In vivo*, wine phenols have been shown to arrest the cell cycle, exert antiproliferative activities, inhibit the expression of mutated genes and the activity of enzymes that promote carcinogenesis, and promote detoxification of xenobiotics. Many of the observed effects are thought to play a role in preventing breast and/or prostate cancers, but this relationship remains unclear.[11]

In general, there exists a broad scientific view that many of the putative health benefits of phenols may arise from their antioxidant activity and their capacity to protect critical macromolecules such as chromosomal DNA, structural proteins and enzymes, LDL, and membrane lipids from damage arising from exposure to active species of oxygen.[12]

However, wine phenols may act in a variety of ways beyond their antioxidant properties. The complex chemical structure of phenols probably involves them in other physiologic processes, which, it has been suggested, may impact favorably on the risks of cancer and cardiovascular disease, immune function, vasodilation, inflammation, bacterial and viral infection,

prostanoid metabolism, enzyme activation, receptor binding, cellular communication, cell proliferation, and in a hormonal role as phytoestrogens.[12]

10.3 Phytoestrogens

It is well known that some plants have hormonal activity, which sometimes gives rise to significant endocrine effects in grazing or experimental animals. These effects could not be linked to the presence of animal steroid hormones in plants but to naturally occurring non-steroid plant constituents, i.e., phytoestrogens, which elicit estrogen-like effects in one or more target tissues in animals.[13] From among the diversity of plant estrogens, isoflavones and coumestans have been identified as the most common estrogenic compounds in plants and were given the name *phytoestrogens*. Plant lignans, precursors of weakly estrogenic compounds in mammalian systems, have now been included among the phytoestrogens.[14] Furthermore, some other flavonoids have also been found to have estrogenic activity.[15] The majority are diphenolic compounds with structural similarities to natural and synthetic estrogens and antiestrogens.

Phytoestrogens bind to and activate estrogen receptors (ER) with increased affinity to ER β rather than to ER α and possibly exert significant effects on some estrogen-responsive tissues in brain, in the cardiovascular and urogenital system, and in bone.[16] ERs are nuclear steroid receptors that bind estrogens and regulate the transcription of estrogen-responsive genes by either binding directly to particular DNA sequences called estrogen response elements (ERE) or interacting with other DNA-bound transcription factors, for example Sp1.[17] Evidence for receptor-mediated antiestrogenic effects of phytoestrogens is lacking, and it is doubtful whether there exists a true receptor-mediated antiestrogenic effect at the cellular level. Hypothetically, *in vivo,* these compounds have the capacity to act as either partial estrogen agonists or antagonists, depending on many factors such as the expression of estrogen receptor subtypes in target cells and concentration of endogenous estrogens. Clinically, therefore, phytoestrogens may exert tissue specific effects, acting as estrogen mimics to initiate estrogen-like actions, or as antagonists to inhibit estrogen action.[16] At high *in vitro* concentrations, phytoestrogens inhibit both estrogen-dependent and -independent cell lines, which suggests that they exert additional mechanisms independent of the estrogen receptor. Receptor-independent mechanisms of hormonal action include, for instance, the inhibition of key steroid enzymes, the stimulation of sex-hormone binding globulin production in liver, and the interference with release of gonadotropins.[13,18] In addition, numerous other non-hormonal biological effects (e.g., antioxidant capacity, antiproliferative and antiangiogenic effects) have been ascribed to these compounds.

Whether phytoestrogens have any biological activity in humans, either hormonal or non-hormonal, has to be investigated. Much of the available data on the absorption and metabolism of dietary phytoestrogens is of a qualitative nature; it is known that dietary phytoestrogens are metabolized by intestinal bacteria, absorbed, conjugated in liver, circulated in plasma, and excreted in urine.[19]

10.3.1 Estrogenic Activity of Wine

The estrogenic effect of wine has been demonstrated very recently in few studies. These studies involve both *in vitro* and *in vivo* systems and strongly support the hypothesis that certain compounds present in red wine can produce measurable estrogenic effects.[20–23]

Cavalier[20] demonstrated the estrogenic effect of wine using a variety of estrogenic markers, including the pituitary hormones luteinizing hormone (LH) (in OVEX rats and postmenopausal women), follicle stimulating hormone (FSH), and prolactin (in postmenopausal women); uterus weight (in OVEX rats) and the estrogen-responsive liver proteins HDL cholesterol and sex hormone-binding globulin (SHBG) (in postmenopausal women).

Klinge et al.[23] evaluated the estrogenic activity of wine (both red and white) extracts, in a yeast estrogen screen (YES) assay, in which yeast expresses copper-inducible estrogen receptor α (ERα) and an estrogen response element (ERE) driven β-galactosidase reporter. In YES, the white wine extracts showed no estrogenic activity. In contrast, red wine extracts showed estrogenic activity equivalent to that of 0.2 n*M* estradiol. Similarly, the white wine extract showed no transcriptional activity with either ERα or ERβ in transiently transfected CHO-K1 cells. In contrast, red wine extracts stimulated ERE-reporter activity in a concentration-dependent manner that was inhibited by 4-hydroxytamoxifen (4-OHT), indicating that the observed transcriptional activity was ER-mediated. The red wine extracts showed significantly higher ERβ versus ERα agonist activity.

In another study, Damianaki et al.[21] measured the antiproliferative effect of red wine concentrate on the proliferation of hormone-sensitive (MCF7, T47D) and -resistant (MDA-MB-231) breast cancer cell lines. Their results indicated that the wine produced a dose-dependent inhibitory effect on cell proliferation. At concentrations lower or equal to 1/100 of wine, all cell lines were inhibited. At higher concentrations (non-compatible but with moderate wine consumption), however, in two of the cell lines (T47D and MDA-MB-231), this inhibition was inverted, and at high concentrations (1/10) a stimulation of cell proliferation was observed. In contrast to wine, the wine polyphenolic extract showed a more potent inhibitory effect in the three cell lines. It is interesting to note that the two hormone-sensitive cell lines (MCF7 and T47D) were more sensitive to the action of wine polyphenols than the hormone-resistant line MDA-MB-231. This result indicated a possible implication of steroid hormone receptors in the action of polyphenols.

Finally, Eng et al.[22] found that red wines were able to inhibit aromatase at all dosages and in a dose-dependent manner. In contrast, white wines did not have any significant inhibitory effect on aromatase. Aromatase is an enzyme (cytochrome P-450) that converts C19 androgens to aromatic C18 estrogen through three hydroxylation steps. Because estrogen has a major effect on the development of breast cancer and aromatase is the last enzyme responsible for estrogen biosynthesis, overexpression of aromatase in breast cancer cells may significantly influence breast cancer progression and maintenance. It is known that flavonoids, which are found in wine, can compete with endogenous estrogens for binding to the estrogen receptor and thus they can act as antiestrogens or weak estrogens and interfere with the onset of breast cancer. Since estrogen is the product of aromatase, wine can behave as inhibitor of aromatase, suppressing estrogen biosynthesis in cells.

10.3.1.1 Wine Phytoestrogenic Compounds

As discussed earlier, wine, especially red wine, is a rich source of phenolic compounds. From all these phenols, the trihydroxy-stilbene resveratrol (Figure 10.2) and certain flavonoid compounds have been reported to have estrogenic effects due to their structural similarity with steroid hormones, particularly estrogens.

The estrogenic effects of flavonoids were first discovered through determining the cause of infertility in Australian sheep eating red clover. The leaves contained up to 5% by dry mass of the isoflavones biochanin A and formononetin. Of the flavonoids, the soy isoflavone genistein has remained the model "phytoestrogen" due to its structural similarity with estradiol, other steroid hormones, and synthetic inhibitors including tamoxifen.[24]

Since some other flavonoids found in wine also possess this structure, it is possible they too have estrogenic activities. However, with the exception of soy isoflavones, few studies have focused on the estrogenicity of the flavonoids *per se*.[25–27] Furthermore, examination of flavonoid structure and correlation with their biological activity has been limited.[24,28,29]

Rosenberg et al.[29] assessed the estrogenic activity of 72 flavonoids and correlate their structure with the potency of their activity. They found that the flavonoid structural requirements for estrogenic activity are the following:

1. The flavonoid structure (diaryl ring) is important for estrogenic activity.
2. Hydroxyl groups appear to be crucial for activity. If hydroxyl groups are not present in the structure, no estrogenic activity is observed.
3. When hydroxyl groups are methylated, activity is diminished.
4. Flavones and flavanones appear to have greater estrogenic potency than flavans.

5. The position of the hydroxyl groups appears to be important. Hydroxyl groups at positions 6, 7, or 4 confer more potent estrogenic activity than hydroxyls at positions 3, 5, or 2.
6. The most potent estrogenic compounds have between 2 and 4 hydroxyl groups. At least one is localized in the 7 position of ring A, and another one in the 4 position of ring B.
7. A hydroxyl or another group at position 3 or 8 interferes with agonist activity on estrogen receptors.
8. Specificity of flavonoids for estrogen receptors is dependent on the position of B ring.

Resveratrol (3, 4, 5-trihydroxystilbene) occurs naturally in grapes. It is synthesized in response to injury, UV irradiation, and fungal attack. Resveratrol content of grapes and wine is influenced by climate, variety, health of the plant, and growing practices. Furthermore, it is particularly affected by vinification techniques such as the duration of maturation, pressing, carbonic maceration, hyperoxidation of the musts, conservation temperature, and pH variability.[30]

Because of its high concentration in grape skin, significant amounts of resveratrol are present in red wine. Relatively recent studies have associated resveratrol with the cardioprotected effect observed among people with moderate wine consumption. For example, it has been reported that resveratrol had protective effects against oxidation of lipoproteins,[31] an important step in atherogenesis. It also inhibited platelet aggregation and altered eicosanoid synthesis.[10] Moreover, resveratrol has been found to possess chemopreventive activity by inhibiting ribonucleotide reductase[32] and cellular events associated with cell proliferation, tumor initiation, promotion, and progression.[33]

The similarity in structure between resveratrol and the synthetic estrogen diethylstilbestrol (DES; 4, 4-dihydroxy-*trans*-, -diethylstilbene) prompted many researchers to investigate whether resveratrol might exhibit estrogenic activity.[34–39] By using several different assay systems, all of the aforementioned researchers agree that resveratrol can be categorized as a phytoestrogen.

10.3.1.2 Wine Flavonoids with Estrogenic Activity

Many assays have been utilized to determine estrogenic activity of purified flavonoid compounds. These include radiolabeled binding assays, measuring steroid-regulated proteins or reporter proteins, and Southern and Northern blotting techniques.[24]

Among the different classes of wine flavonoids, flavonols have been reported to exhibit the highest estrogenic activity. Most common wine flavonols include quercetin, kaempferol, and myricetin (Figure 10.1).

Zava and Duwe[40] have determined estrogenic and anti-estrogenic activities of several flavonoids, including kaempferol and quercetin, using two

methods in estrogen receptor ER breast cancer cell lines. One measured ^{125}I estradiol binding to ER in the nucleus in the absence or presence of increasing concentration of flavonoid, from 100 p*M* to 10µ*M*. The second technique involved measuring the estrogen-regulated secreted protein pS2 after incubating the cells with flavonoid at the above concentrations for 72 h. In both assays, kaempferol was found to have higher affinity (0.012 that of estradiol) than quercetin (0.001). Blocking of pS2 production by tamoxifen further confirmed that binding to the ER was the mechanism observed.

Rosenberg et al.[24] evaluated estrogenic activity of 72 flavonoids using pS2 concentrations in the supernatant as the endpoint. They also found that quercetin exhibited low estrogenic activity.

Miksicek[15] assessed estrogenicity of flavonoids (at 1 µ*M*) using HeLa cells transfected with wild-type, recombinant estrogen cDNA expressed from the plasmid pER-18, and an estrogen-responsive reporter plasmid system measuring production of CAT (pERE-TK-CAT) where significant activity was also seen with kaempferol.

Le Bail et al.[27] used a stably-transfected MCF-7 cell line with a luciferase reporter gene and ERE to determine estrogen agonist and antagonist activities. Significant agonist activities were seen for both kaempferol and quercetin at concentrations as low as 1 µ*M*. Their results agreed with those of Zava and Duwe,[40] since kaempferol was found to be a stronger estrogen than quercetin.

Collins et al.[41] examined, among other flavonoids, the estrogenic activity of kaempferol by growing the yeast strain hER-ERE in the presence of 17β-estradiol and measuring the β-galactosidase activity. They found that this compound exhibited minimal estrogenic activity as compared to estradiol. The concentration necessary for 50% inhibition or displacement of hER binding of 17β-estradiol was 22 µ*M*.

The estrogenic activity of kaempferol was also confirmed by Breinholt et al.,[26] using a recombinant yeast strain stably transfected with the human ER gene, with upregulation of this gene being the measured endpoint. However, the results concerning estrogenic activity of quercetin are contradictory, since they found that this compound was devoid of estrogenic activity. In contrast, Damianaki et al.[21] confirmed estrogenic activity of quercetin using hormone-sensitive cell lines (MCF7).

Few studies have been performed regarding the estrogenic activity of myricetin. Breinholt and Larsen[26] found the myricetin increased the transcriptional activity 4.6-fold above background level, with EC_{50} value 98.3 µ*M*, and it was found to be lower than that of kaempferol (fivefold, 20.4 µ*M*).

The importance of hydroxyl groups at carbon positions 4, 7 was shown here, as both compounds determined to have estrogenic activity possessed hydroxyl groups at these positions.[29] These hydroxyl groups could be considered to be equivalent to the position 3 and 17-hydroxyl groups of estradiol.[27]

In addition, the presence of the hydroxyl group at C-3 position hinders binding, as it is reflected by the lower estrogenic/binding activity that

flavonols exhibited when they were compared to flavanones in the above studies.

The number of the hydroxyl groups seems to affect estrogenic activity. As mentioned earlier, the most potent estrogenic compounds have between two and four hydroxyl groups. The findings of the above studies confirm this hypothesis since kaempferol was the strongest estrogenic compound (four hydroxyl groups) followed by quercetin (five hydroxyl groups) and myricetin (six hydroxyl groups).

Glycosilation of the 3 hydroxyl group of flavanols seems to be responsible for the absence of estrogenic activity in rutin (3-*O*-glycoside of quercetin) as shown by Breinholt and Larsen,[26] Rodenberg et al.,[29] and Le Bail et al.[27]

Regarding wine flavanols (catechin and epicatechin) (Figure 10.1), there exist very few and contradictory results regarding their estrogenic activity. Breinholt and Larsen[26] found that both catechin and epicatechin were devoid of estrogenic activity. In contrast, Damianaki et al.[21] found that in MCF7 cells catechin displaced estradiol from its receptors, at a picomolar range, while epicatechin interacted at the nanomolar range. In T47D cells, only epicatechin interacted with estrogen receptors at the picomolar range.

Both catechin and epicatechin possess the 4 and 7 hydroxyl groups, which are important for estrogenic activity. However, their discriminating structural feature is the lack of an oxygen group at the 4-position of the heterocyclic C-ring. It is possible that the absence of this group together with the lack of a double bond at the 2-3 position of the same ring, which results in lower electron delocalization, also results in lower or absent estrogenic activity in comparison with flavolols. In addition, it seems that the arrangement of the 3-hydroxyl group may affect estrogenic activity, since epicatechin (cis arrangement at the two asymmetric centers with respect to hydrogen on carbons 2 and 3) was found to be a stronger estrogenic compound than catechin (trans arrangement).

10.3.1.3 Resveratrol

Resveratrol is a red wine polyphenol, and not a flavonoid, which nevertheless possesses a similar structure with phenyl hydroxyl groups. As it can be seen in Figure 10.2, two geometric isomers of resveratrol exist, *E* (*trans*-) and *Z* (*cis*-).

Ghem et al.[34] relatively recently reported that resveratrol at concentrations (3 to 10 μ) inhibited the binding of labeled estradiol [^{125}I] to the estrogen receptor and activated transcription of estrogen-responsive reporter genes transfected into human breast cancer cells. This transcriptional activation was estrogen receptor-dependent, required an estrogen report element in the receptor gene, and was inhibited by specific estrogen antagonists. In some cell types (e.g., MCF7 cells), resveratrol functioned as a superagonist (i.e., produced a greater maximal transcriptional activation than did estradiol itself), whereas in others it produced activation equal to or less than that of estradiol. In addition, they reported that resveratrol increased the expression

of native-regulated genes and stimulated the proliferation of the estrogen-dependent T47D breast cancer cells in the absence of estrogen.

Lu and Serrero[35] examined further the effect of resveratrol either alone or in the presence of estradiol on the growth of MCF7 cells at both cellular and molecular levels. They found that resveratrol inhibits their growth in a dose-dependent manner. They demonstrated that at low concentrations (1 μ and above), resveratrol is a partial ER agonist. However, at higher concentrations (5 μ), in the presence of 17-β--estradiol, resveratrol antagonized the growth-promoting effect of estradiol at both cellular (cell growth) and molecular (gene activation) levels.

More recently, resveratrol was reported to compete with [^{3}H] estradiol for binding to rat uterine estrogen receptor with an affinity 5 orders of magnitude lower than does either the synthetic DES or estradiol with an IC_{50} value of 100 μ.[42] Resveratrol was found to be a weak and partial agonist in hERα-transcription assay and in cos-1 cell assays employing transient transfections of ERα or ERβ associated with two different ER-response elements.

In agreement with the above data, Schmitt et al.[39] also found that resveratrol competed with [^{3}H] estradiol for binding to the human ERα under cell-free conditions and induced proliferation of the estrogen receptor-positive MCF7 cell line in a dose-dependent manner at concentrations ranging from 10 n*M* to 10 μ. However, at higher concentrations resveratrol inhibited MCF7 proliferation.

However, because MCF7 cells and uterus reportedly express ERα as well as ERβ, Bowers et al.[43] determined separately the affinity of resveratrol interaction with ERα and ERβ. They found that resveratrol binds ERβ (IC_{50} = 130 μ) and ERβ (IC_{50} = 58.5 μ) with comparable affinity, but with 7000-fold lower affinity than estradiol. Thus resveratrol differs from other phytoestrogens that bind ERβ with higher affinity than ERα. They also reported that resveratrol exhibited estradiol antagonist activity for ERα with select EREs. In contrast, it did not show any antagonist activity with ERβ. Their data indicate that resveratrol differentially affects the transcriptional activity of ERα and ERβ in an ERE sequence-dependent manner.

Although all the investigations described above agreed on the estrogenic properties of resveratrol, they did not study the two resveratrol isomers (*trans*- and *cis*-) separately. These two geometric isomers are both present in wine and may differ significantly in their estrogenic activity.

Basly et al.[37] were the first who studied the difference between the estrogenic activity of *cis*- and *trans*-resveratrol using MCF7 and MVLN cells. They found that although both isomers increased the *in vitro* growth of MCF7 cell lines (at concentrations 10 to 25 μ) and functioned as superagonists of estradiol, *cis*-resveratrol was less effective than the *trans*-isomer. They explained this by the fact that the 3-D molecular models of both isomers compared to estradiol showed that the *trans*-isomer exhibited a plane 3-D structure fitting with estradiol unlike the *cis*-isomer.

All previous studies were focused primarily on the *in vitro* effects of resveratrol in estrogen-sensitive cell lines, neglecting to document its potential

effects in animal models. Henry and Witt[38] studied the *in vivo* effects of resveratrol on reproductive physiology and behavior in adult female rats. They found that in gonodally intact females, resveratrol consumption reduced body weight, disrupted estrous cyclicity, and induced ovarian hypertrophy. However, in ovariectomized females, resveratrol injections (10 to 1000 μg) did not appear to mimic 17 β-estradiol benzoate-induced behavioral responses and had no lasting effects on subsequent estrogen sensitivity or sociosexual behavior. The present experiments reinforce recent *in vitro* findings[34,43] that resveratrol differs from other phytoestrogens by interacting as a possible agonist/antagonist with specific ER subtypes to produce distinct effects in gonadally intact and ovariectomized females.

As underlined by Soleas et al.,[10] the biological efficacy of dietary resveratrol cannot be judged before testing its effects on humans. Moreover, there are many unanswered questions regarding the consumption of resveratrol as a dietary supplement for humans. Accordingly, acquiring knowledge on absorption, metabolism, and biochemical activity in humans is linked to the selectivity of analytical methods and the identification of resveratrol's mechanism of action.

10.4 Antioxidant Activity

10.4.1 Antioxidant Activity of Flavonoids

One of the principal roles that have been proposed as part of the actions of wine phenolic compounds in humans is that of antioxidants. Many attempts have been made to define the term "antioxidant," but the most useful definition is probably that of Halliwell and Gutteridge[44]: "any substance that when present at low concentration compared to those of an oxidizable substrate, significantly delays or inhibits oxidation of that substrate." In assessing an antioxidant, it is important to concentrate on the mechanism of action, since there are many ways to generate free radicals, many ways in which a free radical can be quenched by an antioxidant, many factors which can influence this reaction, and an almost infinite number of possible targets of the free radicals.[45]

Wine flavonoid compounds may exert their antioxidant activity in several ways. They may directly scavenge some radical species by acting as chain-breaking antioxidants. They may suppress lipid peroxidation by recycling other antioxidants, such as tocopherol, by donating a hydrogen atom to the tocopherol molecule. In addition, some flavonoids can chelate pro-oxidant metal ions, such as iron and copper, thus preventing free radical formation from these pro-oxidants while simultaneously retaining their own free-radical scavenging capability.[46] Furthermore, they are effective inhibitors of

pro-oxidant enzymes, such as various cytochrome P450 isoforms, lipoxygenases, etc., which can generate free radicals.[45]

Most flavonoids exhibit antioxidant activity in both aqueous and lipid-phase assay systems, and their free radical scavenging activity can largely be predicted on the basis of their chemical structure. Optimal activity seems to require a double bond between C-2 and C-3 in combination with both the 4-keto group and the 3-hydroxyl group in the C ring, to allow electron delocalization across the molecule; the *meta* 5,7-dihydroxy arrangements in the A ring; and the *ortho* 3,4-dihydroxy moiety in the B ring. However, alterations in the arrangement of the hydroxyl groups and substitution of the contributing hydroxyl groups by glycosylation decreases the antioxidant activity. For metal chelation, the two points of attachment of transition metal ions to the flavonoid molecule are the *o*-diphenolic groups in the 3, 4-dihydroxy positions in the B ring, and the 4-*keto*, 3-hydroxy or 4-*keto* and 5-hydroxy in the C ring of the flavonoids.[46]

In aqueous phase, the antioxidant potentials of polyphenols can be measured by the assay for the trolox equivalent antioxidant capacity (TEAC), which measures the concentration of trolox (the water-soluble vitamin E analogue) solution with an equivalent antioxidant potential to a standard concentration of the compound under investigation. The TEAC reflects the ability of hydrogen-donating antioxidants to scavenge the $ABTS^{+}$ radical cation. TEAC is defined as the concentration of trolox solution with equivalent antioxidant potential to 1 mM concentration of the investigated compound.

Table 10.1 presents the TEAC values obtained for selected wine flavonoid compounds. Quercetin (Figure 10.1) has an identical number of hydroxyl

TABLE 10.1

Trolox Equivalent Antioxidant Activities (TEAC) of Wine Polyphenols[a]

Compound	TEAC (m*M*)
Quercetin	4.72 ± 0.100
Myricetin	3.72 ± 0.300
Epicatechin	2.50 ± 0.002
Catechin	2.40 ± 0.005
Rutin	2.42 ± 0.006
Kaempferol	1.34 ± 0.008
Resveratrol	2.00 ± 0.060

[a] Data taken from Rice-Evans, C.A., Miller, N.J., and Pagana, G., *Free Rad. Biol. Med.*, 20, 933–956, 1996.

groups in the same position as catechin and epicatechin (Figure 10.1), but also contains the 2,3-double bond in the C ring and the 4-*keto* group. This structural advantage confers an enhancement of the TEAC value, approximately twice, due to the altered bonding in the C ring, allowing delocalization between A and B rings and thus stabilization of the aryloxyl radical after hydrogen donation. Kaempferol, which does not have the *ortho*-dihydroxy substitution, and myricetin, which has three hydroxyl groups in B ring, have lower antioxidant activities than quercetin. In addition, blocking the 3-hydroxyl group in the C ring of quercetin as glycoside (while retaining the 3, 4-dihydroxy structure in the B ring) as in rutin, decreases the antioxidant activity.[46]

Many studies have been undertaken in lipophilic systems to establish the structural criteria for activity of polyhydroxy flavonoids in enhancing the stability of fatty acid dispersions, lipids, oils, LDL, and lard toward oxidation.[47–50] Because oxidation of LDL is implicated in the pathogenesis of atherosclerosis, the enhancement of the resistance of LDL oxidation is one of the most common models used by many researchers for investigating the efficacy of flavonoids as antioxidants against radicals generated in the lipophilic phase. The LDL particle contains -tocopherol in its outer monolayer and carotenoids in the inner core. Free radical-mediated peroxidation of the polyunsaturated fatty acids leads to the formation of lipid hydroperoxides. The presence of chain-breaking phenolic antioxidants provides a means of intercepting the peroxidation process by reducing the alkoxyl or peroxyl radicals to alkoxides or hydroperoxides.[46]

In contrast with aqueous phase interactions, the 2,3–double bond is less important for optimum antioxidant activity. A free 3-OH group or 3- and 5-groups present simultaneously are also considered to be important in the lipophilic phase. However, it is not clear whether these differential structural features and their influence as antioxidants can be totally ascribed to a hydrogen-donating antioxidant effect or whether the partition coefficients of the compounds into the lipophilic region and their accessibility to the auto-oxidizing lipids has confounded these effects.[46] Catechin, epicatechin, and quercetin have been shown to conserve endogenous -tocopherol in LDL, and quercetin was the most effective of the compounds studied.[51] In addition, quercetin was more effective than catechin as an antioxidant in protecting the LDL from oxidation in copper-mediated peroxidation systems. Furthermore, catechin, epicatechin, and quercetin have been shown to have powerful antioxidant capacities to approximately the same extents in phospholipid bilayers exposed to aqueous oxygen radicals,[49] although the electron-donating ability of catechin is lower than that of quercetin.

10.4.2 Antioxidant Activity of Resveratrol

Filip et al.[52] studied the antioxidant effect of resveratrol in a mixture of sunflower-rapeseed oil, or in margarine emulsion. They found that

resveratrol behaved as a slight antioxidant against oxidation of triacylglycerols and that it did not achieve the effectiveness of BHT in sunflower and rapeseed oils or of ascorbic acid in the margarine emulsion.

Frankel et al.[31] were the first to demonstrate that *trans*-resveratrol added to human LDL reduced the copper-catalyzed oxidation. At the concentration of 10 μmol/l, the LDL peroxidation was more inhibited by resveratrol than by a polyphenolic extract of red wine. However, as shown by the TEAC values in Table 10.1, resveratrol was a less potent antioxidant than either quercetin or epicatechin.

By measuring the formation of degradative products from polyunsaturated fatty acids porcine LDL oxidation, Belguendouz et al.[53] observed that *trans*-resveratrol mainly acted by chelating copper whereas flavonoids were better free radical scavengers. At the concentration of 1.5 μmol/l, they observed that the lag time was twice as long with resveratrol as with other flavonoids (quercetin, catechin, epicatechin). This high capacity of resveratrol to chelate copper is potentially useful *in vivo,* since LDL are known to have high ability to bind copper. The same group of researchers also found that the chelating capacity of the *cis*-isomer was about half that of the *trans*-isomer, whereas both isomers were equally efficient in scavenging free radicals, suggesting that the spatial position of the hydroxyl groups is of prime importance for the chelation process. However, Basly et al.[37] obtained contradictory results regarding the free radical scavenging properties of the two isomers. By using the stable DPPH (1,1 diphenyl-2-picrylhydrazyl) free radical, they found that the *cis*-isomer was more efficient than the *trans*- one. In contrast, Teguo et al.,[54] by using the same free radical, found that *trans*-resveratrol showed a better free radical scavenging activity than *cis*-resveratrol.

Resveratrol also inhibited the peroxidation of membrane lipids. In rat liver microsomes, Blond et al.[55] showed that in non-enzymatic or in NADPH-dependent peroxidation, the concentration required to produce 50% inhibition was about three times lower with resveratrol than with quercetin. Furthermore, resveratrol was found to prevent metal-induced lipid peroxidation in microsomes and LDL.[56] The authors compared the response of these compounds to that of other polyphenols and found that the presence of the 4-hydroxyl in ring B and the *meta*-hydroxyl structure in ring A were essential for the antioxidant activity of resveratrol.

10.4.3 Antioxidant Activity of Wine

It is well known that the complexity of the polyphenolic composition in red wines constitutes an obstacle regarding the methodology used to assess the antioxidant activity of wines and the interpretation of the results. First, wines contain numerous phenolic compounds in variable amounts, and therefore it is impossible to predict the contribution of each compound to the overall antioxidant activity of a wine. Second, wines are dynamic systems that

change continuously in response to exposure to air, light, etc. Therefore, the oxidation of the polyphenolic constituents, which is related to the antioxidant activity, might change appreciably during aging and storage conditions, and consequently antioxidant ability of red wines depends on the redox equilibrium at the moment of their consumption. This equilibrium is the result of interactions among various types of polyphenols, e.g., copigmentation; in many occasions the structure of polyphenolic components is largely uncharacterized, and their antioxidant functions cannot be revealed in a single antioxidant test.

Early attempts to measure the antioxidant activity of wine were mainly focused on lipid protection. Several methods based on the determination of the secondary products of lipid oxidation such as the thiobarbituric acid method[57] and the tiociannate method[58] have been described in the literature. A different approach is to measure the induction period before the rapid oxidation phase, which occurs in a lipid matrix exposed to conditions of accelerate oxidation.[31] Cao et al.[59] set up a method able to measure directly the oxygen radical absorbance capacity (ORAC) to assess the antioxidant capacity of fruits and vegetables.

A different approach based on the chromatic properties of stable radical cation was applied by Brand-Williams et al.,[60] who used DPPH radical to measure antioxidant ability of wine samples. Minussi et al.,[61] using the colored solution of the ABTS-derived radical cation, were able to measure total antioxidant activity of several wine samples. A similar approach was followed by Fogliano et al.,[62] who used the colored *N,N*-dimethyl-*p*-phenylenediamine (DMPD) radical cation to assess the antioxidant activity of several wines. Recently, the chemiluminescence assay, which is based on the generation of hydroxyl free radicals (OH·), which in turn oxidize luminol that results in light emission, has been applied to evaluate the antioxidant activity of wines.[7]

By using all the aforementioned methods, researchers agree that wines exhibit antioxidant activity and that red wine possesses a stronger antioxidant potential than white wine. However, the results obtained are difficult to compare, since they are expressed in different units or values (e.g., TEAC, quercetin equivalents [QE], gallic acid equivalents [GAE], ascorbic acid equivalents [AAE], amount necessary to decrease by 50% the initial free radical concentration [EC_{50}], % inhibition of LDL oxidation).

In addition, most researchers agree that there exists a correlation between the total antioxidant activity of the wines and their total phenolic content[7,61–68] independent of the method used to measure wine antioxidant activity. This was further supported by Rice-Evans et al.,[47] who found that the total antioxidant activities of a range of red wines varied from 12 to 14 m*M* (TEAC values) for a selected Californian and Spanish wine to about 16 m*M* for an Australian and 23 for a French and an Italian wine. Even though the antioxidant activities of the wines varied over a factor of 2, the ratios of activities to the total phenol content were approximately the same (about a factor of 10), suggesting that there exists a direct relationship between the two.

However, there exist contradictory results regarding the correlation of the principal phenolic groups and individual polyphenols with the *in vitro* antioxidant properties of wines. Misussi et al.,[61] using ABTS radical cations, found strong correlations between epicatechin ($r^2 = 0.9583$) and catechin ($r^2 = 0.9583$) concentrations and antioxidant activities of the wines tested. In contrast, Makris et al.[68] and Arnous et al.[67] did not obtain any significant correlations between these compounds and total wine antioxidant activity measured by the DPPH free radical cation. Furthermore, Frankel et al.[63] found the following correlations between individual phenols and wine antioxidant activity, measured with the LDL oxidation assay: catechin ($r^2 = 0.5776$), myricetin ($r^2 = 0.49$), quercetin ($r^2 = 0.4624$), epicatechin ($r^2 = 0.2025$). However, the differences observed might be due to the LDL oxidation assay, where the system is typically heterogeneous and physical properties, such as lipophilicity, solubility, and partition between the aqueous and lipid phases of LDL, can become important in determining the antioxidant activity.

In addition, significant correlations were obtained between major phenolic classes and total wine antioxidant activity. Simonetti et al.[64] found that wine flavonol content was significantly correlated with antioxidant activity ($r^2 = 0.8593$), in agreement with Arnous et al.[67] ($r^2 = 0.8419$). However, Meyer et al.[65] found that red wine flavonols were significantly correlated with antioxidant activity ($r^2 = 0.54$) and not flavanols.

All the preceding studies indicate that the individual compounds are probably weakly associated with the antioxidant parameters, suggesting that the expression of the antioxidant activity in red wines is rather a consequence of synergism between various phenolics and it is not simply attributed to specific constituents. This was further supported by Rice-Evans et al.,[46] who based their findings on Frankel et al.'s[63] reported figures and determined the mean total antioxidant activity from the calculated antioxidant activities of the individual constituents and, on the basis of the composition of the individual constituents, the contribution to the total antioxidant activity (Table 10.2). The calculated antioxidant activity was only 25% of the measured value. The remaining antioxidant activity was presumably due to unidentified polyphenols and phenolic acids and to synergism between them.

10.5 Wine Antioxidant/Phytoestrogenic Content

Wine is a complex mixture of organic as well as inorganic compounds, the composition of which is influenced by many and various factors. These begin in the vineyard and end in the fermentation cellar. They are related to the oenological environment, including ground climate, geographical origin and grape variety, and oenological practice. In general, red wines have six to seven times more polyphenols than do white ones.[69]

TABLE 10.2

Contribution of Phenolic Constituents to the Total Antioxidant Activity (TAA) of Red Wine

Compound	TEAC[a] (m*M*)	Composition[b] (m*M*)	Contribution to TAA[a]
Quercetin	4.72 ± 0.100	0.02	0.09
Myricetin	3.72 ± 0.300	0.03	0.09
Epicatechin	2.50 ± 0.002	0.28	0.7
Catechin	2.40 ± 0.005	0.66	1.6
Rutin	2.42 ± 0.006	0.01	0.02
Resveratrol	2.00 ± 0.060	0.006	0.01
Total contribution to TAA			4.2
Mean TEAC			16.7

[a] Data taken from Rice-Evans, C.A., Miller, N.J., and Pagana, G., *Free Rad. Biol. Med.,* 20, 933–956, 1996.

[b] Data taken from Frankel, E.N., Waterhouse, A.L., and Teissedre, P.L., *J. Agric. Food Chem.,* 43, 890–894, 1995.

Table 10.3 contains information regarding the concentration of the phytoestrogenic compounds in red wines originating from different countries and varieties. As it was expected, different wines have variable concentration of polyphenols. Taking into consideration the average concentration of the phenolic compounds with estrogenic activity (catechin = 349.9, epicatechin = 152.3, quercetin = 48.9, myricetin = 15.4, resveratrol = 13.6 μ*M*) and the volume of the interstitial fluid (about 40 l),[21] after the ingestion of 250 ml of wine, the circulating concentrations of the estrogenic compounds will be about 2190, 950, 305.6, 96.3, and 85 n*M* for catechin, epicatechin, quercetin, myricetin, and resveratrol, respectively, considering that no metabolism or excretion of the substance will occur (this generalization was based on data from various laboratories and wines in order to obtain rough average values just for calculation reasons). On the other hand, the existence in the serum of polyphenol oxidase, at concentration between 23 and 61 mg/dl, depending on the age and sex (21), makes more than probable the partial metabolism of polyphenols. Therefore, a possible effect of these substances, after ingestion of a moderate quantity of wine, must be studied at low concentrations, at about nanomolar or even picomolar range.

As was seen earlier in this chapter, even at these low concentrations, wine phenolic compounds exhibited estrogenic effects, indicating that moderate consumption of red wine might have a health protective effect. On the other hand, their low circulating concentrations make it difficult to suggest that these substances play a role in the extracellular fluid as antioxidants. However, it can be reasonably assumed that most polyphenols play a major role in the digestive tract by limiting the formation of reactive oxygen species and by scavenging them due to their higher reduction potential. Consequently, vitamins C, E, and -carotene are spared from the attack of reactive oxygen species and, due to their good bioavailability, are readily absorbed and distributed through the tissues, thus increasing the whole

TABLE 10.3

Comparison of the Phenolic Contents (mg/l) of Red Wines from Different Countries

Country	Catechin	Epicatechin	Quercetin	Myricetin	Kaempferol	Resveratrol	Reference
Greece	60.4	40.7	25.7	6.2		1.10	76
							77
France	67.3	31.3	4.1	7.5		8.3	78
							79
							80
Spain (Canary Islands)	76	16.3	23.7	1.7		2.3	81
Italy	37	23	16.0	8.0	0.6	2.8	78
							79
							64
							61
U.S. (California)	191	82	7.7	8.5		1.5	63
Canada	240	82	18.5				78
Australia	39.8	34.1	8.2	2.7		5.8	78
							82
							80
Average	101.6	44.2	14.8	4.9	0.6	3.1	81

body antioxidant status.[64] Indeed Cao et al.[70] confirmed that the overall antioxidant capacity in serum of elderly women was significantly increased following the consumption of red wine.

Furthermore, the health benefits associated with the consumption of wine flavonoids and resveratrol may be attributed to a possible synergism between their antioxidant and phytoestrogenic actions. As it was seen earlier in this chapter, the antioxidants and phytoestrogens share some common structural futures (e.g., the presence of hydroxyl groups in the molecules, the presence of 4, 7 hydroxyl groups, extended electron delocalization in C ring). Thus, it is possible that their antioxidant action may enhance their phytoestrogenic effect.

Little is known about the bioavailability, absorption, and metabolism of the polyphenols in humans, and it is likely that different groups of flavonoids have different pharmacokinetic properties. Generally, absorption and metabolism of polyphenols is influenced by their solubility and chemical structure. Most monomeric and small oligomeric flavonoids are soluble in water and to some extent in lipids. Large polymeric flavonoids are less soluble. Also important are the degree of glycosylation and conjugation with other polyphenols. On this basis, wine catechins, which are not glycosylated and readily water soluble, should be directly absorbed from small intestine.[12] In fact, there exists evidence that catechin is absorbed by the human gut and peak levels in human plasma occur within 2 h of administration.[71] Regarding quercetin, it has been proposed that plasma levels might reach values up to 1 μ,[72] although the findings are conflicting. Other studies have reported that quercetin after oral administration in humans was not detected in plasma, providing indirect evidence for the presence of quercetin conjugates in humans.[73] Soleas et al.[74] presented evidence that in man *trans*-resveratrol was absorbed approximately 20-fold more effectively than catechin. This absorption was dependent on enzymatic sulfation and glucuronidation within the intestinal mucosoa prior to entry into the portal blood.[75] Only a small percentage of the circulating polyphenols and those excreted in human urine was in the free form.[74] It is possible, but not yet proven, that this superior bioavailability would raise the blood concentrations of *trans*-resveratrol after wine consumption to the point where it could offer higher protection to the human body than that reached by the other polyphenols.

Research on the bioavailability of phenolic compounds is very limited and needs to be expanded. In addition, in the future, attention must be given to the identification and quantification of their metabolites in body fluids and tissues. For this purpose, sensitive and selective analytical methods must be developed. With these tools, additional human studies will have to be carried out to understand fully the pharmacodynamics of flavonoids and resveratrol.

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Glossary of Terms and Abbreviations in Phytoestrogen Research

Aberrant crypt foci — Precancerous lesions of the colon.
Acceptable Daily Intake (ADI) — Estimate of the amount of a substance in food or drink, expressed on a bodyweight basis (e.g., mg/kg bodyweight), that can be ingested over a lifetime by humans without acceptable risk.
Accessory proteins — Proteins involved in the correct assembly of other proteins.
Acetyl — The chemical group (-$COCH_3$).
Acetylation — Addition of an acetyl group.
Acetyltransferase — An enzyme that catalyzes addition of acetyl groups.
Activator protein 1 — A transcription factor involved in signaling, growth control, and apoptosis.
Acute — Short-term, in relation to exposure or effect.
Acute toxicity — Effects that occur over a short period of time (up to 14 days) immediately following exposure.
Adaptor — A short DNA sequence between gene segments.
Adenocarcinoma — A cancerous tumor of the walls of organs.
Adenoma — A benign tumor of the organ wall.
Adenomatous polyps — Small precancerous growths in the colon.
Adenosine triphosphate — A phosphorylated nucleoside used by cells to store energy, which may be released during metabolic reactions.
Adipose tissue — Fatty tissue.
Adverse effect — Change in morphology, physiology, biochemistry, growth, development, or lifespan of an organism that results in impairment of functional capacity or impairment of capacity to compensate for additional stress or increase in susceptibility to the harmful effects of other environmental influences.
AF-1 — A measure of the strength of binding of one molecule to another, e.g., of a ligand to a receptor or a substrate to an enzyme.
Affinity — A measure of the strength of binding of one molecule to another, e.g., of a ligand to a receptor or a substrate to an enzyme.
Aglucone — A compound that may be conjugated to form a glycoside.
Agonist — A compound that binds to a receptor to initiate a cellular response.

Alkaline phosphatase — An enzyme present in liver, bone, and other tissues that catalyzes the removal of phosphate groups from organic compounds.
Amniotic fluid — The fluid that surrounds a developing fetus.
Amygdala — A small region in the brain near the hippocampus.
Anaerobic reaction — A reaction occurring in the absence of oxygen.
Androgen — A male sex hormone.
Androstenedione — A male sex hormone.
Angiogenesis — The formation of new blood vessels.
Anhydrosecoisolariciresinol — A lignan found in plants.
Anogenital distance — Distance between the anus and the external genitalia, used as a measure of rodent development.
Antagonist — A compound that negatively affects the activity of an agonist.
Antiatherogenic compound — A compound that counteracts degenerative changes in arterial walls.
Antibody — A protein produced by the immune system that binds to a specific chemical structure.
Antitumor properties — Counteracting tumor formation.
Antral follicle — An ovarian follicle.
APC^{Min} — A mutation (Min) within the mouse APC gene.
API — *See* Activator protein 1.
Apigenin — A flavonoid.
Apolipoprotein — A protein involved in lipid transport.
Apoptosis — The process of programmed cell death associated with normal cell turnover in animals. Inappropriate apoptosis may be a toxic response under certain circumstances.
Apoptotic index — The number of apoptotic cells per 1000 tumor cells.
Area under the curve — A mathematical measurement that describes the plasma concentration of a compound over time.
Aromatase — An enzyme that converts androgens to estrogens.
Aromatase knockout animals — Animals genetically engineered not to possess active aromatase.
Aryl hydrocarbon — An aromatic organic compound.
Arylsulfatase — An enzyme that catalyzes the removal of sulfate from an aryl compound.
Atherogenic diet — A diet that causes degradation of arterial walls.
Atherosclerosis — A disease of the arteries in which fat accumulates to obstruct the flow of blood.
Athymic — Having no thymus gland.
Athyreosis — A condition caused by absence of the thyroid gland.
ATP — *See* Adenosine triphosphate.
Atresia — Degeneration of tissue.
Atrophy — A decrease in size or wasting away of a body part or tissue.
AUC — *See* Area under the curve.
Autoimmune disease — A disease caused when the immune system attacks the body's own tissues.

Autonomic nervous system — Part of the nervous system that controls body functions.
Autophosphorylation — The addition of a phosphate group to an enzyme by that enzyme.
Auxiliary protein — A protein that helps another perform its function.
Base pair — Two complementary nucleotide bases joined and positioned opposite one another in the DNA double helix.
Bax — Bcl-2 associated protein.
Bcl-2 — 8-cell lymphoma 2. A member of the Bcl-2 family of proteins, which inhibit apoptosis.
Bean curd — *See* Tofu.
Bean sprouts — The young shoots of mung or soy beans.
Benign — Not malignant or life-threatening.
Benzo[a]pyrene — A chemical carcinogen.
Bifidobacteria — Bacteria found in the human intestine.
Bilateral breast cancer — Cancer affecting both breasts.
Bile acids — Acids produced in the liver and secreted into the small intestine to aid in the digestion of fats.
Biliary excretion — Elimination of a chemical from the body in the bile.
Binding affinity — *See* Affinity.
Binding cavity — The site in a receptor or enzyme where the ligand or substrate binds.
Bioassay — A biological assay, using a living organism or tissue.
Bioavailability — The proportion of a substance, which reaches the systemic circulation, unchanged after a particular route of administration.
Biochanin A — An isoflavone phytoestrogen.
Biomarker — Observable change (not necessarily pathological) in an organism, related to a specific exposure or effect.
Biostatistics — Statistical analysis that is used to aid the interpretation of biological data.
Biosynthesis — The formation of a biological compound.
Biotransformation — A series of chemical alterations of a compound that occur within the body.
Biphasic — Having two phases.
Blood brain barrier — A barrier that limits the passage of substances between the blood and brain tissue.
BMC — *See* Bone mineral content.
BMD — *See* Bone mineral density.
Body mass index — A measure of body fat that is the ratio of the weight (kg) to the square of its height (m).
Bone mineral content — A measurement of bone mass (g).
Bone mineral density — An indication of bone strength (g/cm2).
Brain-derived neurotropic factor (BDNF) — A chemical that promotes survival and normal functioning of neurones.
Brassicaceae — A family of plants including cabbage, cauliflower, broccoli, and brussels sprouts.

Calbindin — An intracellular protein that transports calcium across intestinal epithelial cells.
Calretinin — A calcium-binding protein.
cAMP — *See* Cyclic AMP.
Cancer — Synonym for a malignant neoplasm, i.e., a tumor that grows progressively, invades local tissues, and spreads to distant sites.
Carcinogenesis — The origin, causation, and development of tumors. The term applies to benign as well as malignant neoplasia.
Carcinoma — A malignant tumor arising from epithelial cells lining, for example, the alimentary, respiratory, and urogenital tracts and from epidermis; also from solid viscera such as liver, pancreas, kidneys, and some endocrine glands. *See also* Tumor.
Cardioprotection — Protection of the cardiovascular system from disease.
Cardiovascular system — The circulatory system of the heart and blood vessels.
Case-control study (*Also* Case comparison study, Case referent study) — A study that starts with the identification of persons with the disease of interest and a suitable control group of persons without the disease.
Castration — Removal or destruction of the testes or ovaries by radiation, surgery, or drugs.
Cathepsin D — An intracellular protease.
Causality — The relationship between cause and effect.
Cdk2 — *See* Cyclin-dependent kinase 2.
Cecum — A section of the gastrointestinal system.
Cell culture — A technique for growing cells under laboratory conditions.
Cell cycle — The sequence of events between cell divisions. The cycle is conventionally divided into GO, GI (G standing for gap), S (synthesis phase during which the DNA is replicated), G2, and M (mitosis).
Cell differentiation — The process of change from one cell type to another.
Cell proliferation — The process of cellular multiplication or growth.
Cerebellum — A part of the brain involved in muscle coordination.
Cerebral cortex — A region of the brain controlling movement and behavior.
Cerebral ventricle — Ventricles located in the brain.
Cervicaladenosis — A disease of the cervix.
c-fos — Proteins involved in intracellular signaling.
CHD — *See* Coronary heart disease.
Chemoprevention — Relating to a compound or activity that will protect an organism from disease.
Cholesterol — A steroid involved in the formation of cell membranes and transport of fat around the body.
Chromatid — One of the usually paired and parallel strands on a chromosome.
Chronic effect — An effect that develops slowly and has a long-lasting course (often but not always irreversible).

Chronic exposure — Continued exposures occurring over an extended period of time or a significant fraction of the lifetime of a human or test animal.

Chrysin — A dietary flavonoid.

CI — *See* Confidence interval.

C-jun — Proteins involved in intracellular signaling.

Clastogen — An agent that produces chromosome breaks and other structural aberrations such as translocation (qv). Clastogens may be viruses or physical agents as well as chemicals. Clastogenic events play an important part in the development of some tumors.

Clearance — Volume of blood or plasma, or mass of an organ, effectively cleared of a substance by elimination (metabolism and excretion) in a given time interval.

C_{max} — Concentration of compound giving maximum response in a biological assay.

CMO — Chief medical officer.

CNS — Central nervous system.

Cognitive function — Relating to mental functions such as memory, attention, and communication.

Cohort — A defined population that continues to exist through time.

Cohort study (*Also* Follow-up, Longitudinal, Prospective study) — The method of epidemiological study in which subsets of a defined population can be identified who may be exposed to a factor or factors hypothesized to influence the probability of occurrence of a given disease. An essential feature of the method is observation of the population for a sufficient number of person-years to generate reliable incidence or mortality rates in the population subsets. This generally implies study of a large population and/or study for a prolonged period of time.

Collagen — An insoluble fibrous protein found in animals. It is the chief constituent of connective tissue and bones.

Confidence interval — Statistical term denoting the range of values within which there is a specified probability (e.g., 95%) of the true result falling.

Confounding factors — A variable related to one or more of the variables defined in the study. Confounding factors may mask an actual association or falsely demonstrate an apparent association.

Congenital — Referring to a condition that is present at, and usually before, birth regardless of causation.

Congenital hypothyroidism — Hypothyroidism that is present from birth.

Conjugated — Joined to another chemical.

Corepressor — Molecule that represses a biological response.

Coronal section — A slice or section of the brain made by cutting from side to side.

Coronary heart disease — Disease in which fatty deposits accumulate along the innermost layers of the coronary arteries. The deposits thicken, which causes narrowing of the arteries and blocks the flow of blood to the heart.

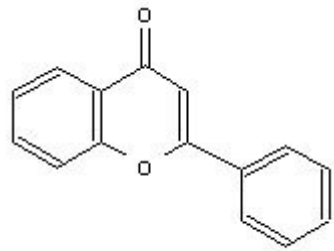
Corpus luteum — A mature ovum that has been discharged from the ovary.

Cortical bone — Dense bone structure composing the outer membrane of the bone.
Cotransfection — The introduction of two different DNA molecules into eukaryotic cells.
Coumestan — One type of phytoestrogen.
Coumestrol — A type of phytoestrogen from the coumestan class.
Craniospinal ganglia — A collection of neurons associated with the spinal cord and cranial nerves.
Creatinine — A waste product of protein metabolism that can be measured in the urine.
Crossover study/trial — A study comparing the effects of two or more treatments in which the subjects, upon completion of one treatment, are switched to another.
Cross-sectional study — A population study based on characteristics of a population at one point in time.
Cross-talk — Communication between two separate signal transduction pathways in the same cell.
Cryptorchidism — A congenital abnormality of the male reproductive system.
c-Src — A member of the non-receptor tyrosine kinase proteins.
C-terminal domain — A region of the estrogen receptor that contributes to its transactivation capacity.
Cyclic AMP — A cyclic mononucleotide of adenosine responsible for the intracellular mediation of hormonal effects on various cellular processes.
Cyclin A — A protein involved in regulating the cell cycle.
Cyclin D_1 — A protein involved in cell division.
Cyclin-dependent kinase 2 — Involved in regulating the cell cycle.
Cyclins — A group of proteins involved in control of the cell cycle.
Cyclooxygenase 2 (COX-2) — An enzyme involved in prostaglandin synthesis.
Cyst — An abnormal development in a body cavity or structure.
Cytochrome — Any of a group of electron transporting proteins containing a heme iron existing in an oxidized or reduced state.
Cytochrome P_{450} (CYP) — An extensive family of proteins involved in enzymatic oxidation of a wide range of endogenous and xenobiotic substances and their conversion to forms that may be more readily excreted. In some cases, the metabolites produced may be reactive and may have increased toxicity. In other cases, the substances may be natural precursors of hormones (e.g., steroids).
Cytometry — A method of counting cells.
Cytoplasm — All the living part of the cell inside the membrane, excluding the nucleus.
Cytosol — Component of the cytoplasm excluding membrane-bound organelles.
Cytotoxic — Toxic to cells.
Daidzein — An isoflavone phytoestrogen.

Decarboxylation — Loss of a carbon dioxide (CO_2).
Decidualization — Process by which cells or tissues go through a period of change to another form.
Dehydroepiandrosterone (DHEA) — A male sex hormone.
Deiodinase — An enzyme that catalyzes the removal of iodine.
Dementia — A condition of deteriorated mentality characterized by a marked decline from the individual's former intellectual level.
Demethylation — Loss of a methyl group ($-CH_3$).
Deoxyribonucleic acid (DNA) — The carrier of genetic information for nearly all living organisms. DNA is composed of two interwound (double helical) chains of linked nucleotides.
Dephosphorylation — Removal of phosphate groups.
DHEA sulfate — The sulfate conjugate of DHEA.
Diastolic blood pressure — The pressure exerted on the walls of the arteries when the heart is in its relaxation phase (diastole).
Diethylstilboestrol — A synthetic estrogen.
Differentiation — Modification of different cells or tissues of the body to undertake particular functions.
Dimer — A polymer consisting of two parts, e.g., a complex of two proteins.
Diphenolic — A compound containing two phenol groups.
Distal femoral metaphysis — The section of the femur closest to the knee that is actively growing.
DNA binding domain — A section of a receptor that binds to DNA.
Dorsolateral prostate — An area of the prostate.
Dose — Total amount of a substance administered to, taken, or absorbed by an organism.
Double knockout animals — Animals that have been genetically engineered so that they do not express either estrogen receptor.
Double-blind — An experimental procedure in which neither the subjects nor the experimenters know the makeup of the test and control groups during the experiment.
Down-regulation — A decrease in number or activity.
E. coli — *See Escherichia coli*.
EC_{min} — The lowest effective concentration of a compound that produces a measurable response.
Edema — A swelling of tissue through an increase in its fluid volume.
Efficacy — The ability of a substance to elicit a response following binding to a receptor.
EGF receptor — Epidermal growth factor receptor.
Electrophoresis — A method to separate charged molecules.
Embryo — The developing human individual from the time of implantation to the end of the eighth week after conception.
Endemic — Widespread in a locality or region.
Endocrine disruptor (*Also* Endocrine modulator) — A chemical, which can be either naturally occurring or man made, that causes alterations in the hormonal activity of an organism.

Endocrine system — A system of glands that secrete a variety of hormones.
Endogenous — Within the body.
Endometriosis — The presence and growth of functioning endometrial tissue in places other than the uterus.
Endometrium — Membrane lining the uterus.
Enterodiol — A lignan metabolite.
Enterocolitis — Inflammation of the small and large intestines.
Enterohepatic circulation — Recycling of a substance by transport through bile via the gut and liver.
Enterolactone — A lignan metabolite.
Enteropathy — A disease of the gastrointestinal tract.
Eosinophilia — An increased number of cell type (eosinophils) in the circulation.
Eosinophils — A type of white blood cell.
Epidemiology — Study of the distribution, and in some instances the causal factors, of disease in communities and populations.
Epidermal growth factor — A polypeptide hormone that stimulates cell proliferation especially of epithelial cells by binding to receptor proteins on the cell surface.
Epididymus — A structure at the back of the testes composed mainly of ductules leading from the testis to the vas deferens.
Episodic memory — A type of long-term memory.
Epithelial cells — Cells that make up the epithelium.
Epithelium — The tissue covering the outer surface of the body, the mucous membranes, and cavities of the body.
Equimolar — At the same molar concentration.
Equol — A metabolite of daidzein.
ER — *See* Estrogen receptor
ERE — *See* Estrogen response element.
ER-negative — Having no estrogen receptors.
ER-positive — Having estrogen receptors.
Erythrocyte — Red blood cell.
Escherichia coli — Common bacterium found in human and mammalian digestive tracts.
Ester — A bond between an organic acid and an alcohol.
Estradiol — A female sex hormone.
Estradiol benzoate — A synthetic analog of estradiol.
Estriol — A female sex hormone.
Estrogen — Sex hormone or other substance capable of developing and maintaining female characteristics of the body.
Estrogen receptors — An intracellular protein that binds estrogen and estrogen-like compounds and mediates subsequent cell responses.
Estrogen response element — A DNA sequence in the promoter region of an estrogen receptor responsive gene that is recognized by and binds to the DNA-binding domain of estrogen receptors.
Estrogenicity — The effect of an estrogen.

Estrone — A female sex hormone.
Estrous cycle — Periodic sexual impulse of some animals.
Ethinyloestradiol — A synthetic estrogen used in contraceptive pills.
Etiology — The cause of disease and mode of operation. *See also* Transactivation.
Euthyroid — Having a normally functioning thyroid.
FACS analysis — *See* Fluorescence activated cell sorter analysis.
FDA — Food and Drug Administration.
Femur — A bone in the leg.
Fermented soybeans — *See* Natto.
Fetoprotein — Fetal protein that binds to and deactivates maternal hormones to protect the fetus.
FFQ — *See* Food frequency questionnaire.
Flavone — A compound possessing the chemical structure:



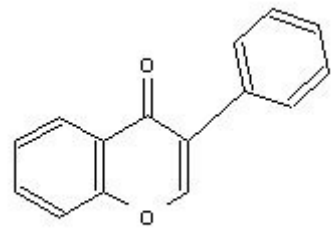
Flavonoid — A general term referring to a compound of similar structure to or derived from a flavone.
Flaxseed — The seeds from the flax plant.
Fluorescence — Luminescence caused by absorption of radiation followed by emission in the form of light.
Fluorescence-activated cell sorter analysis — A technique that separates, classifies, and quantifies cells and antibodies.
Focal adhesion kinase (FAK) — A protein required for cell movement and invasion.
Follicle stimulating hormone — A hormone (gonadotropin) secreted by the pituitary gland that promotes sex hormone production in the gonads.
Follicular phase — The first phase of the menstrual cycle. It lasts from the onset of menses until ovulation.
Food frequency questionnaire — A questionnaire used to obtain qualitative descriptive information about usual food consumption patterns.
Forestomach — A specialized part of the stomach consisting of two compartments.
Formononetin — An isoflavone phytoestrogen.
Fos — A transcription factor.
Free androgen index — A measure of the total testosterone:SHBG ratio in men.
Frontal cortex — A part of the brain thought to be where higher level thinking takes place.
FSH — *See* Follicle stimulating hormone.
Go phase — Stage of the cell cycle where cells no longer replicate.
G_1 phase — Stage in the cell cycle before S-phase that prepares the cell for replication.
G_2 phase — Stage in the cell cycle before S-phase that prepares the cell for replication.

Galactosemia — The presence of galactose in the blood and a characteristic of a rare genetic disorder in which galactose metabolism is inhibited.
Galactosidase — A class of enzymes that cut the glycosidic bonds between the sugar galactose and another sugar of a different type from galactose.
Gavage — Administration of a liquid via a stomach tube, commonly used as a dosing method in toxicity studies.
GC-MS — Gas chromatography coupled with mass spectrometry.
Gene — A specific sequence of DNA molecule encoding a specific protein product.
Gene expression — The process by which the information in a gene is used to create proteins or polypeptides.
Gene product — A protein or polypeptide coded by a gene.
Genetic polymorphism — A difference in DNA sequence among individuals, groups, or populations (e.g., a genetic polymorphism might give rise to blue eyes versus brown eyes or straight hair versus curly hair). Genetic polymorphisms may be the result of chance processes or may have been induced by external agents (such as viruses or radiation). Changes in DNA sequence, which have been confirmed to be caused by external agents, are generally called "mutations" rather than "polymorphisms."
Genetic predisposition — Having a genotype that increases the risk of developing a disease but does not make it certain that it will develop. Additional factors are required before the disease appears.
Genistein — An isoflavone phytoestrogen.
Genome — All the genetic material in the chromosomes of a particular organism.
Genotoxin — Chemical that damages DNA.
Genotype — The genetic constitution of an organism.
Germ cell — Reproductive cells in the testes.
Gestation — The period from the conception of a fetus until birth.
Glomerular — Relating to glomerulus, particularly renal glomerulus.
Glucose — A type of sugar.
Glucosidase — An enzyme that removes glucose from glucose conjugates.
Glucoside — A compound conjugated to glucose.
Glucuronic acid — A sugar added during metabolism to facilitate excretion of a compound.
Glucuronidase — An enzyme in the gut that hydrolyzes conjugated glucuronides.
Glucuronidation — The addition of glucuronic acid to a molecule making it more water-soluble and allowing subsequent elimination.
Glucuronosyl transferase — An enzyme that facilitates addition of glucuronic acid to a compound.
Glycitein — An isoflavone phytoestrogen.
Glycoproteins — Proteins conjugated to carbohydrates.
Glycosylation — Addition of sugar molecules.
GnRH — Gonadotropin releasing hormone.
Goiter — A noncancerous enlargement of the thyroid gland.

Goitrin — A compound found in plants from the Brassicaceae family.
Goitrogen — A compound that causes goiter.
Gonadectomized — Having the gonads (ovaries or testes) removed
Gonadotropin — Hormone secreted from the pituitary that stimulates production of sex hormones from the gonads.
Gut microflora — Bacteria found in the gut.
Gyrase — An enzyme that catalyzes the breaking and rejoining of bonds linking nucleotides in DNA to generate DNA helices.
Half-life ($t_{1/2}$) — Time in which the concentration of a substance will be reduced by half, assuming a first-order elimination process.
HDL — *See* High density lipoprotein.
Heat shock proteins — Proteins synthesized in response to increased temperature.
HeLa cells — A continuously cultured human malignant cell line derived from a cervical carcinoma.
Hepatic — Pertaining to the liver.
HER-2 (Human epidermal growth factor receptor 2) — A protein receptor that is produced in excess amounts in some women with breast cancer.
Heterodimer — A complex of two different proteins.
High density lipoprotein — A lipoprotein composed of a high proportion of protein with little triglyceride and cholesterol. HDL is associated with reduced probability of developing atherosclerosis.
Hippocampus — A region of the brain involved in spatial orientation, the functioning of the limbic system. It is also involved in the establishment of memory patterns.
Histology — Study of cells under the microscope.
Homeobox — A conserved DNA sequence that codes for a protein involved in binding to DNA.
Homeostasis — Maintenance of a normal body state.
Homodimer — A complex of two molecules of the same protein.
Homologous — Corresponding or alike in certain critical attributes.
Hormone — A molecule secreted into the blood that is carried to specific target cells/organs to produce a specific physiological response.
Hormone replacement therapy (HRT) — The administration of estrogen to women with reduced levels of the hormone following menopause or surgical removal of the ovaries.
Hot flushes — A symptom associated with the menopause in which there is a sudden flow of heat to the skin.
HPLC-UV — High-performance liquid chromatography coupled with ultraviolet detection.
HRT — *See* Hormone replacement therapy.
Hydrolysis — A chemical reaction involving water.
Hydrophilic — Water attracting or attracted to water.
Hydrophobic — Water repelling or repelled by water.
Hydroxyestradiol — An estrogen metabolite.
Hydroxyestrone — An estrogen metabolite.

Hydroxylation — Addition of hydroxyl group (-OH).
Hydroxysteroid dehydrogenase — A class of enzymes that catalyzes the conversion of oxo groups to hydroxyl groups on steroids and *vice versa.*
17-hydroxysteroid oxidoreductase I — An enzyme that converts sex hormones to more potent forms.
17-hydroxysteroid oxidoreductase II — An enzyme that converts sex hormones to less potent forms.
Hypercholesterolemia — A condition associated with heart disease in which abnormally high concentrations of cholesterol are present in the bloodstream.
Hyperlipidemia — An abnormally high amount of lipid (fat) in the circulating blood.
Hyperplasia — An increase in the size of an organ or tissue due to an increase in the number of cells.
Hypertension — High blood pressure.
Hypocholesterolemia — The presence of reduced cholesterol in the bloodstream.
Hypoestrogenic symptoms — *See* Menopausal.
Hypogonadism — Functional deficiency of the gonads.
Hypolipidemic — A reduction of lipids in the blood plasma.
Hypophysectomy — Removal of the pituitary gland.
Hypospadias — A congenital abnormality of the male reproductive system.
Hypothalamus — A region of the brain that secretes hormones and regulates the anterior pituitary.
Hypothyroidism — Reduced activity of the thyroid gland.
IC_{50} — The concentration of a chemical estimated to cause inhibition of a biological endpoint by 50%.
IDFA — Infant Dietetic Foods Association.
Idiopathic — Denoting a disease or condition for which the cause is not known.
IGF-1 — Insulin-like growth factor 1. A hormone involved in muscle growth.
IGFBP3 — Insulin growth factor binding protein 3. The major carrier for IGF-1 in human serum.
Immunoassay — An assay system using antibodies to measure the concentrations of analytes.
Immuno(cyto)histochemical — Technique that uses antibodies as a means of detecting molecules in tissues.
Immunosuppressive — Causing or characterized by immunosuppression.
In situ hybridization — Use of DNA or RNA to detect the presence of the complementary sequences.
In utero — Within the uterus.
In vitro — Outside the living system.
In vivo — In the living body.
Inhibin — A hormone that suppresses the release of gonadotropins from the pituitary.

Inhibitory B (IB) — The inhibitory subunit of NF-B. Removal of IB activates NF-B.
Inoculation — Injection of a living or mildly infective pathogen followed by a mild, nonfatal infection resulting in immunity to more virulent forms of the pathogen.
Intelligence quotient (IQ) — A number that shows how a person's intelligence compares with the average.
Intervention study — An epidemiological study in which the experimenter allocates the participants to either an experimental or control group and compares the outcome.
Intraperitoneal — Within the abdominal cavity.
Invasive cancer — *See* Metastasis.
Inverse association — An association in which as one variable increases the other decreases.
Ion exchange chromatography — A separation method using the ionic properties of molecules and their affinity to an ionic resin.
Ipriflavone — A synthetic drug for the treatment of osteoporosis.
Isocaloric — Containing the same level of energy value.
Isoenzyme — A physically distinct form of a given enzyme.
Isoflavone — A compound with a 3-phenyl-4H-1-benzopyran-4-one chemical structure:



Isoflavonoid — A general term referring to a compound of similar structure to or derived from an isoflavone.
Isoforms — Proteins from the same gene that have different amino acid sequences.
Isolariciresinol — A lignan phytoestrogen.
Isotopic labeling — Replacement of an atom with a different isotope.
Isoxanthhumol — A prenylated flavonoid phyioestrogen.
IB — *See* Inhibitory B.
Jun — A transcription factor.
Ki67 — A marker of cell proliferation.
Kinase — An enzyme that transfers a phosphate group between ATP and another molecule.
Knockout animals — Genetically engineered animals in which one or more genes, usually present and active in the normal animal, are absent or inactive.
Lactase deficiency — Lack of the enzyme lactase.
Lactation — A period of milk production in the female.
Lactoferrin — Protein involved in iron transfer.
Lactovegetarian — A person who does not eat meat, meat products, or eggs.
Lariciresinol — A lignan phytoestrogen.

Latency — The interval between a stimulus and a response.
LBD — Ligand binding domain.
LC-MS — Liquid chromatography coupled to mass spectroscopy.
LD_{50} — The dose of a toxic compound that causes death in 50% of a group of experimental animals to which it is administered. It can be used to assess the acute toxicity of a compound but is being superseded by more refined methods.
LDL receptor — Controls the supply of intracellular cholesterol via endocytosis.
LDLC — *See* Low density lipoprotein cholesterol.
Leiomyoma (CHK) — Benign tumor of the smooth muscle.
Leucocyte — White blood cell.
Leukemia — A group of neoplastic disorders (*See* Tumor) affecting blood-forming elements in the bone marrow, characterized by uncontrolled proliferation and disordered differentiation or maturation. Examples include the lymphocytic leukemias, which develop from lymphoid cells, and the myeloid leukemias, which are derived from myeloid cells (producing red blood cells, mainly in the bone marrow).
Leydig cell — A cell type in the testis that produces testosterone.
LH — Luteinizing hormone.
Ligand — A molecule that binds to a receptor.
Ligand binding domain — The part of the receptor to which the ligand binds.
Lignan — A compound with a 2,3 substituted 1,4-dibenzylbutane chemical structure.
Linoleic acid — A fatty acid present in some foods that is required by the body.
Linseed — The seeds of flax, from which linseed oil is obtained.
Lipoprotein — A complex of protein and lipids.
Lipoprotein (a) — One of a family of lipoproteins.
Locomotor activity — Movement of the body.
Longitudinal study — A study in which the same group of people are observed at intervals over a period of time.
Lordosis behavior — A posture adopted by female animals in estrus in the presence of a male.
Low density lipoprotein cholesterol (LDLC) — A lipoprotein in plasma composed of a moderate proportion of protein with little triglyceride and a high proportion of cholesterol. LDLC is associated with an increased probability of developing atherosclerosis.
Luciferase reporter gene — A gene that encodes an easily assayed product (e.g., CAT) and is coupled to the sequence of another gene introduced into cells. The reporter gene can then be used to see which factors activate response elements in the upstream region of the gene of interest.
Lumbar spine — Lower back.
Lumbar vertebrae — Bones of the lower back region.
Luminal — Within the lumen.
Luteal cells — Cells of the corpus luteum.

Luteal phase — The postovulatory phase of the menstrual cycle.
Luteinizing hormone (LH) — A hormone (gonadotropin) secreted by the pituitary that promotes sex hormone production in the gonads.
Luteolin — A plant-derived yellow dyestuff.
Lymphocyte — A type of white blood cell that plays central roles in adaptive immune responses.
Lymphoid — Of, relating to, or being a tissue containing lymphocytes.
Macrobiotic diet — A diet based on the principles of yin and yang and comprising mainly brown rice, whole grains, and vegetables.
Malignancy — *See* Tumor.
Malonyl — A chemical group ($HOOCCH_2COO$-).
Malonylation — The addition of malonyl group to a molecule.
Mammographic density — Breast tissue that has many glands close together. The level of mammographic (breast) density is highly associated with breast cancer risk.
MAPK — A family of enzymes that are involved in cell signaling.
Matatiresinol — A lignan phytoestrogen.
Max_I — Concentration of a compound that gives rise to maximum induction in a biological assay.
MCF-7 — Cell line derived from a human breast tumor.
Median basal hypothalamic and preoptic area (MBH-POA) — Specific regions within the hypothalamus.
Melanoma — A malignant skin tumor.
Menarche — Onset of first menstruation.
Menopause — Cessation of menstruation and ovulation.
Menstrual cycle — The process of ovulation and menstruation.
Messenger RNA (mRNA) — A nucleic acid copy of a DNA sequence that serves the instruction for the synthesis of proteins.
Meta-analysis — In the context of epidemiology, a statistical analysis of the results from independent studies, which aims to produce a single estimate of an effect.
Metabolism — Chemical modification of a compound by enzymes within the body, for example, by reactions such as hydroxylation (*See* Cytochrome P_{450}), epoxidation, or conjugation. Metabolism may result in activation, inactivation, accumulation, or excretion of the compound.
Metabolite — Product formed by metabolism of a compound.
Metastasis — The process whereby malignant cells become detached from the primary tumor mass, disseminate (mainly in the bloodstream or in lymph vessels) and "seed out" in distant sites where they form secondary or metastatic tumors. Such tumors tend to develop at specific sites and their anatomical distribution is often characteristically nonrandom. The capacity to metastasize is the single most important feature of malignant tumors. *See* Tumor.
Methionine — An amino acid.
Methylation — The addition of a methyl group (-CH_3) to a molecule.

Micronuclei — Isolated or broken chromosome fragments which are not expelled when the nucleus is lost during cell division but remain in the body of the cell forming micronuclei. Centromere-positive micronuclei contain DNA and/or protein material derived from the centromere. The presence of centromere-positive micronuclei following exposure to chemicals can be used to evaluate the aneugenic potential of chemicals.
Microsomal enzyme — Enzymes found in endoplasmic reticulum.
Microsomes — The smallest-size particles spun down from cell homogenates in the ultracentrifuge, including broken parts of other fractions.
Migrant studies — The epidemiological study of populations that have moved geographical location.
Miso — A steamed soybean product.
Mitogen — A stimulus that provokes cell division in somatic cells.
Mitosis — The type of cell division that occurs in somatic cells when they proliferate. Each daughter cell has the same complement of chromosomes as the parent cells.
Moiety — One of the portions into which something is, or can be, divided.
Monomer — A molecule consisting of a single unit.
Monophenolic — A chemical structure containing one phenol group.
MRC — Medical Research Council.
mRNA — Single-stranded RNA molecule that specifies the amino acid sequence required for protein synthesis.
MS/MS — Mass spectrometers used in series that can provide structural information as well as quantitative measurement of the concentration of an analyte.
Mucosal — Regarding the mucosa or mucous membranes.
Multivariate analysis — Statistical analysis that allows simultaneous study of two or more dependent variables.
Mutagens — Compounds capable of causing a change in DNA sequence.
Mycoestrogens — Estrogenic compounds produced by fungi.
Myelornonocytic leukemia — Cancer of the bone marrow and white blood cells.
Natto — Food made by fermenting cooked whole soybeans and fried tofu soybean.
Natural killer cell — A type of leucocyte that can recognize and kill virally infected and malignant cells.
Neonatal — The first four weeks of life in humans.
Neoplasm — *See* Tumor.
Neoplastic — Abnormal cells, the growth of which is more rapid than that of other cells.
Neurodegeneration — A loss of nerve cells.
Neurodegenerative — Relating to or characterized by degeneration of nervous tissue.
Neuron — A type of nerve cell.
Neuroprotective factors — Compounds intended to prevent damage to the central nervous system.

Neurotropic factor — A protein that promotes nerve cell growth and survival.
Nipple aspirate — Fluid from the mammary gland nipple.
No observed adverse effect level (NOAEL) — The highest administered dose at which no adverse effect has been observed.
Normocholesterolemic — To possess physiologically normal cholesterol concentrations.
Normotensive — Normal blood pressure, tension, and tone.
Normoxic — At atmospheric pressure.
Northern blot — A technique in molecular biology used to determine mRNA expression.
N-telopeptide — Portions of amino acid sequence of a protein that are removed in maturation.
Nucleotide — The "building block" of nucleic acids, such as DNA and RNA.
Observational study — Describing the design of a scientific study.
Odds ratio — A figure intended to provide a comparison of the presence of a risk factor for disease in a sample of diseased subjects and nondiseased controls.
Olfactory bulb — Structure in the central nervous system involved in the sense of smell.
Omnivorous — Eats both animal and plant matter.
Oncogene — A gene carried by a tumor virus or cancer cell that is solely or partly responsible for tumorigenesis.
Ononin — Isoflavone found in plants.
Ontogeny — The history of development and growth of an individual from the fertilized egg to maturity.
Open field activity — Exercise to study animal behavior.
Organogenesis — The formation of organs.
Osteoblast — A bone-forming cell that secretes the bone matrix.
Osteocalcin — Polypeptide produced by osteoblasts involved in the formation of bone.
Osteoclasts — Cells involved in the degradation of bone.
Osteopenia — Loss of bone.
Osteoporosis — Loss of bony tissue, resulting in bones that are brittle and liable to fracture.
Ovariectomy — Removal of the ovaries.
Ovary — Female reproductive organs where eggs are developed.
Oviduct — A duct that releases eggs from the ovary.
Ovulation — The release of a mature oocyte from the ovary.
Ovum — A female reproductive cell that once fertilized develops into an egg.
Palpitations — Irregular or forceful beatings of the heart.
Paracrine factors — Chemicals involved in communication between cells in the same tissue.
Pathogenesis — The biological mechanisms underlying the clinical manifestation of disease.
Pathophysiology — Study of the disease due to the disturbance of the systems of the body.

PCR — *See* Polymerase chain reaction.
Perimenopausal — The period of time before, during, and after menopause.
Perinatal — The period of time before, during, and after birth.
Pharmacokinetics — How a chemical interacts with the body in terms of absorption, distribution, metabolism, and excretion.
Phenol — A singly hydroxylated aromatic compound.
Phenotype — The visible properties of an organism.
Phosphorylation — Addition of a phosphate group to a molecule.
Physiological — Of or relating to physiology.
Phytochemical — A chemical derived from a plant.
Phytoestrogen — Any plant substance or metabolite that induces biological responses in vertebrates and can mimic or modulate the actions of endogenous estrogens usually by binding to estrogen receptors.
Phytosterols — Compounds naturally produced by plants with similar structure to steroids.
Pituitary gland — A gland at the base of the brain that produces hormones.
Placebo — An inert substance used in experiments testing the efficacy of another substance.
Placental transfer — The movement of a compound from the peripheral blood to the placenta.
Plasma — The fluid portion of blood free from red blood cells.
Platelets — Blood cells involved in blood clotting.
Polyclonal antibody — Antibodies derived from a number of lymphocytes.
Polymerase chain reaction (PCR) — A method to amplify specific sequences of DNA.
Polymorphism — The existence of variation of a genetic characteristic of a population.
Polyp — A small growth on a membrane.
Postpubertal — After puberty.
Postmenopausal — After menopause.
Postnatal — Occurring after birth.
Postnatal day (PND) — Day following birth.
Postpartum — Occurring after birth.
Posttranscriptional — Occurring after genetic transcription.
Potency — Ability of a ligand to elicit a response that is determined by its binding affinity and efficacy.
Preimplantation — The period between fertilization and implantation.
Premature menarche — Premature breast development.
Premenopausal — Before menopause.
Preneoplastic lesions — Abnormal cell growth that may lead to a benign or malignant tumor.
Prenylated flavonoid — A flavonoid including a prenyl group.
6-Prenylnarigenin — A prenylated flavonoid phytoestrogen.
8-Prenylnarigenin — A prenylated flavonoid phytoestrogen.
Preputial separation — Separation of the covering skin of the penis.
Progesterone — A female sex hormone.

Progestin — A female sex hormone.
Prolactin — A hormone secreted by the anterior pituitary to stimulate the production of milk in mammals.
Proliferative index — A measure of the number of dividing cells in a tissue or in culture.
Promoter — A site on DNA to which RNA polymerase will bind and initiate transcription.
Prospective study — A method of epidemiological study in which a defined population is identified and then followed up over time with ascertainment of exposures and/or subsequent disease or mortality.
Prostaglandins — A group of fatty acids that have hormone-like actions.
Prostate gland — A male gland that produces seminal fluid.
Prostate seminal vesicle — Areas in the prostate gland that produce seminal fluid.
Prostate specific antigen (PSA) — An antigen made by the prostate gland and found in the blood that may indicate prostate cancer.
Prostatic fluid — A fluid produced by the prostate that forms part of semen.
Prostatic hypertrophy — An increase in prostate size.
Proteases — Enzymes that break down proteins.
Protein hydrolysate formula — An infant formula based on cow's milk protein that has been broken down into smaller pieces that are easier to digest and less likely to cause allergic symptoms.
Protein tyrosine kinases — Enzymes that phosphorylate specific tyrosine residues on proteins.
Proto-oncogenes — Normal cell genes involved in the regulation of the cell cycle.
Proximal radius — Relating to part of the forearm.
pS2 — An estrogen-induced protein used as a marker of functioning ER status in breast cancer.
PSA — *See* Prostate specific antigen.
Pulmonary — Of the lung.
Quartile — One-fourth of the total number.
Quercitin — A type of flavonoid.
Quintile — One-fifth of the total number.
Raloxifene — Synthetic antiestrogen.
Ras — An oncogene involved in signal transduction and gene transcription and capable of causing cancer.
Recall bias — A systematic error in epidemiological studies due to differences in accuracy or completeness of recall to memory of past events or experiences.
Receptor — Proteins that bind ligands to initiate a change in the working of a cell.
Receptor subtypes — Receptors activated by similar ligands but which have sufficient differences in their pharmacological response or molecular structure to justify being classified separately.

5-Reductase — An enzyme involved in the conversion of testosterone to 5-dihydrotestosterone.
Regression analysis — The relationship between two variables, which estimates the average increase in one variable that is associated with a change of size of one unit in the other variable.
Relative risk — The proportion of diseased people among those exposed to the relevant risk factor divided by the proportion of diseased people among those not exposed to the risk factor. This should be used in cohort studies where those with and without disease are followed to observe which individuals become diseased.
Resorption — Loss of a substance through physiologic or pathologic means.
Reticuloendothelial system — A system in the body that defends against infection and disposes of cell breakdown products.
Retrospective study — A study in which people are enrolled and then have their history of risks, infections, or disease determined.
RT-PCR — Reverse-transcriptase polymerase chain reaction, PCR utilizing reverse transcriptase (RT), an enzyme that catalyzes the synthesis of cDNA from an RNA template.
Running wheel activity — An experimental method used to examine behavior.
Secoisolariciresinol — A lignan phytoestrogen.
Semen — Fluid that contains sperm.
Seminal vesicles — Small glands located near the prostate that produce seminal fluid.
Seminiferous tubule lumen — The cavity in the testis where sperm cells are formed.
Sensorimotor function — A nerve conveying both sensory and motor signals.
Serine — An amino acid.
Sertoli cell — Cells in the testes that support sperm production.
Serum — The fluid that separates from clotted blood or blood plasma that is allowed to stand.
Sex hormone binding globulin — A hepatic glycoprotein that binds endogenous sex hormones in plasma.
Sex hormones — Steroid hormones that control sexual development, including androgens and estrogens.
Sexual differentiation — The process a fetus undergoes to become either female or male.
Sexually dimorphic nucleus — An area in the medial preoptic area of the forebrain. It is larger in males than females.
SHBG — *See* Sex hormone binding globulin.
Signal transduction — The process whereby an extracellular signal is transmitted across the plasma membrane of a cell to activate the intracellular biochemical pathways that lead to the cell's response.
Sitosterol — A plant steroid.
Solubility — The extent to which one substance dissolves into another.

Solvent — A liquid that dissolves another substance or substances to form a solution.
Soy — Derived from the soybean; also referred to as "soya."
Soybean — The edible seed of *Glycine max.*, a dicotyledonous plant of the legume family native to Asia.
Sperm — A reproductive cell produced by males.
Spermatid — An intermediate cell type formed during spermatogenesis.
Spermatogenesis — The production of sperm.
Spermatozoa — Mature male germ cells.
S-phase — Stage of the cell cycle where the cell synthesizes DNA prior to mitosis.
Splenic — Of or relating to the spleen.
Spliced isoform — A sequence of mRNA that may change to produce a different gene product.
Squamous metaplasia — An alteration of plate-like cells.
Src — A protein involved in intracellular signaling.
Stem cell — A type of cell from which specific tissue type cells are produced.
Steroid hormone receptors — A family of cellular receptors.
Steroidogenesis — Production of steroids.
Subcutaneous injection — Injection of a compound under the skin.
Sulfatase — An enzyme that catalyzes the removal of sulfate.
Sulfotransferase — An enzyme that catalyzes the addition of sulfate to a compound.
SULT1A1 — Sulfotransferases convert the unconjugated form of estrone to estrone sulfate.
SULT1A2 — Sulfotransferases convert the unconjugated form of estrone to estrone sulfate.
SV40 — Symbol for simian vacuolaring virus No. 40.
Synergism — Interaction of one agent with another to produce increased activity that is greater than the sum of the effects of the two agents separately.
Systemic arterial compliance — A measure of the elasticity of the arterial wall.
Systolic blood pressure — The pressure exerted on the walls of the arteries during the contraction phase of the heart.
T_3 — *See* Tri-iodotyrosine.
T_4 — *See* Thyroxine.
Tamoxifen — Synthetic antiestrogen used in the management of breast cancer.
T-cells — Any of several lymphocytes that differentiate in the thymus.
TEBs — Terminal end buds present in the mammary gland.
Teleost — Type of fish.
Tempeh — Cake made by fermenting soybeans with rice or mullet.
Terminal end buds — Specialized structures at the end of growing ducts in breast tissue. They are sites of intensive cell proliferation.
Tertile — One-third of the total number.

Testes — Male reproductive organs that produce sperm and male sex hormones.
Testosterone — A male sex hormone.
Textured vegetable protein (TVP) — A meat-like substance used to boost the nutritional content of meals. TVP usually contains defatted soy flour.
Threonine — An amino acid.
Thymic — Of or relating to the thymus.
Thyroglobulin (TG) — A protein in the thyroid gland from which the thyroid hormones (thyroxine and tri-iodotyrosine) are synthesized.
Thyroid binding globulin — A plasma protein involved in binding thyroid hormone.
Thyroid gland — An endocrine gland involved in the regulation of metabolism.
Thyroxine (T_4) — A hormone secreted by the thyroid gland.
Tofu (soybean curd) — A foodstuff obtained by the coagulation of fresh soymilk.
Tolerable daily intake (TDI) — Regulatory value equivalent to the acceptable daily intake (ADI).
Topoisomerase — An enzyme that reduces supercoiling in DNA by breaking and rejoining the two strands of the DNA molecule either simultaneously or separately.
Toxicodynamics — The interaction of a chemical with its site of toxic action.
Toxicokinetics — The fate of chemicals in the body, including a mathematical account of their absorption, distribution, metabolism, and excretion.
TRAMP — Transgenic adenocarcinoma in mouse prostate. Transgenic animal model of prostate cancer.
Transactivation — Stimulation of transcription by factors binding to DNA and activating adjacent proteins.
Transcription — Synthesis of mRNA from DNA.
Transcription factor — A protein involved in the transcription of genes.
Transfection — The incorporation of exogenous DNA into a cell.
Transgenic — Any animal into which cloned genetic material has been transferred.
Translocation — The transfer of cellular components to different positions.
Translocator — A membrane protein controlling the transfer of a substance across a cell membrane.
Triacylglycerol — Glycerol esterified at three hydroxyl groups by a fatty acid.
Triglycerides — The form in which fats are stored in the body.
Tri-iodotyrosine (T_3) — A hormone secreted by the thyroid gland.
TRPM2 — Testosterone repressed prostate message 2, a gene involved in apoptosis.
TSH — Thyroid stimulating hormone.
Tumor — An abnormal mass of tissue that results from excessive cell division that is uncontrolled and progressive.
Tumor T-antigen — Proteins coded by viral genes that are expressed early in the replication cycle.

Tumorigenesis — The development of a tumor.
TVP — *See* Textured vegetable protein.
Tyrosine — An amino acid.
Ulna — One of the bones that compose the forearm, below the elbow.
Unopposed estrogen therapy — HRT products containing only estrogen.
Uterine adenocarcinoma — A cancer that involves cells in the lining of the uterus.
Uterotrophic assay — Biological assay used to measure estrogenic activity, in which the ability of chemicals to stimulate uterine growth is monitored.
Vaginal adenocarcinoma — A cancer that involves cells in the lining of the vagina.
Vaginal canalization — Formation of channels in the vagina during fetal development.
Vaginal cornification — Formation of keritin in cells of the vagina.
Vaginal epithelium — Cells covering the surfaces of the vagina.
Vasculature — Blood vessels.
Vasorelaxation — Relaxation of blood vessels.
Vegan — A person who consumes no animal products.
Vegetarian — A person living on a diet of grains, legumes, nuts, seeds, vegetables, and fruits with or without the use of dairy products and eggs. A vegetarian does not eat meat, poultry, game fish, shellfish, or crustacea and avoids all animal by-products. In addition, a lactovegetarian does not eat eggs.
Veno-occlusive disease — A disease in which blood vessels in the liver become swollen and clogged.
Ventral prostate — Part of the prostate gland.
Vertebrate — Organisms characterized by the possession of a well-formed bony or cartilaginous vertebral column or backbone enclosing the spinal cord.
Very low density lipoprotein (VLDL) — A plasma lipoprotein produced primarily by the liver.
Vitellogenin — A protein synthesized in hepatocytes following estrogen stimulation.
Vulvar carcinoma — Cancer of the external female genitalia.
Westernization — Describes the adoption of a lifestyle typically seen in the United States and Europe.
Whey — Watery liquid left when milk forms curds.
WHO — World Health Organization.
Xanthohumol — A prenylated flavonoid phytoestrogen.
Xenobiotic — A foreign chemical not normally found in the body.
Xenoestrogen — A compound with estrogenic properties not normally found in the body.
Zearalanol — A mycoestrogen.
Zearalenone — A mycoestrogen.
Zinc finger — A protein structure that binds zinc, often found in proteins that bind to DNA.

Units of Measurement

mg — Milligram (10^{-3} grams or 0.001 grams).
µg — Microgram (10^{-6} grams or 0.000001 grams).
ng — Nanogram (10^{-9} grams or 0.000000001 grams).
pg — Picogram (10^{-12} grams or 0.000000000001 grams).
mole (mol) — Molecular weight of a compound expressed in grams.
µM — Micromolar (10^{-6} moles or 0.000001 moles).
nM — Nanomolar (10^{-9} moles or 0.000000001 moles).
pM — Picomolar (10^{-12} moles or 0.000000000001 moles).
mU — Milliunits, of enzyme activity, expressed in terms of the turnover of the appropriate substrate of the enzyme.
mV — Millivolts.
ppb — Parts per billion (1 part in a thousand million).
ppm — Parts per million.
w/w—Weight/weight, to indicate that measures of weight are used in the preparation of a mixture.