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Phytochemical functional foods

**Edited by
Ian Johnson and Gary Williamson**



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1

Introduction

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Phytochemicals are biologically-active, non-nutritive secondary metabolites which provide plants with colour, flavour and natural toxicity to pests. The classification of this huge range of compounds is still a matter of debate, but they fall into three main groups:

- phenolic compounds (including flavonoids and phytoestrogens);
- glucosinolates;
- carotenoids.

Many thousands of phenolic compounds have been identified. They include monophenols, the hydroxycinnamic acid group which contains caffeic and ferulic acid, flavonoids and their glycosides, phytoestrogens and tannins. Flavonoids are widely distributed in plants where they have a role in plant colour, taste and smell. Some have antioxidant properties whilst others are phytoestrogens. Phytoestrogens are diphenolic compounds which exert weak estrogen activity. They include the glycosides genisten and daidzin, found principally in soya products, and lignan found in cereal seeds such as flax. Glucosinolates occur widely in brassica vegetables, imparting, for example, the pungent odour in mustard and horseradish. Carotenoids comprise a wide variety of red and yellow compounds, chemically related to carotene, found in plants. Around 500 carotenoids have been identified in fruits and vegetables. They include β -carotene, a pre-cursor to vitamin A, but also non-nutritive compounds such as lycopene and lutein.

There is now a growing body of evidence to suggest that phytochemicals may have a protective role against a variety of chronic diseases such as cancer and cardiovascular disease. Part I reviews this body of evidence, its strengths and its weaknesses. Chapter 2 discusses the ways in which phenolic

2 Phytochemical functional foods

compounds may help to prevent cardiovascular disease. Chapter 3 provides an overview of the links between a range of phytochemicals and the risk of cancer. Against this background Chapter 4 looks in more detail at the possible protective role of glucosinolates against cancer, a particularly active and promising area of recent research. The following two chapters concentrate on phytoestrogens. Chapter 5 surveys the current scientific evidence for their wide range of potential functional benefits (a topic also reviewed in Chapter 10), whilst Chapter 6 focuses on the particular topic of bone health. Part I concludes with chapters on carotenoids and flavonoids, and with a broader review of the role of phytochemicals in gastrointestinal health, complementing the earlier reviews of cardiovascular disease and cancer.

Against this background, Part II looks at key issues in developing phytochemical functional products. Chapter 10 discusses problems in assessing prevailing and optimal levels of intake, using phytoestrogens as a case study, complementing the discussion of bioavailability in Chapter 7. Since phytochemicals can have harmful as well as beneficial health effects, Chapter 11 reviews ways of testing the safety of phytochemical products, whilst Chapter 12 looks at the critical role of clinical trials in validating functional claims. The final group of chapters looks at production issues, beginning with Chapter 13 which discusses the genetic enhancement of phytochemicals. Chapter 14 looks at the next step in the chain, covering such issues as extraction of phenolic compounds from plant material, and is complemented by Chapter 16 which looks more generally at how to make the most of phenolic compounds at various stages in production. Chapter 15 discusses in more detail the impact of food processing operations on phytochemical functionality. The book concludes by looking at the example of a particular phytochemical product: rice bran.

Part I

The health benefits of phytochemicals

Nutritional phenolics and cardiovascular disease

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2.1 Introduction

Arteriosclerosis is a chronic pathogenic inflammatory-fibro-proliferative process of large and medium-sized arteries that results in the progressive formation of fibrous plaques, which in turn impair the blood flow of the vessel. These lesions can either promote an occlusive thrombosis in the affected artery or produce a gradual but relentless stenosis of the arterial lumen. In the first case, an infarction of the organ supplied by the afflicted vessel occurs, such as in a heart attack, when a coronary artery is affected, and in a thrombotic stroke when a cerebral artery is suddenly blocked. In the second case, the stenosis of the vessel leads to a progressive and gradual damage of the affected organ part.

A number of subtle dysfunctions occur at the cellular and molecular levels in the early stages of disease progression associated with the loss of cellular homeostatic functions of endothelial cells, smooth muscle cells and macrophages which constitute the major cell types in the atheroma environment. These events include the modification of the pattern of gene expression, cell proliferation and apoptosis.

In the last few decades, several epidemiological studies have shown that a dietary intake of foods rich in natural antioxidants correlates with reduced risk of coronary heart disease;^{1,2} particularly, a negative association between consumption of polyphenol-rich foods and cardiovascular diseases has been demonstrated. This association has been partially explained on the basis of the fact that polyphenols interrupt lipid peroxidation induced by reactive oxygen species (ROS). A large body of studies has shown that oxidative modification of the low-density fraction of lipoprotein (LDL) is implicated

in the initiation of arteriosclerosis. More recently, alternative mechanisms have been proposed for the activity of antioxidants in cardiovascular disease, which are different from the 'simple' shielding of LDL from ROS-induced damage. Several polyphenols recognised for their antioxidant properties might significantly affect cellular response to different stimuli, including cytokines and growth factors.

2.2 LDL oxidation and atherogenesis

At cellular level each stage of atheroma development is accompanied by the expression of specific glycoproteins by endothelial cells which mediate the adhesion of monocytes and T-lymphocytes.^{3,4} Their recruitment and migration is triggered by various cytokines released by leukocytes and possibly by smooth muscle cells.⁵ Atheroma development continues with the activation of macrophages, which accumulate lipids and become, together with lymphocytes, so-called fatty streaks.^{3,4,6} The continuous influx, differentiation and proliferation finally leads to more advanced lesion and to the formation of the fibrous plaque.⁶

It is accepted that oxidation of LDL is a key event in endothelial injury and dysfunction.⁷ Oxidised LDL (oxLDL) may directly injure the endothelium and trigger the expression of migration and adhesion molecules.^{8–10} Monocytes and lymphocytes interact with oxLDL and the phagocytosis which follows leads to the formation of foam cells, which in turn are associated with the alteration of the expression pattern of growth regulatory molecules, cytokines and pro-inflammatory signals.⁶ The proposed role of oxLDL in atherogenesis, based on studies *in vitro*, is shown in Fig. 2.1.

LDL, modified by oxidation, glycation and aggregation, is considered a major cause of injury to the endothelium and underlying smooth muscle. LDL, entrapped in the subendothelial space, can undergo progressive oxidation (minimally modified-LDL, mm-LDL).¹¹ Once modified, LDL activates the expression of molecules entitled for the recruitment of monocytes and for the stimulation of the formation of monocyte colonies (monocyte chemotactic protein, MCP-1; monocyte colony stimulating factor M-CSF) in the endothelium.^{12–14} These molecules promote the entry and maturation of monocytes to macrophages, which further oxidise LDL. Modified LDL is also able to induce endothelial dysfunction, which is associated with changes of the adhesiveness to leukocytes or platelets and the wall permeability.^{14,15} Dysfunctional endothelium also displays pro-coagulant properties and the expression of a variety of vaso-active molecules, cytokines, and growth factors.^{16,17} LDL, oxidised *in vitro* by several cell systems or by cell-free systems (transition metal ions or azo-initiators), is recognised by the scavenger receptor of macrophages.¹⁸ The increasing affinity of LDL for the scavenger receptor is associated with changes in its structural and biochemical properties, such as the formation of lipid hydroperoxides, oxidative modification and

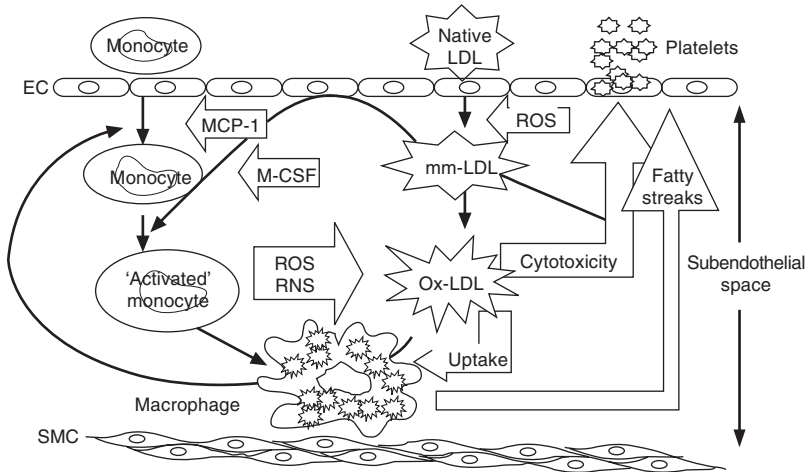


Fig. 2.1 Sequence of events in atherogenesis and role of low-density lipoprotein. Native LDL, in the subendothelial space, undergoes progressive oxidation (mmLDL) and activates the expression of MCP-1 and M-CSF in the endothelium (EC). MCP-1 and M-CSF promote the entry and maturation of monocytes to macrophages, which further oxidise LDL (oxLDL). Ox-LDL is specifically recognised by the scavenger receptor of macrophages and, once internalised, formation of foam cells occurs. Both mmLDL and oxLDL induce endothelial dysfunction, associated with changes of the adhesiveness to leukocytes or platelets and to wall permeability.

fragmentation of apoprotein B-100 and an increase of negative charge.¹⁹ The exact mechanism of LDL oxidation *in vivo* is still unknown, but transition metal ions, myeloperoxidase, lipoxygenase, and nitric oxide are thought to be involved.⁷

2.3 Polyphenols and cell response

Plants produce a variety of secondary products containing a phenol group, i.e. a hydroxyl group on an aromatic ring. These compounds are of a chemically heterogeneous group that includes simple phenols, flavonoids, lignin and condensed tannins. About 4000 plant substances belong to the flavonoid class, of which about 900 are present in the human diet. The daily intake of flavonoids in Western countries has been estimated to be about 23 mg per day.¹ No analogous calculation has been done for phenolic acids, but it is likely to be quite similar in the Western diet.

Many studies have been undertaken to establish the structural criteria for the activity of polyhydroxy flavonoids in enhancing the stability of fatty acid dispersions, lipids, oils, and LDL.^{20,21} As for phenolic acids, the inhibition of oxidation by flavonoids is related to the chelation of metal ions via the

ortho-dihydroxy phenolic structure, the scavenging of alkoxyl and peroxy radicals, and the regeneration of α -tocopherol through reduction of the tocopheryl radical.²⁰ The contribution of flavonoids and phenolic acids to the prevention and possibly to the therapy of cardiovascular disease can also be found on metabolic pathways other than the antioxidant capacity. As previously mentioned, arteriosclerosis is characterised by early cellular events and by the dysregulation of the normal cellular homeostasis.¹⁷ Molecular mechanisms, by which polyphenols may play a role either in the etiopathology or in the pathophysiology of arteriosclerosis, will be discussed here, with particular regard to the modulation of gene expression regulated by the transcription factor nuclear factor-kappa B (NF- κ B), and to the induction of either apoptotic or proliferative responses.

2.4 Polyphenols and activated NF- κ B

The transcription factors of the nuclear factor- κ B/Rel family control the expression of a spectrum of different genes involved in inflammatory and proliferation responses. The typical NF- κ B dimer is composed of the subunits p50 and p65, and it is present as its inactive form in the cytosol bound to the inhibitory proteins I κ B. Following activation by various stimuli, including inflammatory or hyperproliferative cytokines, ROS, oxidised LDL and bacterial wall components, the phosphorylation and proteolytic removal of I κ B from the complex occurs. The activated NF- κ B immediately enters the nucleus where it interacts with regulatory κ B elements in the promoter and enhancer regions, thereby controlling the transcription of inducible genes.^{22,23} A spectrum of different genes expressed in arteriosclerosis have been shown to be regulated by NF- κ B, including those encoding TNF- α (tumour necrosis factor alpha), IL-1 (interleukin-1), the macrophage or granulocyte colony stimulating factor (M/G-CSF), MCP-1, c-myc and the adhesion molecules VCAM-1 (vascular cell adhesion molecule-1) and ICAM-1 (intercellular adhesion molecule-1).²⁴ In the early stages of an atherosclerotic lesion, different types of cells (macrophages, smooth muscle cells and endothelial cells) interplay to cause a shift from the normal homeostasis and a vicious circle may be triggered, exacerbating dysfunction. Figure 2.2 shows a sketch of the regulation of NF- κ B activation by oxidants/antioxidants. Some of the major genes involved in the atherogenesis are also listed.

Several lines of evidence, including the inhibition by various antioxidants, suggest that NF- κ B is subject to redox regulation. Because of its pivotal role in inflammatory response, a significant effort has focused on developing therapeutic agents that regulate NF- κ B activity. In this scenario polyphenols may play an important role, either by directly affecting key steps in the activation pathway of NF- κ B, or by modulating the intracellular redox status, which is, in turn, one of the major determinants of NF- κ B activation.^{6,25} Consistently, experimental data are accumulating regarding polyphenolic

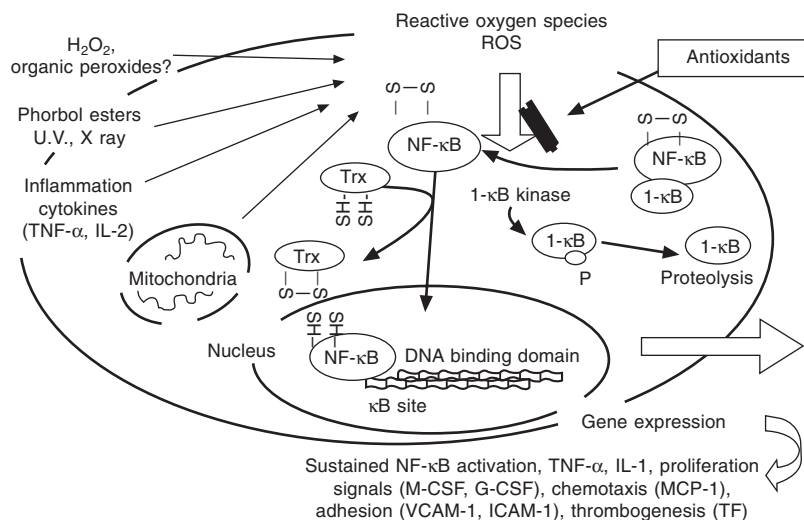


Fig. 2.2 Simplified scheme of oxidant/antioxidant regulation of NF-κB activation. Different stimuli, leading to an increase of ROS generation inside the cell, activate the phosphorylation of IκB inhibitory protein and the subsequent proteolysis. Thioredoxin (Trx) may reduce activated NF-κB proteins facilitating nuclear translocation. Once released from IκB, the NF-κB complex translocates into the nucleus and the binding to DNA domain in the promoters and enhancers of genes such as TNF- α , IL-1, proliferation and chemotactic factors, adhesion molecule. Some of these genes, in turn, may further induce NF-κB activation, leading to a vicious circle if the regulatory cellular system escapes from control.

compounds as natural phytochemical antioxidants that possess anti-inflammatory properties by downregulating NF-κB. Some of the most relevant findings about this aspect are summarised in Table 2.1.

2.5 Other aspects of polyphenols as modulators of signal transduction

Several studies have demonstrated that depending on their structure, flavonoids may be inhibitors of several kinases involved in signal transduction, mainly protein kinase C (PKC) and tyrosine kinases.^{26–29} Agullo *et al.*³⁰ tested 14 flavonoids of different chemical classes and reported that myricetin, luteolin and apigenin were efficient inhibitors of phosphatidylinositol 3-kinase, PKC and tyrosine kinase activity. The authors also observed a structure–function in that the position, number and substitution of hydroxyl groups on the B ring and the saturation of C₂–C₃ bonds affect flavonoid activity on different kinases. Wolle *et al.*³¹ examined the effect of flavonoids on endothelial cell expression of adhesion molecules. A synthetic flavonoid, 2-(3-amino-phenyl)-8-methoxy-chromene-4-one, an analog of apigenin, markedly inhibited

Table 2.1 Flavonoids and flavonoid-related compounds suppressing NF- κ B activity in cell culture studies

Name	Concentration (duration)	Inducers	Cell lines	Ref.
Apigenin	25 μ M (4 h)	TNF α , TNF α + IFN γ	HUVEC	[Gerritsen <i>et al.</i> , 1995]
Caffeic acid phenethyl ester (<i>propolis</i>)	25 μ g/ml (2 h)	TNF α PMA Ceramide-C8 Okadaic acid H ₂ O ₂	U937	[Natarajan <i>et al.</i> , 1996]
Epigallocatechin-3-gallate (green tea)	15 μ M (Co-incubation with inducer)	LPS	Mouse peritoneal macrophages	[Lin <i>et al.</i> , 1997]
	100 μ M (2 h)	LPS	RAW 264.7	[Yang <i>et al.</i> , 1998]
Genistein (soy, clover)	148 μ M (1–2 h)	TNF α , Okadaic acid	U937, Jurkat, HeLa U937	[Natarajan <i>et al.</i> , 1998]
<i>Ginkgo biloba</i> extract	100–400 μ g/ml (18 h)	H ₂ O ₂	PAEC	[Wei <i>et al.</i> , 1999]
Quercetin (wine, onion)	265 μ M (1 h)	TNF α	U937	[Natarajan <i>et al.</i> , 1998]
	10 μ M (co-incubation with inducer)	H ₂ O ₂	HepG ₂	[Musonda and Chipman, 1981]
Silymarin (<i>Silybum marianum</i>)	12.5 μ g/ml (24 h)	Ultraviolet	HaCaT	[Saliou <i>et al.</i> , 1999]
		Okadaic acid LPS TNF α TNF α	HepG2 Würzburg U937, HeLa, Jurkat	[Saliou <i>et al.</i> , 1998] [Manna <i>et al.</i> , 1999]
Taxifolin (pine bark)	303 μ M (24 h)	IFN γ	RAW 264.7	[Park <i>et al.</i> , 2000]
Theaflavin-3,3'-digallate (black tea)	10 μ M (co-incubation with inducer for 1 h)	LPS	RAW 264.7	[Lin <i>et al.</i> , 1999]

M E Gerritsen, W W Carley, G E Ranges, C P Shen, S A Phan, G F Ligon and C A Perry (1995) *Am J Pathol* **147**, 278.

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C Saliou, M Kitazawa, L McLaughlin, J-P Yang, J K Lodge, T Tetsuka, K Iwasaki, J Cillard, T Okamoto and L Packer (1999) *Free Rad Biol Med* **26**, 174.

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S K Manna, A Mukhopadhyay, N T Van and B B Aggarwal (1999) *J Immunol* **163**, 6800.

Y C Park, G Rimbach, C Saliou, G Valacchi and L Packer (2000) *FEBS Lett* **465**, 93.

Y L Lin, S H Tsai, S Y Lin-Shiau, C T Ho and J K Lin (1999) *Eur J Pharmacol* **367**, 379.

TNF- α -induced VCAM-1 cell surface expression in a concentration-dependent fashion, but had no effect on ICAM-1 expression. The inhibition correlated with decreases in steady state mRNA levels, resulting in a reduction in the rate of gene transcription rather than changes in mRNA stability. No effects on NF- κ B activation were observed either by mobility shift assay or by reporter gene assay, indicating that the modulation of VCAM-1 gene expression is due to a NF- κ B-independent mechanism. More recently, Nardini *et al.* reported that both caffeic acid and the procyanidin-rich extract from the bark of *Pinus maritima* inhibit *in vitro* the activity of phosphorylase kinase, protein kinase A and protein kinase C.³² Taken together, these studies opened an important issue in the ability of polyphenols to modulate the expression of genes responsible for pro-atherogenic processes with or without altering the activity of NF- κ B, which can be considered fundamental for other cellular functions.

Hu *et al.*³³ reported that oncogene expression (c-myc, c-raf and c-H-ras) *in vivo*, induced by nitrosamine treatment, is inhibited in mouse lung by tea drinking. The same authors also reported that topical pre-treatment with the tea flavonoid (–)-epigallocatechin gallate significantly inhibits oncogene expression induced by phobol myristate acetate (PMA) in mouse skin.³³ Similarly, c-fos expression, cell growth and PKC activity induced by PMA in NIH3T3 cells were inhibited by the natural flavonoid apigenin, as reported by Huang *et al.*³⁴ Green tea polyphenol extract stimulates the expression of detoxifying enzymes through antioxidant responsive element in the cultured human hepatoma cell line HepG2.³⁵ This activity seems to be mediated by potentiation of the mitogen activated protein kinases (MAPKs) signalling pathway, suggesting an indirect activity of polyphenols in the regulation of cellular responses to oxidative injury. Lin *et al.*³⁶ reported that both curcumin and apigenin inhibit PKC activity induced by PMA treatment in mouse skin. The same inhibitory effect can be observed in mouse isolated fibroblasts pretreated with curcumin. Apigenin, kaempferol and genistein reverted the transformation of the morphology of the v-H-ras transformed NIH3T3 line. The authors suggest that both PKC activity and oncogene expression may be the mechanism by which polyphenols exert their anti-tumour activity.³⁶ The flavonoid silymarin inhibits the expression of TNF- α mRNA induced by either 7,12-dimethylbenz(a)anthracene or okadaic acid in the SENCAR mouse skin model.³⁷ This inhibitory activity, which is associated with a complete protection of mouse epidermis from tumour promotion by OA and results in a significant reduction (up to 85%) of tumour incidence induced by 7,12-dimethylbenz(a)anthracene,²⁶ may also be relevant in the atherogenesis, since TNF- α plays a central role in the vicious circle of macrophage-endothelial cell dysfunction.^{24,38}

The cell-to-cell interaction following the expression of adhesion molecules (ICAM-1, VCAM-1 and selectin) in endothelial cells induced by cytokines treatment has been reported to be blocked by hydroflavones and flavanols.³⁹ Apigenin, the most potent flavone tested in this study, inhibited the expression

of adhesion molecules, the expression of both interleukin-6 and interleukin-8 induced by TNF- α and interleukin-1-induced prostaglandin synthesis. Apigenin was found to have no effect on the nuclear translocation of NF- κ B, but significantly inhibited the expression of the reporter gene β -galactosidase driven by NF- κ B elements in SW480 cells induced by TNF- α , suggesting that NF- κ B transcriptional activation was affected.³⁹ Also the adhesion of cytokine treated lymphocytes to endothelial cells was blocked by pretreatment of endothelial cells with apigenin.³⁹ Finally, the same study reports apigenin to have a strong anti-inflammatory activity *in vivo* on carragenin-induced rat paw edema and on delayed type hypersensitivity in the mouse. Taken together, these data suggest that both flavonoids and phenolic acids may have important effects in diseases involving leukocyte adhesion and trafficking and oxidant-induced gene expression.

2.6 Indirect evidence for polyphenol activity in atherogenesis

An indirect effect of flavonoids and phenolic acids on NF- κ B activation, and therefore on NF- κ B-driven gene expression, may be inferred from two kinds of study: one addressing the modulation of NF- κ B activity by other antioxidant molecules (α -tocopherol, thiolic antioxidants such as *N*-acetyl-cysteine, lipoic acid, pyrrolidinedithiocarbamate), and others addressing the role of flavonoids and phenolic acids in the antioxidant network. α -Tocopherol and lipoic acid inhibit NF- κ B in different cellular models,^{40–42} and several studies describe the ability of flavonoids and phenolic acids to exert a significant tocopherol and glutathione sparing effect either under basal homeostatic conditions or following oxidative challenge.

Roy and co-workers demonstrated that the adhesion of lymphocyte to endothelial cells is regulated by the thiolic antioxidant α -lipoic acid and by α -tocopherol.⁴³ Similarly, an enhancement of the endogenous levels and a protective effect on α -tocopherol after peroxynitrite treatment by the procyanidin-containing extract from pine bark was reported by Virgili *et al.*⁴⁴ The same complex mixture of procyanidins has been reported to enhance the activity of the enzymatic machinery which regulates the GSH redox status in endothelial cells.^{45,46} In fact, a significant increase in GSH (reduced glutathione) levels, an increased activity of the GSH redox enzymes (GSH reductase and GSH peroxidases) and an increase in the enzymatic activity of both SOD (superoxide dismutase) and catalase have been reported and proposed by Wei and collaborators to be mediated by an increase of protein synthesis.⁴⁶ The important role of GSH in the antioxidant network usually results in a greater resistance to pro-oxidant cytotoxicity and, in general, leads to a greater resistance of cells to dysfunction.¹⁷

Proliferation of vascular smooth muscle cells is one of the most important features of arteriosclerosis.⁶ Vascular smooth muscle cells display a unique

susceptibility to antioxidants which indicates that they respond differently from other types to changes in the redox status. In fact, hydrogen peroxide has been demonstrated to stimulate the proliferation of vascular smooth muscle cells while inhibiting the proliferation of vascular endothelial cells.⁴⁷ However, the effect of antioxidants on smooth muscle cell proliferation is still unclear. α -Tocopherol inhibits the proliferation of smooth muscle cells by preventing the activation of PKC.⁴⁸ Two structurally different thiol-containing compounds, *N*-acetylcysteine (NAC) and pyrro-lidinedithiocarbamate (PDTC) have been reported to induce apoptosis in cultured vascular smooth muscle cells in a dose- and time-dependent fashion.⁴⁹ In the same report the overexpression of the proto-oncogene *bcl-2* was observed to counter PDTC and NAC-induced apoptosis, suggesting that thiol oxidation status in the cell plays an important role in switching on the apoptotic program.

2.7 Conclusion and future trends

Dietary consumption of polyphenols is associated with a lower risk of degenerative diseases. In particular, protection of serum lipids from oxidation, which is a major step in the development of arteriosclerosis, has been demonstrated. More recently, new avenues have been explored in the capacity of polyphenols to interact with the expression of the human genetic potential. The understanding of the interaction between this heterogeneous class of compounds and cellular responses, due either to their ability to interplay in the cellular antioxidant network or directly to affect gene expression, has increased.

One main line of future research could be in the inhibitory/activating effect on key enzymes involved in the pathogenesis of arteriosclerosis. In particular, enzymes regulating signal transduction involved in phosphorylation of proteins, such as PKC and tyrosine protein kinase, seems to be somehow modulated by different polyphenols and may represent a possible target for polyphenol activity.

The ability of polyphenols to modulate redox-sensitive pathways of cellular response in endothelial cells, lymphocytes and smooth muscle cells has also been observed. Although some data is already available on NF- κ B, AP-1 and other transcription factors sensitive to the cellular redox status in response to oxidatively modified LDL, the cellular response to lipoproteins modified by the exposure to reactive nitrogen species, is still largely unknown. The unravelling of the mechanisms of the regulation of transcriptional control of gene expression will possibly be a promising future line of investigation.

In conclusion, polyphenols seem to be able to affect the expression of genes involved in the pathogenesis of atherogenesis. Cytokines and adhesion molecules appear to be among the most important genes expressed during the pro-inflammatory situation which precedes the formation of the atheroma, and have also been reported to be affected, at least in part, by phenolics. We

can therefore foresee that a considerable effort will be addressed to the study of the mechanisms through which polyphenols affect the control of the expression of these genes. These studies will give a solid background for the understanding of the molecular mechanisms of the beneficial effects of polyphenols on human health.

2.8 List of abbreviations

- IkB: inhibitory protein kappa B
- ICAM-1: intercellular adhesion molecule 1
- IL-1: interleukin-1
- LDL: low density lipoprotein
- MAPKs: mitogen activated protein kinases
- MCP-1: macrophage chemotactic protein 1
- M-CSF: macrophage colony stimulating factor
- mmLDL: minimally modified LDL
- NAC: *N*-acetylcysteine
- NF- κ B: nuclear factor-kappa B
- oxLDL: oxidised LDL
- PKC: protein kinase C
- PMA: phobol myristate acetate
- ROS: reactive oxygen species
- TNF- α : tumour necrosis factor alpha
- AM-1: vascular cell adhesion molecule 1

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Phytochemicals and cancer: an overview

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3.1 Introduction

The precise reasons for the high levels of death from cancer in developed countries are controversial. Since cancer is largely a disease of old age, its prevalence will inevitably rise with the average longevity of the population, but other factors seem to be at work in developed countries. Even in the nineteenth century it was possible for Tanchou (Tanchou, 1843) to propose that increasing rates of cancer were a characteristic of urban societies. International studies of age-corrected rates of cancer continue to support this view (WHO, 1997). A classic illustration of the historical association between increased urbanisation and industrialisation and cancer rates is provided by Japan. Up until the 1970s rates of breast and bowel cancer were four to five times lower than in the USA and many countries of Northern Europe, whereas stomach cancer was several times more common. Since 1970 rates of breast and bowel cancer have risen steeply in Japan, but stomach cancer, as in many other industrialised countries, has declined. The explanation for these changes must lie in some aspect of environment or lifestyle. However, despite decades of epidemiological and clinical research, we are still far from understanding the factors that determine the risk of cancer at sites other than the lung.

The epidemiological evidence suggests that diet is a significant factor in the development of cancer. In their classic epidemiological study, Doll and Peto (1981) estimated that diet was responsible for as many as 35% of cancers in the West. An encyclopaedic report on nutrition and cancer by the World Cancer Research Fund (1997) has confirmed the central importance of diet as a major determinant of many forms of cancer across the globe. The interactions between diet and the biological processes leading to the

development of cancer are extremely complex, but one can envisage three general factors that are potentially important in any human population:

- the presence of carcinogenic compounds in foods;
- the adequacy of nutrient intake;
- the adequacy of intake of non-nutrient anti-carcinogenic food components.

The presence in food of carcinogenic compounds which play an active role in damaging cells and inducing tumours is a topic largely beyond the scope of this chapter. There are many proven carcinogens in our diets (Helferich and Winter, 2000). One example is the exposure to ethanol from alcoholic drinks. In this case the decision to drink alcohol lies in the hands of the consumer, and there is evidence that alcohol has some protective effects against heart disease which may well outweigh its adverse effects on cancer (Gaziano *et al.*, 1999). In most cases the human body is equipped with efficient defences against such compounds, and the level of exposure is usually too low to be of relevance to health. Consumers have expressed particular concern about the health risks of contaminants and additives in modern highly-processed foods. However, both these areas are highly regulated with little evidence of high levels of exposure or adverse health effects (Kantor, 2002; Shaw and Vannoort, 2001).

The second issue is the adequacy of nutrient intake, and the possibility that certain deficiencies might influence an individual's susceptibility to cancer. Epidemiological studies suggest that in many Western countries the risks of cancer tend to be greater at the lower end of the socio-economic scale amongst groups with a poorer overall diet. The analysis of nutrient intake and its effects amongst different groups is complex, as is establishing an optimal intake for such groups. An adequate supply of a nutrient is determined by a mix of food intake, bioavailability (the fraction of the ingested nutrient available for use by the body) and bioefficacy (the efficiency of the final absorption and conversion of the ingested nutrient to its active form) (Northrop-Clewes and Thurnham, 2002). The majority of dietary studies suggest that there remains considerable room for improvement of the diet of populations in Western Europe and other developed countries, including nutrient intake (Trichopoulou and Naska, 2002). Many studies have linked vitamin A and, notably, its pre-cursor, β -carotene, with a lower incidence of cancer, though there remains debate about optimal levels of intake (see Section 3.8). Research has also suggested that good vitamin D status might, in conjunction with calcium nutrition, lower the risk of colon cancer; that other vitamins such as vitamin C and vitamin E may act as antioxidants; and that long-term use of folate supplements may also reduce the risk of cancer (Northrop-Clewes and Thurnham, 2002). These nutrients are discussed in more detail in Section 3.6.

Finally, susceptibility to cancer may be increased by an inadequate intake of biologically active food components, which, though not classified as nutrients, may nevertheless exert important anti-carcinogenic effects over

the lifespan of an individual. Much the strongest and most consistent message to emerge from epidemiological studies over the last two decades has been that the risk of almost all the most important carcinomas seems to be increased substantially by a low consumption of plant foods, and of fruits and vegetables in particular (World Cancer Research Fund, 1997). The very extensive review by Block *et al.* (1992) showed clearly that individuals with the lowest intake of fruit and vegetables experienced a risk of developing cancer which was about twice that of those with the highest intake. The fraction of the population exposed to risks of this magnitude was typically between 20 and 33%. One possible criticism of this conclusion is that it was based largely on case-control studies which are generally agreed to be more prone to bias than cohort studies. However, although some large prospective trials have subsequently failed to provide the expected level of support for a protective effect of fruits and vegetables against specific tumours (Kim, 2001), the general conclusion that fruits and vegetables are protective against cancer in Western societies remains remarkably robust (Gerber *et al.*, 2002; Riboli and Norat, 2001). The evidence from three major studies is summarised in Table 3.1.

Vegetables and fruits are rich in micronutrients, many of which are essential for the maintenance of tissue integrity and a properly functioning immune system. The antioxidant nutrients have received particular attention because of their putative ability to reduce oxidative damage to DNA, caused by free radicals of endogenous origin (Hennekens *et al.*, 1984). However, intervention studies with antioxidant vitamin supplements have produced disappointing results, and there is still no firm evidence that the antioxidant micronutrients are of central importance in the anti-carcinogenic effects of fruits and vegetables (Ruffin and Rock, 2001). Epidemiological studies show that levels of micronutrients in plasma correlate inversely with risk of cancer (Ziegler, 1991), but micronutrients are also excellent markers of fruit and vegetable intake (Negri *et al.*, 2002). Diets that are high in varied types of plant foods contain an immense variety of other biologically active constituents that can be shown to inhibit carcinogenesis in animals and *in vitro* systems. Discovering the mechanisms that underlie the beneficial effects of fruits and vegetables in human populations is turning out to be far more difficult than seemed probable two decades ago. Nevertheless substantial progress has been made. In this chapter the broad principles of carcinogenesis and its inhibition are considered and the various ways in which nutrients and phytochemicals can influence the initiation and development of this group of diseases are reviewed.

3.2 What is cancer?

The existence of cancer and the distinction between benign and malignant tumours were recognised by the early Greek physicians, who coined the term 'carcinoma', derived from the Greek *karkinos*, meaning 'crab', alluding to the creeping crab-like behaviour of a spreading tumour. The development of

Table 3.1 Analysis of the level of evidence of protection provided by studies on fruit and vegetables and cancers (from Gerber, 2000)

Cancer sites	CNERNA (France 1996)	World Cancer Research Fund (USA 1997)	COMA Food and Nutrition Policy (UK 1998)
Mouth and pharynx	consistent	convincing	fruit: weakly consistent vegetables: inconsistent
Larynx	consistent	probable	moderately consistent
Oesophagus	consistent	convincing	strongly consistent
Lung and respiratory tract	consistent	convincing	fruit: moderately consistent vegetables: weakly consistent
Stomach	consistent	convincing	moderately consistent
Colon-rectum	vegetables: moderately consistent	vegetables: convincing	vegetables: moderately to weakly consistent
Pancreas	consistent	probable	consistent but limited
Liver	ND	vegetables: possible	ND
Breast	inconsistent	green vegetables: probable	green/yellow vegetables: moderately consistent
Ovary	inconsistent	possible	inconsistent
Endometrium	inconsistent	insufficient	inconsistent
Cervix	ND	possible	consistent but limited
Prostate	inconsistent	vegetables: possible	vegetables: moderately consistent but limited
Kidney	ND	vegetables: possible	ND
Bladder	ND	probable	moderately consistent but limited
Thyroid	ND	possible	ND

ND = not determined.

microscopy eventually led to the recognition that tumours contained cells that differed fundamentally in appearance and behaviour from those of the surrounding tissue. Oncology, the scientific investigation and clinical treatment of tumours, was founded in the early years of the twentieth century. However, it is only since the 1980s that the development of the cell and molecular sciences has enabled biologists to begin to acquire a deeper understanding of tumour biology. Much of this insight has been gained through the use of isolated tumour cells grown *in vitro*, and of animal models of carcinogenesis, which enable tumours to be studied within the complex environment of living tissue. Both of these approaches have their limitations, and we are still far from a full understanding of cancer in human beings.

All cancers are diseases of abnormal cell proliferation, development and death. During the earliest stages of human life all of the embryonic cells divide constantly and differentiate to form the specialised tissues and organs.

Throughout infancy and childhood, cell proliferation continues at whatever rates are necessary to fulfil the requirements of growth. As maturity is reached, organs such as the central nervous system, muscles and skeletal tissues cease to grow, and cell division becomes minimal. However, certain tissues continue to proliferate throughout life. These include the blood-forming tissues, the epithelia which line the surfaces of the body exposed to the environment, the glandular tissues which produce secretions, and the sexual organs which produce new reproductive cells. Cancer can affect virtually any organ of the body, but tissues such as those of the lungs and gut, which have characteristically high rates of cell division and chronic exposure to the external environment, are particularly vulnerable.

A tumour can be defined as any focal accumulation of cells beyond the numbers required for the development, repair or function of a tissue. Tumours may be benign or malignant. The former are usually relatively slow growing, but, more importantly, the cells tend to retain much of the specialisation and spatial localisation of the tissue from which they are derived. In contrast, malignant cells are characterised by a loss of differentiation, faster growth, and a tendency to invade surrounding tissues and migrate to other organs to form secondary tumours or metastases. Cancer may be defined as the development, growth and metastatic spread of a malignant neoplasm. Malignant tumours derived from epithelial cells are called carcinomas, and those derived from connective or mesenchymal cells are called sarcomas. It is usually the secondary tumour that is lethal, so the early diagnosis of malignant primary tumours is essential for effective treatment.

3.3 The nature of tumour growth

Regardless of their function in the body, all cells carry a complete set of genetic instructions for the development and function of the whole organism. The subset of genes which is expressed by any particular cell type determines its phenotype, the precise details of the structure, specialised functions and life cycle of the cell which enable it to exist in harmony with other cells as part of a tissue. The events that occur during the early stages of cancer development usually involve damage to the DNA coding for such crucial genes.

With the exception of certain cancers of childhood which often affect growing tissues such as the brain or bones, carcinogenesis – the development of cancer from normal cells – is usually a relatively slow process which occupies a substantial proportion of the lifetime of an individual. Tumour cells invariably contain a number of mutations affecting genes controlling the rate at which cells divide, differentiate or die, or the efficiency with which DNA damage is repaired (Anderson *et al.*, 1991; Fearon and Vogelstein, 1990). Such mutations may be inherited through the germ-line, and these form the basis for a number of recognised familial cancer syndromes. However,

most of the genetic abnormalities detectable in sporadic cancers, which are far more common, are somatic mutations acquired during carcinogenesis.

Such damage may result from exposure to radiation or chemical mutagens, or through the effects of molecular species such as oxygen free radicals generated by the normal metabolism of the body. Whatever the source of the DNA damage, however, the defining characteristic of a pro-carcinogenic mutation is that it favours the proliferation and survival of an abnormal population of cells that have the potential for further evolution towards the malignant state (Nowell, 1976). Chemical carcinogens such as those present in tobacco smoke tend to be electrophiles – substances that can react easily with electron-rich regions of cellular proteins and DNA. The products formed by such interactions with DNA are called adducts. These are stable compounds which disrupt the syntheses of new DNA when the cell net divides, so that the sequence of genetic code in that region is damaged and the new cell carries a mutation. Many chemical carcinogens must be activated to an electrophilic form before they can act and, ironically, this often occurs as part of the sequence of events employed by the cell to detoxify the parent molecule or pro-carcinogen.

Many of the target genes that undergo mutation during carcinogenesis have been identified, and their functions and interactions with other genes are at least partially understood (Anderson *et al.*, 1992). The proto-oncogenes were first identified through their near-homology to the critical DNA sequences present in certain cancer-causing viruses which, when inserted into mammalian cells, would transform them into tumours. These so-called viral oncogenes have evolved through the 'capture' and exploitation of mammalian genes by viruses. In their original form such genes are essential components of normal mammalian cellular physiology and are expressed, usually to facilitate increased cellular proliferation, only at critical stages in the development or function of a tissue. When such 'proto-oncogenes' are activated inappropriately within the mammalian genome, without the intervention of a virus, they are termed 'oncogenes'. This can occur because of a mutation to the control sequence for the gene, causing over-expression of the normal product, or a mutation in the coding sequence itself, giving rise to a product that functions normally but which cannot be broken down. For example, the K-ras gene, which codes for a protein-regulating cell proliferation, is mutated and hence abnormally expressed early in the development of approximately 40% of human colorectal carcinomas (Bos *et al.*, 1987).

In contrast to the proto-oncogenes, over-expression of which creates conditions that favour tumour growth, it is the loss of expression of a tumour-suppressor gene that facilitates development of malignant characteristics in a cell. The p53 gene is a good example (Donehower and Bradley, 1993). The p53 product is a protein of molecular weight 53 kD, which functions as a regulator of cell proliferation and as a mediator of programmed cell death in response to unrepaired DNA damage. The absence of p53, or its presence in a mutated and therefore non-functional form, allows cells bearing other forms

of DNA damage to continue dividing rather than undergoing apoptosis (Baker *et al.*, 1989; Gerwin *et al.*, 1992). There are familial forms of cancer caused by an inherited p53 defect, and acquired mutations of this gene are among the most common genomic abnormalities found in a variety of human cancers.

According to the 'two hit hypothesis' for the functional role of tumour-suppressor genes, mutations at both alleles are required to fully inactivate the tumour-suppressor activity of such genes (Knudson, 1989). However, another important mechanism for the induction of genetic abnormalities has attracted attention in recent years. Cytosine bases in the DNA backbone can acquire a methyl group which, if they lie within the promoter region of a gene, can cause it to be 'silenced' or, in effect, switched off (Kass *et al.*, 1997). This is a normal mechanism for the regulation of gene expression, but it is becoming clear that abnormal DNA methylation can also occur and be transmitted across successive cell divisions. This provides a so-called 'epigenetic' mechanism for the inactivation of genes regulating tumour suppression or DNA repair, which can contribute to the complex series of events leading to the development of a tumour (Jones and Laird, 1999).

3.4 Models of carcinogenesis

The simplest experimental model of carcinogenesis is the three-stage model (Pitot and Dragan, 1994) consisting of:

- initiation;
- promotion;
- progression.

At the initiation stage, a single cell is thought to acquire a mutation and then divide repeatedly so that the mutation is passed on to a clone of daughter cells, thus forming a focal lesion that can survive and grow at the expense of neighbouring cells. During promotion, the normal constraints on proliferation and spatial organisation within the affected tissue are disrupted further. The appearance of further mutations to proto-oncogenes and tumour-suppressor genes leads to a progressive loss of differentiation and orderly growth. The genes involved in this transition to cancer, and the functions they perform, are under intensive investigation and are particularly well characterised in the intestinal epithelium (Stappenbeck *et al.*, 1998). At the progression stage the lesion has made the transition to malignancy and can give rise to secondary tumours at remote sites. Animal models have been used to identify specific carcinogenic substances which can act as:

- mutagens at the initiation stage but do induce malignancy on their own;
- promoters which cannot initiate tumours, but do accelerate tumour development after initiation;
- complete carcinogens which can do both.

As we shall see later, this approach has also been used to identify inhibitors of carcinogenesis and to delineate their mode of action. The difficulty with animal models of carcinogenesis is that they usually require the application of large doses of carcinogens and promoters to groups of rodents, so that a high tumour yield is obtained during the course of the experiment. Such techniques are a poor model for induction of human cancers because these are usually caused by very prolonged exposure to a complex array of unknown carcinogenic stimuli over the course of a lifetime. However, there is no doubt that much of the fundamental understanding of tumour biology that has been gained from animal studies applies also to human disease.

3.5 Diet and gene interactions

As we have seen, carcinogenesis is a prolonged multi-stage process which usually occurs over many years. Because of its complexity there are, in principle, many critical steps at which food-related substances or metabolic processes may interact with the sequence of events so as to accelerate, delay or even reverse it. Diet-related anti-carcinogenesis can usefully be classified into:

- blocking mechanisms, which operate during the initiation phase of carcinogenesis;
- suppressing mechanisms, which delay or reverse tumour promotion at a later stage (Johnson *et al.*, 1994; Wattenberg, 1990).

A schematic illustration of these concepts and a summary of the mechanisms through which they may act is given in Fig. 3.1.

The principal blocking mechanism through which dietary constituents are thought to act is modulation of the Phase I and Phase II biotransformation enzymes which are expressed strongly in the gastrointestinal mucosa and in the liver and which act as a first line of defence against toxic substances in the environment (Greenwald *et al.*, 1990). Phase I enzymes, such as the cytochrome P450 complex, catalyse oxidation, reduction and hydrolytic reactions, thereby increasing the solubility of potentially toxic compounds. However, this phase may also create electrophilic intermediates and hence activate pro-carcinogens. Phase II enzymes, such as glutathione S-transferase, act on the products of Phase I metabolism to form conjugates, which generally reduces their reactivity and increases their excretion. The biological activity of a carcinogen will therefore often depend upon the relative activities of the Phase I and II enzymes involved in its metabolism. Pharmacological and dietary treatments can be used to block Phase I enzymes and enhance Phase II activity, so as to minimise the activation of carcinogens and increase their excretion. There is good evidence from experimental animal studies that this strategy can reduce DNA damage and tumour yield (Primiano *et al.*, 1995).

Experimental animal studies have also shown that some substances can

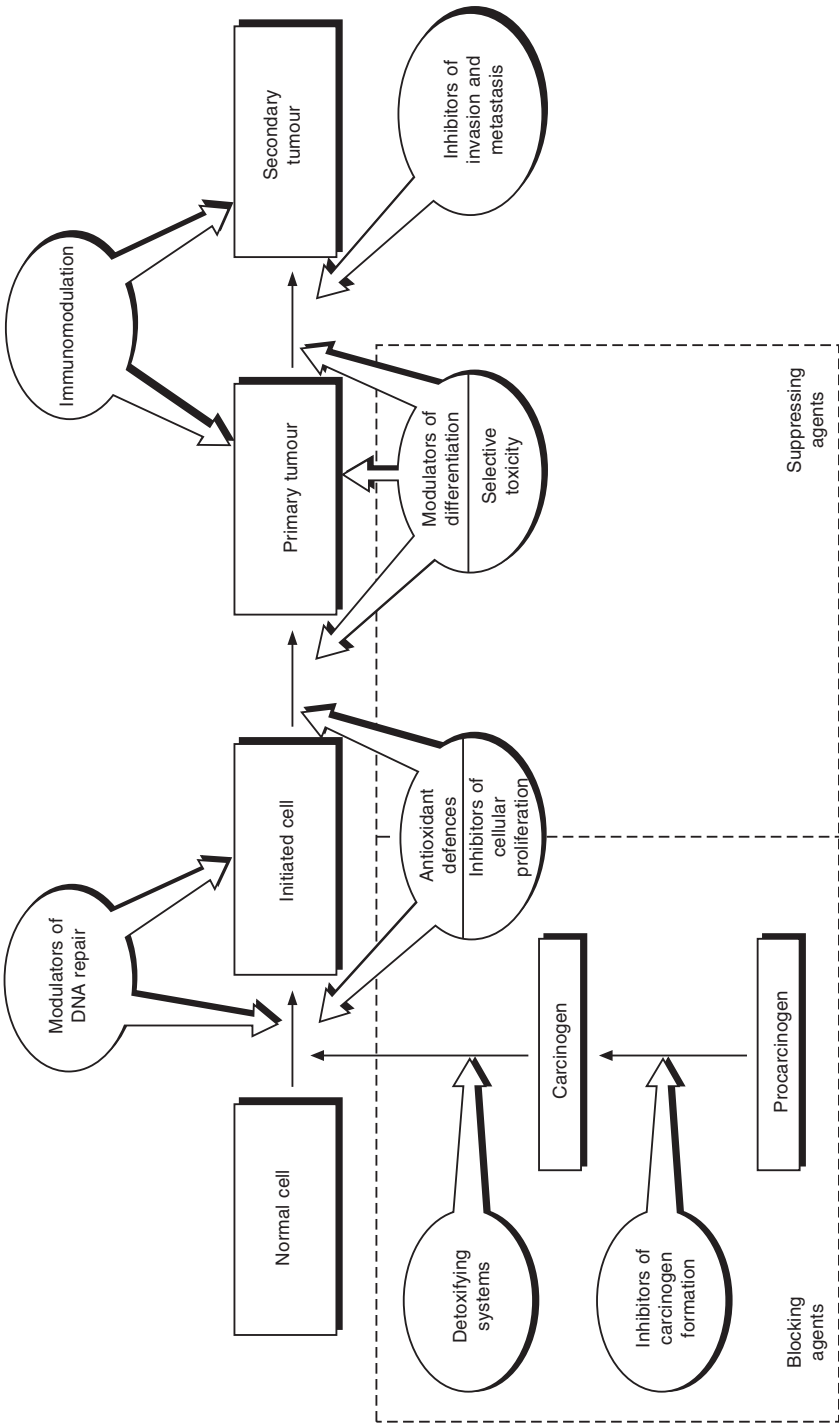


Fig. 3.1 Hypothetical sites of interaction between anti-carcinogenic substances in the diet and the progressive stages of carcinogenesis. Blocking agents are those acting to prevent initiation, whereas suppressing agents act to inhibit the development of tumours from initiated cells. (Reproduced from Johnson *et al.*, 1994)

inhibit or suppress the appearance of tumours, even when given days or weeks after exposure to a chemical carcinogen (Wattenberg, 1981). The mechanism of action cannot therefore involve protection against DNA damage, but instead must be due to some reduction in the rate at which initiated cells develop into tumours (Fig. 3.1). Suppression of carcinogenesis may involve inhibition of mitosis and increased expression of the differentiated phenotype, which serves to reduce the clonal expansion of initiated cells. It may also involve an increased susceptibility to undergo programmed cell death or apoptosis, which can eliminate pre-cancerous cells from the tissue (Chan *et al.*, 1998; Smith *et al.*, 1998).

3.6 Cancer risk and particular nutrients

Prolonged energy and protein malnutrition, or specific micronutrient deficiencies, may increase an individual's risk of cancer, perhaps because of an adverse effect on the immune system. However, life expectancy in societies with large malnourished populations is low, and infectious diseases are more likely to be the principal causes of illness and mortality. In prosperous Western societies, over-consumption of energy, coupled with inadequate exercise, appears to be a major risk factor for cancer. The World Cancer Research Fund report on diet and cancer made some general recommendations on food supply, eating and related factors (World Cancer Research Fund, 1997). For individuals, the general advice was to consume nutritionally adequate and varied diets based predominantly on fruits, vegetables, pulses and minimally processed starchy foods. Overweight, defined as body mass index (BMI: weight in kg/height in metres) in excess of 25 is associated with a rise in the relative risk of most cancers, and frank obesity is particularly associated with cancers of the breast and endometrium (WHO, 1997). For these reasons the report recommended that BMI should be maintained between 18.5 and 25. The committee did not consider that fat consumption was directly associated with cancer risk, but it did recommend that fat should contribute no more than 30% of total energy consumption, so as to reduce the risk of weight gain.

The general recommendations on energy and fat intake are similar to those for the avoidance of heart disease. However, the recommendation to consume a variety of fruits and vegetables is based partly on the putative presence of diverse protective factors in plant foods. This concept does provide, at least in principle, a rationale for the functional health benefits of plant foods beyond the simple provision of nutrients at a level that prevents symptoms of deficiency.

3.6.1 Vitamin D

Vitamin D is the name given to a group of fat-soluble compounds essential for maintaining the mineral balance of the body. Vitamin D is also known as

calciferol and as the anti-rachitic vitamin, and its principal function is to regulate calcium and phosphate metabolism. It has two main forms: ergocalciferol or vitamin D₂ (plant origin) and cholecalciferol or vitamin D₃ (animal origin). Vitamin D is produced from endogenous sources, synthesised in the skin from 7-dehydrocholesterol (7DHC) in a reaction catalysed by the ultra-violet (UV) light and exogenous sources from the diet. There are only a few natural food sources: egg yolk, oily fish, butter and milk. Margarines and spreads are also fortified with vitamin D. However, the biggest source results from exposure to sunlight (Maxwell, 2001). There are at least 37 metabolites of vitamin D (Norman, 1990) but only three – 25 hydroxyvitamin D (25-OHD), 1,25 dihydroxyvitamin D₃ (1,25-OHD) and 24, 25 dihydroxyvitamin D (24,25-OHD) – have any important biological activity.

Mortality rates from colon cancer are highest in those areas that receive the least amount of sunlight. A prospective study of 26,000 volunteers investigated the association between 25-OHD and the risk of colon cancer. In those with 25-OHD concentration of 50 nmol/L (20 ng/ml) or more, the risk of colon cancer was decreased threefold. Confounding factors such as consumption of milk, meat or fat in the diet were not considered. However, these observations and previous epidemiological and laboratory studies suggest that good vitamin D status in conjunction with calcium nutrition might lower the risk of colon cancer (Garland *et al.*, 1989).

3.6.2 Vitamin C

Vitamin C occurs as L-ascorbic acid and dihydroascorbic acid in fruits, vegetables and potatoes, as well as in processed foods to which it has been added as an antioxidant. The only wholly undisputed function of vitamin C is the prevention of scurvy. Although this is the physiological rationale for the currently recommended intake levels, there is growing evidence that vitamin C may provide additional protective effects against other diseases including cancer, and the recommended dietary allowance (RDA) may be increased in the near future. Scurvy develops in adults whose habitual intake of vitamin C falls below 1 mg/d, and under experimental conditions 10 mg/d is sufficient to prevent or alleviate symptoms (Bartley *et al.*, 1953). The RDA is 60 mg per day in the USA, but plasma levels of ascorbate do not achieve saturation until daily intakes reach around 100 mg (Bates *et al.*, 1979). Most of the ascorbate in human diets is derived from natural sources, and consumers who eat five portions, or about 400–500 g, of fruits and vegetables per day could obtain as much as 200 mg of ascorbate.

Ascorbate is probably the most effective water-soluble antioxidant in the plasma. It scavenges and reduces nitrite, thus inhibiting the formation of carcinogenic N-nitroso compounds in the stomach, and *in vitro* studies suggest that it plays a protective role against oxidative damage to cell constituents and circulating lipoproteins (Frei *et al.*, 1989). The evidence suggests that vitamin C can act both as an antioxidant and a pro-oxidant. Ascorbic acid

can readily donate electrons to quench a variety of reactive free radicals and oxidant species and is easily returned to its reduced form by electron donors such as glutathione, flavonoids, tocopherol and NADPH (Zeigler *et al.*, 1996; Xu and Wells, 1996). Vitamin C is believed to be of fundamental importance as an antioxidant in tissues. The evidence suggests that it protects against plasma lipid and low-density lipoprotein peroxidation by scavenging peroxy radicals from the aqueous phase before they can initiate lipid peroxidation, which it does by regenerating oxidised vitamin E to the active reduced form (Thurnham, 1994). *In vitro* vitamin C is rapidly lost when plasma is exposed to peroxy radicals, cigarette smoke or activated neutrophils, and its disappearance is accompanied by the onset of lipid peroxidation (Halliwell, 1994).

Vitamin C can also be violently pro-oxidant. *In vitro* it has been shown to release iron from ferritin and stimulate lipid peroxidation (Halliwell, 1994). The reducing properties of vitamin C are responsible for the conversion of Fe^{3+} to Fe^{2+} which is extremely important in the absorption of iron, but within the tissues could be potentially harmful because Fe^{2+} is a potent free-radical catalyst able to produce hydroxyl radicals (Stadtman, 1991). Stadtman and colleagues have argued that much ongoing protein damage involves metal ion-dependent OH generation, hence a small rise in OH generation over a lifetime could increase the incidence of age-related cancer (Tanner, 1976). In the healthy human body, most transition metal ions are safely sequestered and are available to catalyse free radical reactions. However, metals are always in transit within and between cells. It is possible therefore that interactions of metal ions with ascorbate could contribute to oxidative damage (Halliwell, 1994). It is probable nevertheless that the antioxidant properties of ascorbate predominate in healthy people most of the time.

The epidemiological evidence is consistent with a protective effect of vitamin C against cancers of the stomach, pharynx and oesophagus in particular (Block, 1991). The evidence for causality remains inconclusive because of the sheer complexity of the composition of fruits and vegetables, which are the main source of the vitamin in the unsupplemented diet. Byers and Guerrero considered the collective evidence from a large series of case-control and cohort studies in which intakes of fruits and vegetables, and of vitamin C and E from food or from supplements, were determined (Byers and Guerrero, 1995). There was a strong and consistent protective effect of fruits and vegetables against cancers of the alimentary tract and lung and a correlation with estimated vitamin C intake based on fruit and vegetable composition. However, there were considerable confounding effects of other dietary constituents and the evidence for a protective effect of vitamin C from supplements was less convincing. Supplementation trials with vitamin C using biomarkers of oxidative damage to DNA bases to measure levels of oxidative DNA damage *in vivo* showed little evidence of a beneficial effect, except where vitamin C intakes were low. In addition, no conclusive evidence of a protective effect of vitamin C in studies on strand breaks, micronuclei or

chromosomal aberrations was found (Halliwell, 2001). There is some evidence that diet-derived vitamin C may decrease gastric cancer in some populations, but whether this is due to its antioxidant or other properties is uncertain.

3.6.3 Vitamin E

The major lipid-soluble antioxidant is vitamin E, first isolated from wheatgerm oil and obtained principally from nuts, seed oils and cereals. Vitamin E is actually a collective term for eight compounds: α -, β -, γ - and δ -tocopherol, and α -, β -, γ - and δ -tocotrienol, but RRR- α -tocopherol accounts for 90% of endogenous vitamin E activity in humans. All the tocopherols and tocotrienols contain a hydroxyl-bearing aromatic ring structure, which enables them to donate hydrogen to free radicals, and thus act as biological antioxidants. The unpaired electron which results from hydrogen donation is delocalised into the ring structure of the tocopherol, rendering it relatively stable and unreactive. Chain reactions initiated by hydroxyl radicals can be broken by the formation of a stable radical as a result of interaction with vitamin E (Burton *et al.*, 1983). Vitamin E is readily incorporated into cell membranes, which, being rich in polyunsaturated fatty acids, are highly susceptible to damage by free radicals derived from metabolic activity. In humans, frank symptoms of vitamin E deficiency are only seen in premature infants or malabsorption states, but intakes higher than are required to protect against deficiency may provide additional protection against free-radical mediated DNA damage.

Epidemiological studies show a strong inverse correlation between risk of cancer and vitamin E intake at the population level, but the association is not corroborated by studies of individuals taking supplements (Byers and Guerrero, 1995). Moreover, a well-controlled investigation designed to test the hypothesis that dietary supplementation with vitamins C and E would reduce the recurrence of adenomas in patients who had undergone polypectomy showed no evidence of a protective effect (Greenberg *et al.*, 1994). Similarly a prolonged placebo-controlled intervention with vitamin E or vitamin E and β -carotene failed to prevent the development of lung cancer in smokers (Albanes *et al.*, 1995). A more recent review has suggested that, despite the use of high doses of vitamin E, large changes in the vitamin content of blood and liver, and extended periods of study in animals and humans (smokers and non-smokers), vitamin E does not appear to have affected repair products of oxidative DNA damage, sister-chromatid exchanges in peripheral lymphocytes or DNA adducts in lymphocytes (Morrissey and Sheehy, 1999).

3.6.4 Folate

In historical terms, folates are among the most recently identified of the vitamins. Wills was the first to describe a form of anaemia associated with pregnancy and malnutrition which could be cured by yeast or liver extract (Wills, 1933; Wills *et al.*, 1937). The active constituent of these dietary

supplements was eventually isolated as folic acid (pteroylglutamic acid), a water-soluble substance containing a pteridine ring linked to para-aminobenzoic acid and glutamic acid. Naturally occurring folates originate from green plants and yeast cells, and are plentiful in liver and kidney. They are usually reduced and substituted in the pteridene moiety, and contain up to seven glutamate residues. Dietary folates are deconjugated to the monoglutamic form at the surface of the intestinal mucosa, actively transported and mostly metabolised by the epithelial cells to the main circulating form which is 5-methyltetrahydrofolic acid. The principal metabolic role of folates and their derivatives is to act as coenzymes in reactions involving transfer of single carbon groups during the synthesis of amino acids and DNA. This accounts for their vital role in growth support, pregnancy and the production of blood cells. It has been conclusively established that an inadequate supply of folates during the early stages of embryonic development increases the risk of neural tube defects (Scott *et al.*, 1990), and a number of foods, including breakfast cereals and bread, are now routinely enriched with folic acid. Growing interest in the relationship between human folate status and the long-term risk of disease will probably ensure that this trend continues.

It is well established that folate-deficient diets are associated with increased risk of hepatic cancer in animal models (Dizik *et al.*, 1991). Rats fed diets deficient in methyl donating groups have higher rates of cell proliferation, increased DNA damage and a higher susceptibility to experimentally induced cancers, which appears to result from changes in gene expression associated with abnormalities of DNA synthesis (Pogribny *et al.*, 1995). The precise relationship between folate metabolism and carcinogenesis is unclear, but the link may lie in the role that folate coenzymes play in the control of DNA methylation. In mammals and many other organisms the cytosine nucleotides in the DNA backbone frequently become methylated by the enzyme DNA-methyltransferase (DNA-MTase) after replication. As mentioned earlier, the methylation pattern of the cytosine residues is now believed to be an important determinant of gene expression. Much remains to be learned about this topic but, in general, loss of methylation could cause abnormal expression of oncogenes controlling cell proliferation, whereas inappropriate methylation of cytosine-rich regions of DNA in the promotor regions of tumour-suppressor genes could cause loss of function (Baylin *et al.*, 1998).

Issa *et al.* (1994) demonstrated that methylation of CpG islands in the estrogen receptor (*ER*) gene occurs in a very high proportion of colorectal tumours, and that the same site-specific abnormality occurs progressively with age in the otherwise normal colorectal mucosa of human subjects with no colorectal neoplasia. The same authors have also shown that expression of *ER* in tumour cells slows mitosis and should perhaps be regarded as a tumour-suppressor gene, the silencing of which may be an early 'field' event inducing hyperproliferation and predisposing the colorectal mucosa to induction of neoplasia. There is no direct evidence that human folate metabolism is involved with these phenomena, but there is circumstantial evidence that inadequate

folate nutrition is a risk factor for cancer, particularly of the bowel and cervix (Freudenheim *et al.*, 1991).

Ma *et al.* (1997) have explored the relationship between risk of colorectal carcinoma and a common mutation affecting the activity of the enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) in a large cohort study. The presence of a homozygous mutation was shown to reduce the risk of colorectal cancer in men with adequate folate levels, but the protection was absent in men with low overall folate status. One possible explanation for this effect is that low levels of MTHFR expression shunt folates into DNA synthesis, thereby helping to maintain normal patterns of DNA methylation. Low levels of folate might favour hypomethylation of cytosines, and possibly cause a compensatory upregulation of DNA-MTase, leading to hypermethylation of CpG islands, but this remains highly speculative. Nevertheless the growing epidemiological evidence that inadequate folate nutrition increases the risk of cancer, whereas long-term use of folate supplements reduces the risk (Giovannucci *et al.*, 1998), will ensure that interest in the preventative role of folate-supplemented foods will continue.

3.7 Phytochemicals

The discovery that, in industrialised societies, diets deficient in fruits and vegetables can effectively double the risk of developing many different types of cancer has focused renewed attention on the beneficial properties of these foods (Block *et al.*, 1992; Patterson *et al.*, 1990; Southon and Faulks, 2002). As we have seen, plant foods are rich in micronutrients, but they also contain an immense variety of biologically active secondary metabolites providing colour, flavour and natural toxicity to pests and sometimes humans (Johnson *et al.*, 1994). The chemistry and classification of such substances is still a matter for much research and debate, but this has not prevented attempts to isolate and exploit substances that have variously been termed 'protective factors', 'phytoprotectants', 'phytochemicals' and 'nutraceuticals'. Phytochemical compounds include:

- non-nutrient carotenoids;
- compounds containing phenol rings (phenolic compounds), notably flavonoids;
- plant-derived estrogens (phytoestrogens);
- a group of sulphur compounds from brassica vegetables (glucosinolates).

Other compounds include the plant-derived sterols (phytosterols) and other sulphur-containing compounds found in onions and their relatives. Only a few of the more important examples will be discussed in the following sections.

As mentioned earlier, cancerous mutations can occur as a result of oxidative damage to DNA caused by free radicals generated as a damaging side-effect

of aerobic metabolism (Feig *et al.*, 1994). Superoxide radicals are formed by the addition of an electron to molecular oxygen. These highly reactive species can then acquire a further electron and combine with protons to form hydrogen peroxide. In the presence of transition metal ions such as Fe^{2+} and Cu^{2+} , hydrogen peroxide can break down to give even more highly reactive hydroxy radicals which may damage DNA directly, or participate in self-propagating chain reactions with membrane lipids. Plant and animal cells defend themselves against these effects by deploying so-called antioxidant compounds to trap or quench free radicals and hence arrest their damaging reactions. A variety of defence systems based on both water- and lipid-soluble antioxidant species and on antioxidant enzymes are deployed throughout the intra- and extracellular environment, at the sites most vulnerable to pro-oxidant damage. Many of those in the human body are dependent upon antioxidants derived from the diet.

The theory that free radicals are a major cause of human cancer and that the risk of disease can be reduced by increased consumption of food-borne antioxidants has prompted an enormous growth of interest in antioxidant nutrients and other antioxidant substances in food (McLarty, 1997). It is worth noting, however, that the role of mutagenesis due to oxygen free radicals in the pathogenesis of human cancers remains largely hypothetical (Feig *et al.*, 1994), and attempts to prevent cancer by intervention with high doses of antioxidant vitamins have, as discussed earlier, been largely unsuccessful (Albanes *et al.*, 1995; Greenberg *et al.*, 1994).

3.8 Carotenoids

The α -tocopherol, β -carotene (ATBC) Cancer Prevention study was a randomised-controlled trial that tested the effects of daily doses of either 50 mg (50 IU) vitamin E (all-racemic α -tocopherol acetate), or 20 mg of β -carotene, or both with that of a placebo, in a population of more than 29,000 male smokers for 5–8 years. No reduction in lung cancer or major coronary events was observed with any of the treatments. What was more startling was the unexpected increases in risk of death from lung cancer and ischemic heart disease with β -carotene supplementation (ATBC Cancer Prevention Study Group, 1994). Increases in the risk of both lung cancer and cardiovascular disease mortality were also observed in the β -carotene and Retinol Efficacy Trial (CARET), which tested the effects of combined treatment with 30 mg/d β -carotene and retinyl palmitate (25,000 IU/d) in 18,000 men and women with a history of cigarette smoking or occupational exposure to asbestos (Hennekens *et al.*, 1996).

The third study was the Physicians Health Study, in which 22,071 US male physicians were randomised to get either 50 mg β -carotene or 325 mg aspirin, or both, or neither, every other day for 12 years. There was no evidence of a significant beneficial or harmful effect on cancer or cardiovascular

disease, but the number of smokers in the study was too small to be certain whether β -carotene was harmful in the group or not (Hennekens *et al.*, 1996).

The antioxidant activities of carotenoids and other phytochemicals in the human body can be measured, or at least estimated, by a variety of techniques, *in vitro*, *in vivo* or *ex vivo* (Krinsky, 2001). Many studies describe the use of *ex vivo* methods to measure the oxidisability of low-density lipoprotein (LDL) particles after dietary intervention with carotene-rich foods. However, the difficulty with this approach is that complex plant foods usually also contain other carotenoids, ascorbate, flavonoids, and other compounds that have antioxidant activity, and it is difficult to attribute the results to any particular class of compounds. One study, in which subjects were given additional fruits and vegetables, demonstrated an increase in the resistance of LDL to oxidation (Hininger *et al.*, 1997), but two other showed no effect (Chopra *et al.*, 1996; van het Hof *et al.*, 1999). These differing outcomes may have been due to systematic differences in the experimental protocols or in the populations studied (Krinsky, 2001), but the results do indicate the complexity of the problem, and the hazards of generalising too readily about the putative benefits of dietary antioxidants.

There is strong epidemiological evidence that a relatively low level of β -carotene in the plasma is associated with an increased risk of lung cancer. Zeigler *et al.* (1996) reviewed epidemiological studies of diet and lung cancer and reported that a high carotenoid intake was associated with a reduced risk in eight cohort studies and 18 out of 20 retrospective studies. Such associations can be interpreted as evidence for a protective effect of β -carotene but, in the absence of more direct evidence, they do not prove a causal link. To test the hypothesis directly, three major intervention trials were launched with a view to testing the protective effects of carotenoids against lung cancer in human volunteers.

One other study deserves a mention. The Cancer Prevention Study II was a prospective investigation using a very large cohort of over one million adult Americans, in which the effects of commercial multivitamin supplements and vitamins A, C or E on mortality were studied, during a follow-up period of seven years. The results were complex in that the use of multivitamins plus vitamins A, C and/or E significantly reduced the risk of lung cancer in both former smokers and life-long non-smokers, but vitamins A, C and E apparently increased the risk in current smokers.

Overall these studies suggest a tendency for certain antioxidant vitamins, and particularly β -carotene, to increase the risk of lung cancer in smokers or other individuals who are already at a high risk of developing the disease. The reasons for this have not been established, but there seems to be a strong possibility that high doses of β -carotene may promote the development of already established pre-cancerous lesions (Hughes, 2001). If this is so, then the promotional effects may be mediated by an interaction between β -carotene and the immune system. There is no doubt that immune cells are particularly sensitive to oxidative stress. Immune mechanisms are mediated largely via

interactions between membrane-bound receptors that occupy lipid-domains that are rich in polyunsaturated fatty acids. Lipid peroxidation alters the fluidity of such membranes and impairs the expression and function of receptors, and this in turn disrupts cellular signal pathways. Although optimal levels of carotenoids and tocopherols undoubtedly confer protection against membrane peroxidation, it is possible that the high levels of β -carotene used in some of the intervention trials may have exerted pro-oxidant effects. Such a mechanism might be particularly likely in the lungs, where the oxygen tension is relatively high (Hughes, 2001; Palozza, 1998).

In two of the β -carotene intervention trials, subjects who had high plasma β -carotene levels at entry experienced a relatively low risk of lung cancer during follow-up (McDermott, 2000). The dilemma posed by these trials, therefore, is that, whilst a high dietary intake of this substance in the plasma appears to protect against cancers of the lung and other sites, supplementation at higher levels seems to have an adverse effect. How then can carotenoids be used to optimise immune function and minimise the risk of cancer? Perhaps the answer lies in the complexity of plant foods, and the fact that naturally occurring β -carotene is associated with a rich variety of other biologically active phytochemicals including other carotenoids, flavonoids and other polyphenols. High levels of supplementation may interfere with the absorption of other dietary micronutrients, or unbalance delicate interactions between these substances in the target tissues. Future studies may be better conducted using enriched food sources rather than single substances at supra-physiological levels. Carotenoids are discussed in more detail in Chapter 7.

3.9 Flavonoids

A huge variety of biologically active phenolic compounds containing one or more aromatic rings are found naturally in plant foods, where they provide much of the flavour, colour and texture. The simpler phenolic substances include:

- monophenols with a single benzene ring, such as 3-ethylphenol and 3,4-dimethylphenol found in fruits and seeds;
- the hydroxycinnamic acid group which contains caffeic and ferulic acid;
- the flavonoids and their glycosides which include catechins, proanthocyanins, anthocyanidins and flavonols;
- the tannins which are a complex and poorly defined group of water-soluble phenolics with high molecular weights.

The daily intake of phenolic substances may be as high as 1 g per day, but the quantity of defined flavonoids in the diet probably amounts to no more than a few tens of milligrams per day.

As long ago as the 1930s Rusznyák and Szent-Györgi proposed that the flavonoids were an essential dietary factor contributing to the maintenance

of capillary permeability (Rusznayk and Szent-Györgi, 1936). This is no longer thought to be true, but recent interest in dietary antioxidants and metabolically active phytochemicals has focused renewed attention on the possible beneficial effects of flavonoids (Hollman and Katan, 1997; Manach *et al.*, 1996). Flavonoids are very effective antioxidants, and it has been proposed that they protect against cardiovascular disease by reducing the oxidation of low-density lipoproteins. There is some epidemiological evidence for this, but flavonoids are generally poorly absorbed from food, and their effect on the overall antioxidant capacity of the plasma remains to be established.

Flavonoids and other phenolic substances may exert local anti-carcinogenic effects in the intestine where, in addition to acting as intraluminal antioxidants, they may induce Phase II xenobiotic metabolising enzymes and suppress the production of biologically active prostaglandins by inhibiting the arachidonic acid cascade (Formica and Regelson, 1995). They may also inhibit mitosis by inhibiting intracellular protein kinases (Yoshida *et al.*, 1990). Although briefly under suspicion as a natural carcinogen (MacGregor, 1984), the ubiquitous flavonol quercetin is now regarded as a possible protective factor against cancers of the alimentary tract (Deschner *et al.*, 1993). Flavonoids are discussed in more detail in Chapter 8.

3.10 Phytoestrogens

The phytoestrogens are diphenolic compounds derived from plant foods which bear a structural similarity to mammalian estrogens (Setchell and Cassidy, 1999). The glycosides genistin and daidzin, and their methylated derivatives biochanin A and formononetin, which are found principally in soya products, are broken down by the intestinal microflora to yield genistein, daidzein and, in some individuals, equol. These compounds are absorbed into the circulation, and they or their breakdown products can be detected in human urine (Axelson *et al.*, 1982). The lignan pre-cursors matairesinol and secoisolariciresinol occur more commonly in cereal seeds such as flax. They are also degraded in the gut to yield the active lignans enterolactone and enterodiol. These compounds exert weak hormone-like activity and may bind to estrogen receptors *in vivo*, thereby effectively blocking the more potent activity of endogenous estrogens.

In human feeding trials with soy products, isoflavones have been shown to modify the menstrual cycle, and there is much interest in the possibility that these compounds could suppress the growth of hormone-dependent tumours of the breast and reproductive organs (Setchell and Cassidy, 1999). There are also epidemiological associations suggesting a protective effect of soy-based diets against prostate cancer in males (Denis *et al.*, 1999), but once again the causal mechanisms have not been proven and there is a strong possibility of confounding by other dietary factors.

Genistein may also suppress tumour growth by other non-estrogenic mechanisms including suppression of cell turnover by inhibition of protein kinases involved in the regulation of mitosis. On the other hand, it is less widely recognised that genistein is an inhibitor of topoisomerase II, an enzyme that helps to maintain the structure of DNA during mitosis. Both synthetic topoisomerase poisons and genistein are known to be mutagenic *in vitro*, but the biological significance of this is unclear (Kaufman, 1998). There is no epidemiological evidence to suggest any adverse effect of soy products in humans, but caution is obviously necessary when considering the incorporation of such biologically active compounds into functional foods. Phytoestrogens are discussed in more detail in Chapters 5 and 6.

3.11 Glucosinolates

Interest in glucosinolates stems from epidemiological experimental evidence showing that brassica vegetables such as cabbage, sprouts, kale and broccoli seem to offer particularly strong protection against cancer of the lung and gastrointestinal tract (Steinmetz and Potter, 1996; Verhoeven *et al.*, 1996). The brassicas, and a few other edible plants drawn from the order *Capparales*, are the source of all the glucosinolates in the human diet. Around 100 different compounds have been identified, all of which possess the same fundamental structure comprising β -D-thioglucose group, a sulphonated oxime moiety and a variable side-chain (Fenwick *et al.*, 1983).

Glucosinolates occur throughout the plant, although the concentration varies between tissues, and they are stable under normal conditions. However, when the plant tissue is physically damaged, for example by food preparation or chewing, they come into contact with an enzyme – myrosinase – which is released from intracellular vacuoles. Myrosinase hydrolyses the glucosinolates to release glucose and an unstable product which then undergoes further degradation to release a complex variety of breakdown products. The most important from the nutritional point of view are the isothiocyanates, a group of hot and bitter compounds, commonly termed ‘mustard oils’. These compounds, which are often volatile with an acrid smell, are the principal source of flavour in mustard, radishes and the milder vegetables (Fenwick *et al.*, 1983). High levels of glucosinolates reduce the palatability of plant tissues for generalist herbivores such as birds and molluscs (Giamoustaris and Mithen, 1995). Glucosinolates with an aliphatic side-chain containing a β -hydroxy group yield isothiocyanates which spontaneously cyclise to form stable oxazolidine-2-thiones. These compounds are goitrogenic to domestic livestock, and this is an important limiting factor in the commercial exploitation of brassica feedstuffs (Heaney and Fenwick, 1995).

There is ample evidence from both animal experiments and tissue cultures studies to show that brassica vegetables and their constituents selectively induce Phase II enzymes. Evidence for the induction of Phase II enzymes by

two classes of glucosinolate breakdown products, the isothiocyanates and indole-3-carbinole, has been systematically reviewed by Verhoeven *et al.*, (1997). Particular attention has been paid to induction of Phase II enzymes by sulphoraphane, an isothiocyanate derived from broccoli (Talalay *et al.*, 1995), but other isothiocyanates derived from other common brassica vegetables probably exert comparable levels of biological activity (Hecht, 1999).

Wattenberg (1981) showed that both cruciferous vegetables and benzyl isothiocyanate could inhibit the appearance of tumours in experimental animals long after the initial exposure to a carcinogen. Suppressing mechanisms are still poorly understood, but one possibility is that glucosinolate breakdown products modulate the level of apoptosis in target tissues. Isothiocyanates have been shown to induce apoptosis in tissue culture, and in the colorectal crypts of the rat after treatment with the carcinogen dimethylhydrazine, an effect which is associated with a reduction in pre-cancerous lesions (Smith *et al.*, 1998). Glucosinolates are discussed in more detail in Chapter 4.

3.12 Other nutritional factors

Apart from recognised nutrients and the emerging plethora of potentially biologically active secondary plant metabolites or phytochemicals, a variety of other food-borne factors that are difficult to classify may play some role in the prevention of cancer. Epidemiological evidence suggests, for example, that consumption of a relatively high ratio of fish and poultry to red meat significantly decreases the risk of bowel cancer (Willet *et al.*, 1990). The reasons for this are unclear. Such diets may provide a favourable balance of amino acids or minerals, a relatively low intake of potentially pro-oxidant iron, or a relatively high intake of certain polyunsaturated fatty acids. However, far more research will be necessary before any underlying principles can be exploited for use in the context of functional foods.

Dietary fibre, which comprises all the non-digestible structural carbohydrates of plant cell walls and any associated lignin, provides a further example of a complex food-borne factor which cannot be classified as a nutrient, and which continues to generate debate over such issues as definition and analytical techniques. However, whatever the unresolved complexities, dietary fibre has a lengthy history and had proved itself eminently suitable as a component of functional food products long before the term was even coined.

3.13 Conclusion and future trends

Great progress has been made towards a better understanding of the relationship between diet and cancer since Doll and Peto published their study on the causes of human cancer in 1981 (Doll and Peto, 1981), but the practical

application of this knowledge in the fight against human disease remains frustratingly limited. As should now be clear, cancer is not a single disease arising from one causal event. In most cases the victim acquires the disease only after years of exposure to a host of environmental factors which will have interacted with his or her unique genome, throughout a large fraction of their lifespan. Even in the case of carcinoma of the lung, which is the most frequent cause of death from cancer in most Western countries, and which has a known and avoidable cause, it has required many years of patient epidemiological investigation to establish this relationship beyond doubt. Even in the case of lung cancer, it is still not possible to predict any individual smoker's particular level of risk with certainty. The problem of diet and cancer is immensely more difficult because of the variety of diseases involved and the complexity of human diets, and because the task requires the recognition and understanding of an array of protective factors rather than any single source of carcinogens.

As we have seen, some of the most compelling evidence for a protective effect of diets against cancer to emerge in recent years is that for fruit and vegetables (Block *et al.*, 1992; Steinmetz and Potter, 1991; Southon and Faulks, 2002). Despite the difficulties in disentangling the effects of diet from other aspects of lifestyle such as smoking, exercise and alcohol consumption, most authorities agree that, compared to those at the other end of the scale, the highest consumers of fruits and vegetables in most populations have about half the risk of developing most types of cancer. In an age of convenience foods and pre-cooked meals, many consumers find a high consumption of fresh vegetables difficult to achieve. At first sight this seems to provide an excellent opportunity for the development of functional food products which could provide the protective effects of fresh vegetables without the need for greatly increased bulk or frequency of consumption. The difficulty lies in the sheer complexity of plants and the bewildering variety of diseases to which the protective effects seem to apply. Diet is inescapably complex, and food often seems to exert biological effects greater than the sum of its parts. Much further research is required to warrant the complex connection between phytochemicals in foods and the risk of cancer.

3.14 References

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Food-borne glucosinolates and cancer

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4.1 Introduction

The evidence that fruits and vegetables protect against almost all important human cancers, other than those of the nervous system and lymphatic tissues, is strong and consistent, but rather imprecise, and generally gives very little indication as to which particular foods of plant origin are likely to be most effective¹. In order to develop public health strategies, to provide advice to consumers and, ultimately, to optimise the composition of new varieties of fruits and vegetables, much more information about the mechanism of action of particular plant constituents is needed². In this chapter we review the putative benefits and mechanisms of action of one group of biologically active compounds that are peculiar to brassica vegetables. The glucosinolates are a large family of sulphur-containing compounds that function as natural pesticides³ in plant families of the order *Capparales*⁴. In the human diet, glucosinolates and their breakdown products are obtained predominantly from cruciferous vegetables, and from various condiments such as mustard and horseradish⁵. Historically they have been considered as potential natural toxicants. Their goitrogenic effects, observed principally in livestock animals fed on brassica fodder crops, have been recognised for many years^{6,7}. Genotoxic and cytotoxic effects of some glucosinolate breakdown products have also been observed, principally in cell culture⁸⁻¹⁰, but also in some animal studies^{11,12}. Conversely, it has also been apparent since the 1960s that these compounds can suppress the development of cancer in a variety of experimental models¹³.

Epidemiological data on the protective effects of plant foods provides convincing evidence that a high consumption of brassica vegetables is inversely

associated with a variety of important cancers, including those of the lung and intestinal tract^{13–15}, and perhaps also the prostate¹⁶. These epidemiological studies are supported by an increasing body of experimental evidence that provides plausible mechanisms for the putative anti-carcinogenic effects of the glucosinolates. Before moving on to consider the biological effects of glucosinolates in the human diet, it is first necessary to describe something of their complex biochemistry, and their occurrence and functional activity in plants used as human food.

4.2 Sources, structures and metabolites of the glucosinolates

Beta-thioglucoside-N-hydroxysulphates (glucosinolates) occur in 16 families of dicotyledenous angiosperms. In Western industrialised countries the main sources of glucosinolates in the diet are the cruciferous vegetables – principally *Brassica spp* – which include cabbages, sprout, kale and broccoli, and *Raphanus spp*, the radishes^{17,18}. The generic structure of the glucosinolates (Fig. 4.1) consists of a sulphonated oxime moiety linked to glucose, and a highly variable side-chain, derived predominantly from one of the three amino acids, methionine, tryptophan or phenylalanine, or various from branched amino acids⁵. It is the variability of the side-chain that accounts for the large number of different glucosinolates. At the time of writing over 120 different glucosinolates have been identified, and no doubt many more remain to be discovered. Several different glucosinolates are present within any one type of vegetable, and the actual levels vary considerably with variety and growing conditions. Because glucosinolate breakdown products confer flavour, and in the case of certain forage crops, toxicity, they are of considerable interest to plant breeders¹⁹.

Glucosinolates remain stable in the intact plant, principally because they are confined within the vacuole, physically isolated from the membrane-associated hydrolytic enzyme myrosinase (EC 3:2:3:1). However, on disruption of the cell structure during attack by insect pests, food processing or chewing, the enzyme and substrate are brought together. The glucosinolate undergoes rapid hydrolysis, yielding glucose and an unstable aglycone, which can undergo a variety of spontaneous reactions to yield various intermediates, including isothiocyanates, thiocyanates and nitriles. Under domestic conditions, or in the alimentary tract, the most common reaction is a Lossen rearrangement, which yields isothiocyanates²⁰ (Fig. 4.1). These compounds provide the hot and acrid flavours associated with watercress, mustard, coleslaw, radish and Brussels sprouts. Table 4.1 lists the common names of some of the most studied glucosinolates, together with the chemical name and their dietary sources.

The actual level of glucosinolates in the human diet depends upon many

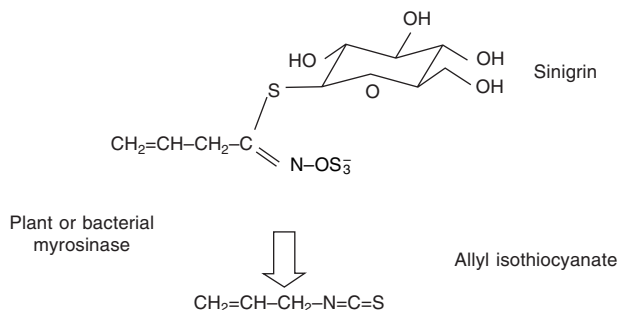


Fig. 4.1 Sinigrin is an aliphatic glucosinolate that occurs at significant levels in the human diet, notably in mustard and Brussels sprouts. When brought into contact with myrosinase, derived either from plant cells or from colonic bacteria, it is broken down to yield a variety of products including the acrid, volatile, biologically active compound allyl isothiocyanate.

factors including the processing and storage conditions that the vegetables have undergone prior to consumption²¹. Rodrigues and Rosa have described the behaviour of glucosinolates in a commercial broccoli variety (*Brassica oleracea* L. var. *Tokyodome*) in some detail²². The highest levels of glucosinolates were present in the primary and secondary inflorescences of the mature plant. Storage at room temperature caused a 75% decline in glucosinolate levels, but losses could be minimised by refrigeration or freezing. In contrast, Hansen *et al.*²³ described a 42% increase in total glucosinolates in another variety of broccoli ('Marathon'; *Brassica oleracea* L. var. *italica*) stored for seven days at 10°C in air. However, modifying the carbon dioxide content of the atmosphere could vary the behaviour of the glucosinolates. In contrast to broccoli, the levels of the glucosinolate sinigrin in white cabbage remain relatively stable during long-term storage²¹.

Myrosinase is a relatively thermolabile enzyme, which is readily denatured at temperatures above about 50°C, or lower, if the ambient pressure is raised²⁴. Thus cooking will inactivate myrosinase to varying degrees, depending on temperature, duration and other factors²⁵. Intact glucosinolates are therefore present in cooked vegetables and will not be susceptible to degradation during mastication and gastric digestion. However, cooking can lead to loss of glucosinolates and their metabolites through leaching into the cooking water, or loss of volatile isothiocyanates. Qualitatively, one can state that to maintain maximal glucosinolate content of brassica vegetables they should be eaten raw with minimal processing and preparation. However, since the thermal and physical effects of cooking conditions influence both the activity of myrosinase, through denaturation of the enzyme, and the level of glucosinolates and their breakdown product in the plant tissue, through thermal breakdown and leaching, the overall effects are extremely complex. Precise estimates of the behaviour of these compounds through the food chain are difficult to achieve, but Dekker *et al.*²⁶ have developed a predictive mathematical approach to this complex issue.

Table 4.1 Glucosinolates that have been studied in relation to their anti-carcinogenic characteristics and their common dietary sources

Glucosinolate common name	Side chain*	Vegetable source
epigoitrin	2-hydroxybut-3-enyl <i>eHBITC/eHBN</i>	† Sea kale/star mustard/ Abyssinian mustard
glucobrassicin	indol-3-ylmethyl <i>IMITC/IMN</i>	Brussels sprouts/broccoli/ cabbage/mustard/garden cress/ cauliflower
glucocheirolin	3-methylsulfonylpropyl- MSoPITC/MSoPN	Horseradish
glucoiberin	3-Methylsulfinylpropyl- <i>MSPITC/MSPN</i>	Brussels sprouts/broccoli/ cauliflower
gluconapin	but-3-enyl – <i>BEITC/BEN</i>	Brussels sprouts/red cabbage
gluconasturtum	phenylethyl- <i>PEITC</i>	Watercress/turnip
glucoraphanin	4-Methylsulfinylbutyl- <i>sulforaphane</i>	Broccoli
glucoraphenin	4-methylsulfinyl-3-butenyl – <i>MS3BITC/MS3BN</i>	Brassica sp.
glucosinalbin	p-hydroxybenzyl- <i>pHBITC/</i> <i>pHBN</i>	Mustard/garden cress
glucotropeolin	benzyl- <i>BITC</i>	Garden cress/horseradish/ mustard
neoglucobrassicin	1 Methoxyindol-3-ylmethyl	Brussels sprouts/broccoli
progoitrin	2-hydroxybut-3-enyl <i>HBITC</i>	Brussels sprouts/red cabbage
Sinigrin	2-propenyl (allyl)- <i>AITC</i>	Brussels sprouts/cabbage/ mustard/garden cress/cauliflower
_____	Phenylhexyl- <i>PHITC</i>	
_____	4-phenylbutyl- <i>PBITC</i>	Horseradish
_____	Phenyl- <i>PITC</i>	Horseradish

*isothiocyanates (ITC) or nitrile (N) abbreviation is included when discussed elsewhere in the text.

†plant source not necessarily part of the human diet.

4.3 Digestion and absorption

From the previous discussion it should now be clear that the route of uptake and assimilation of glucosinolates and their breakdown products depends crucially upon the timing and site of myrosinase activity during the consumption and digestion of brassica vegetables (Fig. 4.2). Since limited chopping induces only small amounts of glucosinolate loss at the cut surfaces of vegetables, large fragments, such as whole leaves or broccoli florets, will undergo only minimal loss of glucosinolates. If such vegetables are eaten raw, both intact glucosinolates and active myrosinase will be ingested simultaneously. The enzyme and its substrates will then be brought together within the oropharyngeal space, and glucosinolate breakdown products will be released into the stomach

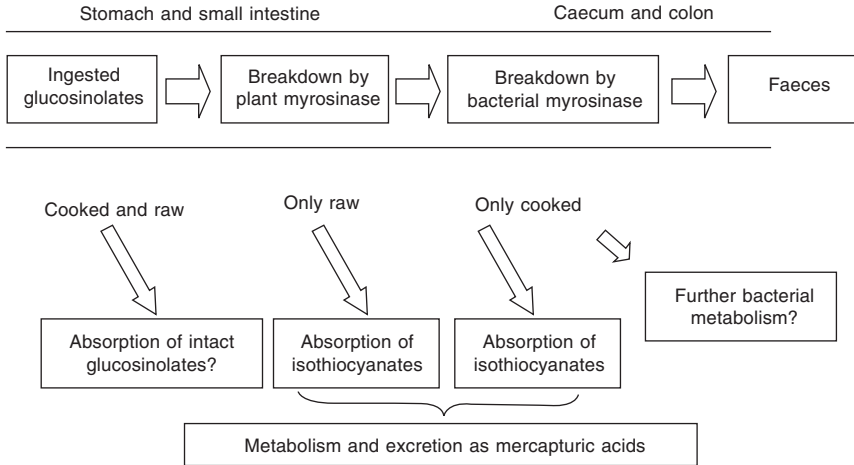


Fig. 4.2 A schematic summary of the possible fates of glucosinolates and their breakdown products following consumption of brassica vegetables. Glucosinolates may be consumed in cooked or raw vegetables. If cooked, the enzyme myrosinase is denatured, and an unknown quantity of intact glucosinolates may be absorbed in the upper gastrointestinal tract. If eaten raw, brassica vegetables retain their active myrosinase, enabling glucosinolate breakdown products to be released during mastication and digestion. Isothiocyanates are absorbed, metabolised, and excreted in urine as mercapturic acids. The colonic bacteria can also break down glucosinolates, releasing isothiocyanates and other metabolites from them into the faecal stream from which at least some are absorbed³³.

and small intestine. Isothiocyanates will then become available for absorption and should be detectable in the plasma quite soon after consumption. However, if the vegetables are cooked, myrosinase will be deactivated and any glucosinolates that are not leached, or destroyed by thermal degradation will be released into the gut lumen intact. In principle they are then available for passive absorption, or for eventual degradation by the microflora of the large bowel (Fig. 4.2).

There is good evidence that isothiocyanates are absorbed in the small intestine, conjugated with glutathione, and then metabolised to mercapturic acids. These appear in the urine as dithiocarbamates, which can be analysed and used as a convenient biomarker of total isothiocyanate intake and metabolism²⁷. Shapiro *et al.* (2001) studied the appearance of isothiocyanate metabolites in urine following the consumption of cooked and uncooked broccoli sprouts²⁸. The test-meals consisted of fresh uncooked sprouts containing both intact glucosinolates and active myrosinase, and cooked sprouts containing either intact glucosinolates or free isothiocyanates. Excretion of isothiocyanate metabolites increased significantly after consumption of sprouts, but the bioavailability of the free isothiocyanates was estimated to be about six times greater than that of intact glucosinolates. The positive relationship between intake and excretion of isothiocyanates was approximately linear over a 25–200 μm dose range. As might be expected, chewing the raw sprouts thoroughly to increase the exposure of the glucosinolates to myrosinase

improved the uptake of isothiocyanates from raw sprouts considerably. Recently the same group has described a method for the determination of dithiocarbamates in plasma, serum and erythrocytes, which enables them to study the pharmacokinetics of isothiocyanate absorption in detail²⁹. Volunteers consumed 200 μM of mixed isothiocyanates (sulphoraphane, iberin and erucin) from broccoli sprouts. Peak plasma concentrations of between 1 and 2.3 μM were achieved within one hour after feeding. The cumulative excretion at 8 h after consumption was about 58% of the ingested dose.

A number of studies have also confirmed that cooking significantly modifies the bioavailability of glucosinolates and their breakdown products from more conventional food sources. Getahun and Chung³⁰ fed watercress as a source of phenethylisothiocyanate (PEITC), and used urinary excretion of metabolites to assess absorption and metabolism. When volunteers ate 350 g of cooked watercress, containing 475 μmol glucosinolates, the excretion of isothiocyanates ranged from 1.2–7.3%. In contrast, when they ate 150 g of raw watercress, which retained its myrosinase activity, the excretion of isothiocyanates ranged from 17.2–77.7% of the 972 μmol of glucosinolates consumed. Shapiro *et al.*³¹ showed that the uptake and excretion of free isothiocyanate derived from horseradish was rapid and consistent with first order kinetics in the small intestine. When broccoli was eaten, the recovery of isothiocyanates as urinary metabolites was only about 10% of the quantity eaten, but this increased to 47% when the glucosinolates were degraded by treatment with myrosinase.

The fate of intact glucosinolates in the upper alimentary tract is not known with any certainty. Glucosinolates have been shown to cross the intestinal mucosa by passive transfer *in vitro*³², and studies with germ-free animals suggest that uptake may also occur in the small intestine *in vivo* (S. Rabot, personal communication). However, nothing is known about the fate or biological significance of any glucosinolates that may be absorbed intact by humans. It is clearly established that the bacterial microflora of the human colon do express myrosinase activity, and that intact glucosinolates reaching the colon are first hydrolysed, and then further degraded to a range of metabolites³³. Significant quantities of isothiocyanate metabolites are excreted in the urine of healthy human volunteers after eating brassica vegetables, even when myrosinase has been completely inactivated by cooking^{30,31}. This route of assimilation falls to negligible levels when the numbers of colonic bacteria are reduced by bowel preparation and antibiotics³¹. Rabot *et al.*³⁴ isolated a strain of *Bacteroides thetaiotaomicron* (II8) from human faeces, which was capable of degrading glucosinolates. In germ-free rats inoculated either with this bacterium alone or with an intact human microflora, consumption of sinigrin led to a considerably higher excretion of allyl isothiocyanate compared to germ-free animals, and a correspondingly lower faecal excretion of intact sinigrin³⁵.

Even if only a relatively small fraction of ingested glucosinolates reach the circulation as the result of metabolism by the gut microflora, degradation of glucosinolates in the faecal stream may be important as a source of

isothiocyanates and other breakdown products that can act directly on the colonocytes which line the intestinal mucosa³³. It is of some interest therefore to estimate the concentrations of these compounds within the colonic lumen. In their studies on rats mono-associated with *Bacteroides thetaiotaomicron*, Elfoul *et al.* showed that when the animals were gavaged with 50 µm/l sinigrin, they produced a peak concentration of 200 nm/l allyl isothiocyanate in the colonic lumen³⁵. Assuming that the same concentration ratio (1: 250) is achievable in humans, a hypothetical meal consisting of 10 Brussels sprouts might have a wet weight of approximately 100 g and provide 400 µm of glucosinolates, giving an intraluminal concentration of isothiocyanate of around 1.6 µm/l. This concentration of isothiocyanates is in the same order of magnitude as that needed to induce biologically important effects on colorectal cell lines *in vitro*.

4.4 Glucosinolate breakdown products and cancer

To the consumer, the most obvious biological effect of the glucosinolate breakdown products is to provide flavour to food. There is no doubt that these compounds are essential to the sensory qualities of our familiar brassica vegetables³⁶. Intact glucosinolates are themselves perceived as bitter by taste panels. In a detailed study of Brussels sprouts it has been shown that sinigrin, and to a lesser extent gluconapin and glucobrassicin, are the main determinants of bitterness in cooked sprouts³⁷. The minimum concentration at which 50% of the panel could detect sinigrin was about 100 mg/l. More recent studies have confirmed that both sinigrin and progoitrin contribute to the perceived bitterness of Brussels sprouts, and that varieties with low levels of these glucosinolates are generally rated as more acceptable by consumer panels³⁸. Pungency, as distinct from bitterness, is a characteristic of the volatile isothiocyanates, for which the trivial name is 'mustard oils'. These compounds produce sensations that can be described as 'acrid' and 'corrosive' and, though they are directly responsible for the special qualities of mustard and radishes, their consumption inevitably tends to be self-limiting.

The chemical and sensory properties of glucosinolate degradation products depend upon the structure of the side-chain in the glucosinolate from which they are derived. Thus the methylthioalkyl glucosinolates break down to release the more volatile and pungent isothiocyanates, whereas methylsulfinylalkyl glucosinolates yield much less of these volatile isothiocyanates with a less acrid flavour⁴. As with many biologically active phytochemicals, foods rich in bitter or acrid glucosinolates and their breakdown products are a minority taste. There has been a strong tendency in recent years for plant breeders to produce new varieties of vegetable with reduced bitterness¹⁹. However, breeding programmes aimed at improving consumer acceptability may also be reducing the beneficial effects of vegetables for human health³⁹.

The adverse effects of glucosinolate breakdown products derived from rapeseed glucosinolates on thyroid function, growth and fertility in livestock have been recognised for decades, and an immense amount of work has been done to investigate and control the problem^{40–43}. In contrast, there is little or no evidence that brassica vegetable consumption exerts adverse effects on otherwise well nourished humans, although it must be admitted that there has been relatively little research on the issue⁴⁴. In the absence of any convincing evidence for an adverse effect of glucosinolates and their breakdown products, much more attention is now devoted to the epidemiological evidence in favour of a protective effect of cruciferous vegetables against cancer^{14,15}.

Within the UK about 90% of cancers are *carcinomas* – solid tumours of epithelial origin – and of these 80% are associated with epithelia lining the bladder, lungs and alimentary tract. Nevertheless there are large geographical variations in the incidence of carcinomas of these tissues, indicating that in principle they are preventable. Even amongst smokers, fruits and vegetables appear to play an important role as determinants of lung cancer risk. As is discussed in other chapters, carcinogenesis is a prolonged multi-step process⁴⁵. The sequence of events can usefully be regarded as comprising an *initiation phase*, in which progenitor cells acquire a critical DNA lesion enabling them to survive and undergo clonal expansion, and a *promotion phase* in which the precancerous cells acquire further genetic and epigenetic abnormalities, leading to altered expression of genes regulating proliferation, differentiation and apoptosis. The initiation phase can be *blocked*, either by detoxification of a pro-carcinogen or by the removal of the carcinogen⁴⁶. Once the initial genetic damage has occurred, progression can be *suppressed* by processes that favour reduced cell proliferation, differentiation or deletion of initiated cells by apoptosis. Using animal models of carcinogenesis, Wattenberg demonstrated that a variety of drugs and naturally occurring food-borne substrates could inhibit carcinogenesis by acting as blocking and suppressing agents⁴⁶. Isothiocyanates are prominent amongst the phytochemicals that have been shown to exhibit one or other, and sometimes both, effects in animal models. Recent evidence suggests that certain isothiocyanates may also act against the human gastric microorganism *Helicobacter pylori*. This specialised bacterium has been implicated in the aetiology of gastric cancer, and this effect may constitute a further, possibly synergistic, protective effect of brassica vegetables in the human alimentary tract⁴⁷. Despite the difficulty of studying carcinogenesis in human subjects, there is increasing evidence that these mechanisms operate in human beings consuming brassica vegetables as part of their normal diets.

4.5 Blocking the initiation phase

The human body deploys an array of enzymes that metabolise potentially toxic compounds derived from the environment. Phase I enzymes such as the

cytochrome p450 family are monooxygenases that metabolise lipophilic pro-carcinogens, often converting them to highly carcinogenic epoxides in the process². Phase II enzymes such as the glutathione S-transferase family metabolise these products to form inactive, water-soluble conjugates that are readily excreted in urine. Consumption of foods rich in glucosinolates has been recognised as modifying these metabolic pathways of carcinogen and drug metabolism for over a decade⁴⁸⁻⁵¹. Although Phase I enzymes such as the cytochrome p450 family (CYP) and monooxygenases render xenobiotics more hydrophilic, this process can also activate pro-carcinogens, making them both more active, but also more susceptible to detoxification by Phase 2 enzymes, including GST, quinone reductase and UDP-glucuronyl transferases (UGT).

It is now generally agreed that a shift toward increased Phase 2 enzymes is beneficial in relation to removal of potential carcinogens and pro-carcinogens⁵². Numerous *in vitro* and animal studies have been undertaken to examine the impact of individual isothiocyanates (ITCs) on Phase 2 induction in a range of cell types, in particular hepatic and intestinal tract cells⁵³⁻⁵⁷, and a more limited number on the effects of nitriles^{53,54,58}, the latter being potent inducers of Phase 2 enzymes at doses that had no effect on cytotoxicity. Of particular note is that some indole glucosinolates can induce Phase I enzymes and therefore have the potential to increase carcinogenicity of environmental procarcinogens¹⁸. However, *in vivo* studies using lower levels of glucosinolates do not support a pro-carcinogenic role for ITCs.

Some of the most impressive evidence for the blocking effects of isothiocyanates *in vivo* comes from the work of Hecht and coworkers⁵⁹. Isothiocyanates such as PEITC, benzyl isothiocyanate (BITC) and sulforaphane modify the balance of Phase I and II xenobiotic metabolising enzymes that are expressed in liver, and in epithelial cells including those of the colon. Hecht and colleagues have studied the effects of PEITC on the induction of lung tumours in a rat model by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in considerable detail. In a chronic study with simultaneous administration of NNK and PEITC, lung tumours were induced in 70% of the control rats given only NNK, but only 5% of those co-treated with PEITC. In addition, there was a marked reduction in a biomarker of NNK activation, 4-hydroxy-1-(3-pyridyl)-1-butanone-releasing haemoglobin adducts, in rats given PEITC, and a significant increase in excretion of two NNK metabolites, (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol glucuronide). Taken together, these findings provide good evidence that PEITC, for which watercress is the major source in the human diet, has the potential to block the carcinogenic effects of tobacco smoke.

The involvement of tobacco smoke carcinogens in the aetiology of lung cancer is conclusively established, but the role of specific chemical carcinogens as inducers of colorectal cancer is much less clear. Mutagenic pyrolysis products derived from cooked food have come under suspicion as possible

colorectal carcinogens, however⁶⁰, and recently it has been reported that garden cress (*Lepidium sativum*), which is rich in the glucosinolate glucotropaeolin, the pre-cursor of BITC, inhibits the induction of precancerous colonic lesions (aberrant crypts) by 2-amino-3-methyl-imidazo [4,5-f] quinoline (IQ) in the rat⁶¹. The authors attributed this protective effect to an enhancement of the detoxification of IQ by the Phase 2 enzyme uridine-di-phospho-glucuronosyl transferase (UDPGT).

Of all the Phase 2 enzymes studied, the glutathione S-transferase (GST) family has received most attention in relation to cancer risk. There are numerous GSTs present in mammalian cells, and these have been classified historically into four families of proteins⁶². GST θ and GST μ are the most frequently studied sub-types in the context of cancer risk. A number of polymorphisms of the GSTT and GSTM genes occur in human populations, and these have been linked to changes in cancer risk in a range of tissues including lung, oesophagus, stomach and colon^{63,64}. The induction of GST in human volunteers fed test-meals of red cabbage and Brussels sprouts has recently been reported⁶⁵.

London *et al.*⁶⁶ described the relationship between the concentration of isothiocyanate metabolites in urine and subsequent risk of lung cancer in a large cohort of Chinese men. The presence of deletion-polymorphisms of the *GSTM1* and *GSTT1* genes, which code for different members of the GST enzyme family, were determined at the time of urine sampling. During 10 years of follow-up there were 232 cases of lung cancer, which were compared with 710 matched controls. Individuals with detectable levels of isothiocyanate metabolites in urine experienced about two thirds of the risk of cancer compared to controls. However, further analysis showed that the effect was observable only in subjects with homozygous deletion of *GSTM1* or *GSTT1*, and was strongest in those with deletion of both *GSTM1* and *GSTT1* in whom the relative risk of disease was 28% of the controls. These results, which were sustained when the analysis was restricted to present or past smokers, indicate a very complex interplay between environmental factors, genotype and risk of cancer. The GST enzymes play a major role in the detoxification of environmental mutagens, but they also metabolise anti-carcinogenic phytochemicals, including the isothiocyanates. Thus loss of *GSTT1* and *GSTM1* might be expected to compromise an individual's ability to metabolise and dispose of tobacco smoke carcinogens. This assumption is borne out by the observation that individuals with a null genotype for both *GSTM1* and *GSTT1* were at over two and a half times the risk for lung cancer if they lacked isothiocyanates in their urine, and therefore presumably consumed few brassica vegetables. Similar results, obtained in both Chinese and North American populations, are consistent with the emerging concept of interactions between polymorphisms of *GSTM1*, and *GSTT1* and a protective effect of a diet high in brassicas against lung cancer⁶⁶⁻⁶⁹. A similar finding has been reported for colorectal adenomatous polyps⁷⁰.

In all these studies, isothiocyanates appeared to reduce the risk of neoplasia preferentially amongst persons genetically deficient in *GSTT1* and *GSTM1*.

These observations provide something of a problem in that it cannot be the induction of *GSTT1* or *GSTM1* by isothiocyanates that is the protective mechanism against neoplasia. Rather the absence of these enzymes, which are known to play a major role in the metabolism and excretion of isothiocyanates, presumably favours the continued presence of isothiocyanates in the circulation. How then do the compounds inhibit neoplasia of the lung under these circumstances? One possibility is that other Phase 2 enzymes that detoxify chemical carcinogens are up-regulated by isothiocyanates persisting longer in the circulation of *GSTT1* and *GSTM1* deficient individuals. Alternatively the compounds or their metabolites may have a different mode of action entirely. Isothiocyanates have long been known to exert suppressing effects, as well as blocking activity and substantial evidence has accumulated to suggest that isothiocyanates may also act by selectively inducing apoptosis in preneoplastic cells.

4.6 Suppressing the promotion phase

Many studies in which animal models have been used to study the anti-carcinogenic effects of glucosinolate breakdown products have administered the compounds before or during treatment with the carcinogen, in order to identify blocking activity^{71–73}, whilst others have administered the components throughout the study⁷⁴, and a few have only fed them after initiation^{75,76}. Broccoli, which is rich in the precursor of sulforaphane⁷⁷, has been a particular focus of interest in relation to blocking effects, but a variety of other isothiocyanates have been shown to suppress the proliferation of tumour cells *in vitro*. Pure sinigrin, which is the major glucosinolate in Brussels sprouts, was used in the study on suppression by Smith *et al.*⁷⁶. Allyl isothiocyanate (AITC), the breakdown product of sinigrin, is of particular interest as a potential suppressing agent and, unlike some other isothiocyanates, it has been shown to have only minimal genotoxic effects in *in vitro* assays⁸. In the rodent studies of Futakuchi⁷⁵ and Chung *et al.*⁷³ reductions in the numbers and size of the pre-neoplastic aberrant crypt foci were observed in response to phenethylisothiocyanate and sulforaphane respectively. Similar effects observed by Smith *et al.* were associated with increased levels of crypt cell apoptosis 24–48 h after treatment with 1,2-dimethylhydrazine (DMH)⁷⁶.

Many *in vitro* studies have been undertaken to explore the relative effectiveness of isothiocyanates^{78–81}, and associated compounds⁸², as inhibitors of cell growth and inducers of apoptosis in cell lines, and some of these are summarised in Table 4.2. Clonal survival studies are often used to determine whether isothiocyanates inhibit initial cell anchorage and subsequent growth of sparsely seeded cells. However, differences in cell number following challenge with the isothiocyanate could be due either to decreased cell division or increased cell loss⁸³, and the authors of some *in vitro* studies have failed to recognise this. Other studies have, however, considered the interacting

Table 4.2 Summary of studies comparing the anti-proliferative effects against tumour cell lines of glucosinolate metabolites *in vitro*. The comparisons do not distinguish between increased cell death and reduced rate of cell division

Cell system	Relative 'anti-proliferative' response	Reference
HT29 (colorectal)	BITC > PEITC	Musk <i>et al.</i> (8)
CHO (Chinese hamster ovary)	PEITC > AITC	
SVM(muntjac cells)	AITC > BITC > PEITC >>> PITC	
HeLa	BITC > PITC >> AITC	Hasegawa <i>et al.</i> (88)
HeLa	PHITC > PEITC = PBITC = PMITC >>> PITC	Yu <i>et al.</i> (81)
HT29	AITC > BITC > PEITC >>> sulforaphane	Lund <i>et al.</i> (90)
K562 (erythroleukemic cells) ¹	BITC > MSoPITC >> MS3BITC > eHBITC > pHBITC	Nastruzzi <i>et al.</i> (82)
K562 (erythroleukemic cells) ²	MSoPN > pHBN > eHBN > MS3BN = BN	Nastruzzi <i>et al.</i> (82)
Hepa1c1c7 (murine liver cells) ¹	MSPITC > PEITC > BITC > AITC > pHBITC = HBITC = IMITC	Tawfig <i>et al.</i> (96)
Hepa1c1c7 (murine liver cells) ²	IMN > PEN > BIN > AN > HBN = B EN > MSPN	Tawfig <i>et al.</i> (96)

1. Cells exposed to myrosinase treated parent glucosinolate at neutral pH.

2. Cells exposed to myrosinase treated parent glucosinolate at acid pH.

roles of these factors in the control of cell growth and survival. In one such study, isothiocyanates have been shown to reduce aberrant cell proliferation in mammary tissue both by inhibiting cell proliferation and by inducing apoptosis⁸⁴.

In general, isothiocyanates cause disruption to the cell cycle *in vitro*, but the evidence that they actually induce apoptosis under these conditions is less clear-cut. It is interesting to note, however, that, even with respect to the cell cycle, they do not all act in the same way. Sulforaphane causes a block in cell cycle at G1/S⁸⁵, whereas PEITC and AITC cause a block at G2/M^{86–88}. Like sulforaphane, however, PEITC-NAC (N-acetylcysteine) conjugate appears to block in G1⁸⁹. Studies by Lund *et al.* show that in the case of the colorectal cell line HT29, which lacks wild type p53, treatment with AITC causes the cells to detach from the substratum but, at least in the short-term, they do not then enter apoptosis⁹⁰. Where it does occur, induction of apoptosis by isothiocyanate appears to be a p53-dependent process⁹¹. However, this statement must also be qualified, because the effect appears to depend on which metabolite is considered. For example, sulforaphane does appear to be able to induce apoptosis in HT29 cells⁸⁵, which express a mutated form of the protein.

In some studies it has been shown that ITCs can cause increases in the pro-apoptotic caspase enzymes, caspase 3, caspase 8 or caspase 9^{78,81}. However, in other work, specific caspase inhibitors failed to block cell death or cell detachment from the substratum^{79,92}. This suggests that caspase-activation may only be a bystander event, or may only occur after the initiating event of a block in the cell cycle. Indeed, although expression of c-Jun amino-terminal kinase (JNK) in response to ITCs has been linked to the pro-apoptotic process, it remains entirely possible that this is a parallel signalling pathway, more closely related to the induction of Phase 1 or Phase 2 enzymes than to the apoptotic programme^{93,94}.

Overall, the emerging evidence seems to support a scheme in which the initial response of cells to exposure to ITCs is a change in gene expression for those proteins that may be involved in expression of protective enzymes⁹⁵, or factors affecting cell anchorage. Apoptosis may then occur as a result of exposure to higher concentrations of the inducer, or for a longer period of time. Moreover, the nature of the response, in terms of the balance between apoptosis and enzyme induction, also depends on the ITC involved. Thus while 3-methylsulfinylpropyl induces the Phase 2 enzyme quinone reductase at doses which are not cytotoxic, AITC, which has a minimal effect on Phase 2 induction, does reduce cell number in liver cells⁹⁶. Phenethylisothiocyanate has been reported to induce both JNK and extracellular signal-regulated kinase (ERK) expression in the liver cell line HepG2, whereas sulforaphane only induces ERK and in fact apparently suppresses JNK activity⁹⁷. Similarly AITC causes loss of cell adhesion more effectively than sulforaphane in intestinal cell lines, but does not induce Phase 2 enzymes⁹². In view of the greatly differing levels and types of glucosinolate found in brassica vegetables, these differences between particular glucosinolate breakdown products may have important implications for human health.

4.7 Summary and conclusions

An individual consuming 3–4 portions of broccoli per week, which is the level at which a protective effect against adenomatous polyps has been reported⁷⁰, probably consumes 300–400 mg of glucosinolates. In view of the biological activity of this class of compounds, the consequences for health are of considerable interest, and an outline of the main features of glucosinolate absorption and metabolism in humans is beginning to emerge. The levels of myrosinase activity, and the quantities of the glucosinolates and the glucosinolate breakdown products themselves, vary in a complex way, which depends upon storage conditions and physical processing all the way through the food production chain from farm to the cooked food²⁶. Once ingested, the plant material is disrupted by mastication and gastric motility so that any remaining myrosinase can act on the intact glucosinolates that are still present in the food. Intraluminal ‘digestion’ of glucosinolates by plant myrosinase,

followed by rapid absorption in the stomach and small intestine, appears to be the major route for the delivery of isothiocyanates to the tissues. In the absence of myrosinase activity, some glucosinolates may be absorbed from the human alimentary tract in an intact form, but the biological significance of this, if any, is unknown. Any unabsorbed glucosinolates will be delivered to the colonic microflora, and a sizeable fraction will be broken down to isothiocyanates, some of which are absorbed and metabolised, so that they become available to exert effects on target tissues. There is now strong evidence that both the pharmacokinetics of isothiocyanates in individuals, and their effectiveness as anti-carcinogens, depend upon common genetic polymorphisms of the glutathione-S-transferases.

Glucosinolates influence both the palatability and the nutritional properties of vegetables. However, the levels of glucosinolates in brassica vegetables can be manipulated relatively easily by selective breeding³⁸. The commercial motivation to reduce glucosinolates in order to improve the palatability of vegetables may impair their health benefits. Examples of the opposite trend, to use selective breeding to increase glucosinolate levels in selected varieties, already exist. It must not be forgotten that, like synthetic drugs, biologically active secondary plant metabolites may have adverse side-effects⁹⁸. Further research is needed to define the biological activities of individual glucosinolates in greater detail, so that the balance of benefit, risk and consumer preference can be properly defined.

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Phytoestrogens and health

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5.1 Introduction

Phytoestrogens are, by definition, a group of naturally occurring plant compounds, which exhibit estrogenic properties. The four principal groups of phytoestrogens found in food are the isoflavones, coumestans, prenylated naringenins and lignans. However, only the isoflavones and lignans are commonly found in the UK diet. Soy is a major dietary source of phytoestrogens, and soy-based products contain significant quantities of isoflavones. The highest concentration of lignans has been found in grains, seeds (i.e. linseed) and other fibre-rich foods.

Estimates of phytoestrogen intakes from the UK diet have suggested that the average consumer will ingest less than 1 mg/person/day (Jones *et al.*, 1989). However, on the basis that an estimated 60% of processed foods available in the UK contain soy products, this is likely to be an underestimate. In addition, sections of the population, including infants fed soy-based formulae, vegetarians, vegans and those consuming a diet rich in soy foods, may be exposed to higher concentrations than the average consumer. Figures published in 1995 suggest that 2–3% of UK infants, up to the age of nine months, are fed soy-based infant formula (Department of Health, UK, 1996). In comparison it has been estimated that 25% of infants in the USA are fed soy-based formula (American Academy of Pediatrics, 1998).

Soy-based infant formula contains relatively high concentrations of phytoestrogens. As such, formula fed infants may be exposed to higher concentrations than the average consumer. Mean intakes of phytoestrogens in the UK have been estimated to be 4.5–5 mg/kg bw/day (MAFF, 1998), although other studies have indicated that consumption could potentially be

higher (Setchell, 1998; Rupp *et al.*, 2000; Murphy *et al.*, 1997). Phytoestrogen enriched foods and dietary supplements are marketed on the basis of their potential health benefits. In 2000, the value of the European market for isoflavones was estimated at 106 million euros. This represents approximately 9% of the rapidly expanding phytonutrient market. It has been predicted that the combined phytonutrient market will increase to an estimated 163 million euros by 2008 (Frost and Sullivan, 2002).

In the 1940s, adverse effects on fertility were observed in animals that had been grazing on phytoestrogen-rich plants (i.e. clover) raising concerns that similar effects could also occur in humans. To date, no adverse effects have been reported in populations traditionally consuming large quantities of soy. On the contrary, it has been suggested that phytoestrogens may have a beneficial effect on human health as these populations generally have a much lower incidence of hormone-dependent diseases, such as breast and prostate cancer. Foods and dietary supplements rich in phytoestrogens are now widely marketed on the basis of their potential health benefits. However, the concerns raised about exposure to dietary phytoestrogens led several scientific committees to consider the public health implications of these compounds in the late 1990s. This chapter will examine the biological effects of dietary phytoestrogens and discuss the potential public health implications relating to exposure.

5.2 Mechanisms of phytoestrogen action: receptor and non-receptor mediated

5.2.1 Receptor mediated effects of phytoestrogens

Phytoestrogens may elicit their biological effects by binding to estrogen receptors (ERs). Until recently, only a single ER isoform, ER α , had been identified; however, a second receptor termed ER β has also been identified (Enmark *et al.*, 1997, 1999; Saunders *et al.*, 2000, 2001). It has been shown that the ERs have different intracellular and tissue distribution patterns and are responsible for different biological effects (see Table 5.1). A number of spliced variants of both ER α and ER β have also been identified (Inoue *et al.*, 2000; Ogawa *et al.*, 1998; Vladusic *et al.*, 1998).

The major ER ligand is estradiol, although a number of different ligands have been found to bind to both ERs. It has been demonstrated that phytoestrogens can bind to both the ER α and ER β ; however, they appear to bind preferentially to ER β (Kuiper *et al.*, 1998). Estradiol is used as the standard against which the estrogenic properties of other compounds are measured. There are a number of *in vitro* and *in vivo* methods by which to assess the estrogenic potency of phytoestrogens; however, assessments can vary significantly between methods. *In vivo* assays, such as the uterotrophic assay, provide a more complete view of estrogenic activity and incorporate different biological processes that may influence activity. In contrast, *in vitro*

Table 5.1 Tissue distribution of ER subtypes in humans

Organ/tissue	ER α	ER β
Lung	—	3
Adrenal gland	3	—
Kidney	3	3
Prostate	—	3
Testes	—	3
Heart	3	3
Brain	3	3
Thymus	—	3
Breast	3	3
Uterus	3	3
Endometrium	3	3
Vagina	3	—
Fallopian tube	—	3
Ovary	3	3
Bladder	—	3
Epididymus	—	3
Pituitary	—	3
Liver	3	—
Muscle	—	—
Fat	—	—
GI tract	—	3
Colon	—	3
Small intestine	—	3
Bone	3	3

methods are a simple measurement of direct interaction with the ER and not necessarily predictive of *in vivo* activity.

In general, phytoestrogens have been found to be relatively weak estrogens requiring far higher concentrations to produce an equivalent biological response. The results of both *in vitro* and *in vivo* studies of phytoestrogen potency have estimated the rank order of estrogenic potency to be: estradiol >> coumestrol > genistein, equol > glycitein > 8-prenylnaringenin > daidzein > formononetin, biochanin A, 6-prenylnarinigenin, xanthohumol and isoxanthohumol.

5.2.2 Non-receptor mediated effects of phytoestrogens

In addition to direct interaction with ERs, phytoestrogens may also act indirectly to modulate the concentration of endogenous estrogens.

Effect on estrogen bioavailability

It has been suggested that phytoestrogens may alter the concentration of sex

hormones either by binding to or stimulating the synthesis of sex hormone binding globulin (SHBG). Studies have shown that although phytoestrogens can bind to SHBG, they have a much lower affinity than estradiol (Nagel *et al.*, 1999). *In vitro* studies have shown that phytoestrogens can modulate the concentration of SHBG; however, these effects occur at levels much greater than would be achieved via the diet (Mousavi and Adlercreutz, 1993; Loukovaara *et al.*, 1995). To date however, the evidence that dietary phytoestrogens can alter SHBG in humans is inconclusive and it is not clear whether such interactions would contribute to the estrogenic effect of these compounds (Duncan *et al.*, 1999a; Nagata *et al.*, 1997; Nagata, 2000; Pino *et al.*, 2000; Habito *et al.*, 2000).

Effects on estrogen biosynthesis and metabolism

Phytoestrogens may also modulate the concentration of endogenous steroid hormones by binding to and inactivating the enzymes involved in their biosynthesis and metabolism.

Inhibition of 17 β -hydroxysteroid oxidoreductase

The 17 β -hydroxysteroid oxidoreductase enzymes (HSOR) occur as two distinct isoforms (I and II). They are involved in the conversion of estrone to estradiol. The type I enzyme converts estrone to estradiol (also androstenedione to testosterone) and the type II catalyses the reverse reaction. Phytoestrogens have been shown to inhibit both HSOR enzymes *in vitro*.

Aromatase inhibition

The enzyme aromatase catalyses the conversion of testosterone to estrogen. A number of *in vitro* studies have shown that phytoestrogens can inhibit aromatase (Adlercreutz *et al.*, 1993; Kao *et al.*, 1998; Pelissero *et al.*, 1996). However, the high concentrations required to cause this effect *in vitro* are unlikely to be achieved *in vivo* following dietary exposure.

Inhibition of glucuronidation

Polyphenols and flavanoids in rat liver microsomal fractions have been demonstrated to inhibit glucuronidation of estrone and estradiol *in vitro* (Zhu *et al.*, 1998). In addition, flavonoids have also been found to induce phase I and II enzymes in rats including UDP-glucuronosyl transferase (Seiss *et al.*, 1996). However, the effects of phytoestrogens have not been evaluated for either their inhibition or induction of glucuronosyl transferase activity.

Inhibition of steroid sulphotase and sulphotransferase

Steroid sulphotransferase catalyses the addition of sulphate to steroidal compounds whilst steroid sulphotase catalyses the reverse reaction. *In vitro* studies have demonstrated that a metabolite of genistein, 4-ethylphenol, can inhibit sulphotransferase (Harris *et al.*, 2000). Sulphoconjugates of daidzein have also been found to potently inhibit these enzymes *in vitro* (Wong and

Keung, 1997). It remains to be established whether similar reactions would occur *in vivo*.

Effects on estrogen receptor expression

Phytoestrogens can modulate expression of both sub-types of estrogen receptor (Patisaul *et al.*, 1999, 2001; Wang *et al.*, 1996). However, the data is limited to measurements of mRNA content and it is unclear whether this differential expression results in any functional change.

Effects on estrogen production

The production of endogenous estrogens is controlled in the central nervous system by the secretion of gonadotrophin releasing hormone (GnRH) from the hypothalamus. GnRH acts on the pituitary gland to increase secretion of the gonadotrophins, follicle stimulating hormone (FSH) and lutenising hormone (LH). Following their release, LH and FSH stimulate the maturation of the ova resulting in increased concentration of testosterone to estradiol by the enzyme aromatase. When sufficient levels of estradiol have been reached, estradiol exerts a negative feedback on the hypothalamus inhibiting further secretion of GnRH. Ashby *et al.* (2000) demonstrated that, although the estrogenic effects of phytoestrogens were prevented by co-administration of an estrogen receptor antagonist, they were also prevented following co-administration of either a GnRH or an aromatase inhibitor. These findings suggest that phytoestrogens can stimulate the release of GnRH from the hypothalamus, which subsequently increases the production of endogenous estrogen.

5.3 Other effects of phytoestrogens

Thyroid peroxidase

Thyroid peroxidase (TPO) is an enzyme involved in the production of thyroid hormones. TPO catalyses the iodination of tyrosine residues on thyroglobulin during the synthesis of the thyroid hormones tri-iodothyronine (T3) and thyroxine (T4). *In vitro* studies by Divi *et al.*, (1997) and Divi and Doerge (1996) demonstrated that genistein, daidzein and biochanin A inhibited TPO catalysed hormone synthesis by acting as alternative substrates for iodination. This effect was reversed following the addition of iodine. Inhibition of TPO *in vivo* would reduce the concentration of thyroid hormones produced in the body. However, the concentration of phytoestrogens required to inhibit this enzyme is greater than the concentration likely to be achieved *in vivo* following dietary exposure.

Inhibition of topoisomerase II

Topoisomerase II is a nuclear enzyme involved in replication, transcription,

recombination, integration and transposition (Okura *et al.*, 1988). Inhibition of topoisomerase II may disrupt progression through the cell cycle and initiate apoptosis. *In vitro* studies have shown that genistein can interact with topoisomerase II (Okura *et al.*, 1988; Markovits *et al.*, 1989; Yamashita *et al.*, 1990). There is no direct evidence that phytoestrogens interact with topoisomerase II *in vivo*; however, studies have suggested that inhibition of topoisomerase II induced apoptosis accounts for the chemoprotective effects of genistein in rodents (Record *et al.*, 1997; Zhou *et al.*, 1998). However, the concentration of phytoestrogens required to inhibit this enzyme is greater than the concentration likely to be achieved *in vivo* following dietary exposure.

Inhibition of protein kinases

Protein kinases are essential enzymes for cellular growth and differentiation. They allow cells to respond to external stimuli through signal transduction pathways by catalysing the phosphorylation rate of serine, threonine and tyrosine residues in proteins (Scott and Pawson, 2000). A number of studies have demonstrated that genistein can inhibit tyrosine kinases *in vitro* (Akiyama *et al.*, 1987; Spinozzi *et al.*, 1994; Dalu *et al.*, 1998). However, it has been suggested that tyrosine kinase inhibition can be modulated by the concentration of estradiol and other growth factors, and other studies have indicated that genistein may not consistently inhibit tyrosine kinase (Panno *et al.*, 1996; Twaddle *et al.*, 1999). However, the concentration of phytoestrogens required to inhibit this enzyme is greater than the concentration likely to be achieved *in vivo* following dietary exposure.

Effects on cell growth and differentiation

The effect of genistein on cell growth and development has been investigated in a number of cell lines. Genistein has been shown to exert a biphasic effect on cell growth, increasing cell proliferation at concentrations < 10 μM whilst inhibition of cell growth occurred at concentrations > 10 μM (Zava and Duwe, 1997; Hsieh *et al.*, 1998). Inhibition of the cell cycle was found to occur at the G₂/M phase (Matsukawa *et al.*, 1993; Salti *et al.*, 2000). A study by Constantinou and Huberman (1995) has also demonstrated that genistein can induce differentiation in a number of different cell lines.

Genotoxic effects

Information on the genotoxic potential of phytoestrogens is limited. Although *in vitro* studies have indicated that coumestrol and genistein can cause genotoxic effects, these results occur at concentrations that are much higher than would be achieved in the plasma following ingestion of these compounds (Boos and Stopper, 2000; Kulling *et al.*, 1999).

Antioxidant properties

The antioxidant properties of phytoestrogens have been studied *in vitro* and it has been suggested that these may be related to the chemical structure

(Harper *et al.*, 1999). These studies, however, have used concentrations of phytoestrogens much greater than would be achieved via the diet.

5.4 The health effects of phytoestrogens: osteoporosis, cardiovascular disease and thyroid function

The public health implications of dietary phytoestrogens have received a great deal of attention over the last few years. However, despite substantial data from epidemiological and experimental studies, the biological effects of phytoestrogens remain equivocal.

5.4.1 Osteoporosis

Osteoporosis is a condition in which bone density is decreased, but the composition remains unchanged. Bone mass is lost when an imbalance between osteoblasts and osteoclasts occurs in favour of bone resorption. This imbalance results in structural failure of bone and increases the likelihood of fracture. A range of different factors is required to maintain good bone health, many of which are dependent on estrogen. Osteoclast activity is enhanced by estrogen deficiency and hence osteoporosis becomes a particular problem for postmenopausal women. Hormone replacement therapy has been shown to be an effective treatment in the prevention of bone loss (Felson *et al.*, 1993). The relative rarity of osteoporosis and of hip fractures in populations consuming high levels of soy has prompted investigations into the role of phytoestrogens in osteoporosis.

Studies have demonstrated that treatment with soy or phytoestrogen enriched diets is effective in conserving bone in rodent models of osteoporosis (Anderson and Garner, 1998; Ishimi *et al.*, 2000; Draper *et al.*, 1997). The mechanism of action of phytoestrogens on bone health is unclear but several mechanisms including inhibition of bone resorption and stimulation of bone formation may be involved (Fanti *et al.*, 1998; Ishimi *et al.*, 1999; Picherit *et al.*, 2000). Limited data from studies in postmenopausal women have indicated that phytoestrogen supplements have a small, beneficial effect on bone loss in the lumbar spine (Alekel *et al.*, 2000; Potter *et al.*, 1998; Somekawa *et al.*, 2001).

5.4.2 Cardiovascular system

Cardiovascular disease (CVD) is one of the leading causes of death worldwide. There are a number of established risk factors including serum cholesterol levels, smoking and family history, which are responsible for between 50 and 75% of the CVD cases, with the remainder due to factors that cause atherosclerosis. Estrogen treatment such as hormone replacement therapy is known to protect against CVD by decreasing the levels of low-density

lipoprotein cholesterol (LDLC) and increasing high-density lipoprotein cholesterol (HDLC). A high level of lipoprotein a (Lp(a)) is also acknowledged to be an indicator of CVD, and Lp(a) levels have been shown to be reduced by estrogen treatment (Shewmon *et al.*, 1994).

Mortality rates from CVD are generally lower in Asian populations compared to Western populations (Knight and Eden, 1996). Although many dietary factors are known to play a protective role in CVD and it has been suggested that phytoestrogen content of Asian diets may be responsible for the cardioprotective effect.

The current weight of evidence suggests that soy does have a beneficial effect on cardiovascular health; however, the active component has yet to be identified. Studies have also demonstrated that dietary inclusion of phytoestrogen-rich foods can lower plasma cholesterol levels (Ridges *et al.*, 2001). However, these data do not conclusively demonstrate that phytoestrogens are responsible for the hypocholesterolemic effect, and it is possible that some other component in soy may be responsible (Erdman and Fordyce, 1989; Ling and Jones, 1995).

5.4.3 Thyroid gland

Certain plants including soybeans have been found to contain goitrogenic compounds that can interfere with the activity of the thyroid gland (McCarrison, 1933). In the 1950s and 1960s a number of cases of altered thyroid function were associated with consumption of soy-based infant formulae (Hydovitz, 1960; Shephard *et al.*, 1960; Van Wyk *et al.*, 1959). The alterations reported were largely associated with reduced iodine intake. It is possible, therefore, that infants most likely to be affected are those from geographical areas where dietary iodine intakes are low. Following these reports, changes to soy-infant formulae, including iodine supplementation, were implemented (Fomon 1993). Since these alterations were made, there have been no further reports of goitrogenic effects in peer-reviewed literature.

It has been suggested that by interacting with the thyroid gland phytoestrogens could potentially increase thyroid binding globulin (TBG) thus increasing thyroxine requirements (Duncan *et al.*, 1999). It has also been suggested that phytoestrogen-containing supplements may have a similar effect, although it is not clear whether the level of phytoestrogens in supplements would be sufficient. While it is possible that phytoestrogens may alter the concentration of TBG there is little data to suggest they act by this mechanism to produce clinical effects.

In rodents, increased serum thyroid stimulating hormone (TSH) concentrations have been associated with an increase in thyroid cancer (Thomas and Williams, 1999). Although it is not known whether phytoestrogens can provoke a similar response, significantly elevated TSH levels have been reported in individuals consuming a vegan diet, which may contain increased levels of phytoestrogens. These data suggest that a vegan diet may not contain

adequate iodine and that vegans, may be more susceptible to the goitrogenic effects of soy (Key *et al.*, 1992). In contrast, other studies have indicated that consumption of unfermented soyfoods is associated with a decreased risk of developing thyroid cancer (Horn-Ross *et al.*, 2002). Phytoestrogens have been shown to inhibit TPO, an enzyme involved in thyroid hormone production. This effect was reversed following the addition of iodine (Divi RL *et al.*, 1997).

5.5 The health effects of phytoestrogens: central nervous system and immune function

5.5.1 Central nervous system

Endogenous estrogens are known to be active in a number of areas of the brain. There are indications that estrogens may play a role in mood, locomotor activity, pain sensitivity, vulnerability to neurodegenerative diseases and cognition (McEwan, 1999). In humans, the blood brain barrier is not fully developed at birth and, for this reason, the central nervous system (CNS) may be more sensitive to phytoestrogens *in utero* or at birth. As ERs are expressed in the CNS, phytoestrogens may also be active in this area.

Transfer of phytoestrogens

Studies investigating the transfer of phytoestrogens from the peripheral blood brain barrier to the CNS have indicated that the concentrations of isoflavones in the CNS are several orders of magnitude lower than in the blood stream. This suggests that phytoestrogens do not efficiently cross the blood brain barrier in rodents (Chang *et al.*, 2000; Coldham and Sauer, 2000).

Biochemical effects of phytoestrogens

Exposure to phytoestrogens has been shown to alter the expression of proteins in the brain which are thought to be important in injury and age-related neurodegeneration. The studies, conducted using animal models, reported altered expression of a number of proteins including brain-derived neurotrophic factor (Pan *et al.*, 1999a,b) and calcium binding proteins such as calbindin and calretinin (Lund *et al.*, 2001; Lephart *et al.*, 2000; Taylor *et al.*, 1999). Similar studies have not been carried out using human tissue and the implications for human health are currently unknown.

Behavioural effects of phytoestrogens

Phytoestrogens have also been shown to have behavioural effects in rodents including increases in sexual activity (Patisaul *et al.*, 2001) and a reversal of sex-specific behaviours (Lund *et al.*, 2001; Flynn *et al.*, 2000). In rodents, the sexually dimorphic nucleus of the preoptic area (SDN-POA) is located in the hypothalamic region of the brain. This area of the brain controls

sex-specific patterns of sexual behaviour and is responsible for converting androgens to estrogens and controlling gonadotrophin secretion. The development of the SDN-POA occurs in response to endogenous estrogens, and the size of the region varies according to sex. Phytoestrogens have been shown to masculinise the smaller female SDN-POA by increasing its volume (Faber and Hughes, 1993). It is not known whether an equivalent area exists in humans or whether these findings will have any implications for human health.

The effects of phytoestrogens on cognitive function have also been investigated in humans. However, the results of these studies were inconclusive. White *et al.*, (2000) concluded that tofu consumption in midlife was associated with diminished cognitive function later in life. In contrast, File *et al.* (2001) reported that short-term consumption of high levels of isoflavones (100 mg/day) may improve memory.

5.5.2 Immune system

It has been known for some time that gender and sex hormones can have a substantial impact on the organisation and function of the immune system. Estrogen is known to affect the development and organisation of lymphoid tissues and the activity of various cellular components of immune function. *In vitro*, phytoestrogens have been reported to alter cell proliferation and cytokine production (Atluru and Atluru, 1991; Wang *et al.*, 1997). Limited data from *in vivo* studies suggests that, under certain circumstances, phytoestrogens may have anti-inflammatory properties (Sadowska-Krowicka *et al.*, 1998; Regal *et al.*, 2000). Phytoestrogens have been shown to affect immune function in rodents by altering cell mediated and humoral activity and thymic development (Zhang *et al.*, 1997; Yellayi *et al.*, 2002). However, it is not clear whether these findings have any implications for human health. To date, however, studies investigating the effect of consuming soy infant formulae have not reported any impairment in immune function (Cordle *et al.*, 2002; Ostrom *et al.*, 2002).

5.6 The health effects of phytoestrogens: cancer

The incidence of a number of cancers has been found to be much higher in Western populations compared to Chinese and Japanese populations. Evidence from epidemiological and migrant studies has suggested that differences in the racial characteristics, diet and lifestyle of these populations may play a role in the aetiology of these diseases. There are likely to be many dietary and genetic factors affecting the incidence of disease among these populations. However, the relatively high consumption of soy-based products by Asian populations has attracted much attention due to their chemoprotective effects (Bingham *et al.*, 1998; Cassidy and Faughan, 2000).

5.6.1 Breast cancer

Breast cancer is one of the most common forms of cancer affecting women and, in Western countries, the incidence is rising. The risk of breast cancer increases markedly with age, although a decrease in the rate occurs after the menopause, suggesting that development is hormone-dependent. To date, a number of hormone-related risk factors have been identified (Bingham *et al.*, 1998). Countries such as Japan have relatively low rates of breast cancer, which have been associated with consumption of a diet high in soy foods. Currently, however, the data from epidemiological studies is inconclusive.

In Asian women consuming a diet relatively high in soy foods, menstrual cycle lengths have been found to be generally longer than in those of Western women. It has been suggested that increases in menstrual cycle length may lower an individual's lifetime exposure to estrogen thus reducing the risk of breast cancer. However, the results of human studies investigating the effects of soy and phytoestrogens on these biomarkers of breast cancer are inconsistent. *In vivo* data are generally supportive of a protective role for soy foods in breast cancer and some studies have indicated that phytoestrogens are the protective component of soy. In addition, *in vitro* studies have identified a number of possible mechanisms of phytoestrogen action. However, these effects have only been demonstrated at concentrations much higher than those likely to occur from dietary exposure.

5.6.2 Endometrial cancer

The pattern of hormonal risk factors involved in the development of endometrial cancer is similar to those associated with the development of breast cancer. In addition, there is substantial evidence to suggest that HRT can increase the risk (Beral *et al.*, 1999; Bingham *et al.*, 1998). Compared to the UK, the incidence of endometrial cancer in countries such as Japan is relatively low (Bingham *et al.*, 1998). It has been suggested that dietary factors may be responsible for the reduced incidence, and there is indirect evidence from epidemiology studies which suggests that increased consumption of soy products may lower the risk of endometrial cancer. However, these data are not conclusive. To date, no studies have demonstrated a link between consumption of phytoestrogens and an increased risk of endometrial cancer.

5.6.3 Prostate cancer

In countries such as Japan, although the incidence of latent, small or non-infiltrative prostate cancer is similar to that found in Western countries, the incidence of invasive cancer and associated mortality is far lower (Adlercreutz and Mazur, 1997). In Japan, diets have traditionally included large amounts of soy, and it has been proposed that this may be responsible for the protective effect. To date, however, the results are inconsistent, although there may be an inverse correlation with non-fermented soy foods. The active component

of soy has not been identified, although evidence from both *in vivo* and *in vitro* studies indicates a role for phytoestrogens. However, beneficial effects were found at concentrations much higher than would be achieved from the diet. To date, there have been no long-term studies investigating the effect of phytoestrogens on prostate cancer risk in man.

5.6.4 Colorectal cancer

Colorectal cancer is one of the most common forms of cancer in the UK with 17,000 cases being reported each year. In countries such as Japan, however, the incidence of colorectal cancer has traditionally been lower. Comparisons between cancer rates and diet have often been used as the basis for identifying factors that may have a protective role. The current epidemiological data have produced conflicting results, but there is some evidence to suggest that soy products have a protective effect against the development of colorectal cancer. The active agent in soy has not been identified, although it has been suggested phytoestrogens may be responsible. These suggestions arise from *in vitro* data, which demonstrate that phytoestrogens inhibit growth of human colon cancer cells (Park *et al.*, 2001). To date, however, no epidemiology studies have specifically investigated the role of phytoestrogens.

5.6.5 Stomach cancer

The incidence of stomach cancer varies from country to country around the world. In the UK, stomach cancer is the sixth most common form amongst adults with some 10,500 cases diagnosed each year. It has been suggested that differences in the incidence of stomach cancer may be explained to some extent by differences in diet. The incidence of stomach cancer in countries, consuming large amounts of soy is high. However, the results from epidemiological studies investigating the relationship between soy and stomach cancer are inconsistent, although increased risk of stomach cancer has most often been associated with the consumption of fermented soy products (Messina *et al.*, 1994; Nagata *et al.*, 2000; Hirayama, 1971, 1982; Nomura *et al.*, 1990). To date, there have been no studies specifically investigating the effects of phytoestrogens.

The results from animal studies investigating the effect of soy and isoflavones on stomach cancer have also produced conflicting results (Tatsuta *et al.*, 1999; Kim *et al.*, 1985; Watanbe *et al.*, 1999). The most consistent data comes from *in vitro* studies, which indicate that phytoestrogens can inhibit cell growth of gastric cancer cell lines. However, the concentrations of phytoestrogens used in these studies are much greater than would be achieved from the diet (Matsukawa *et al.*, 1993; Yangihara *et al.*, 1993). Thus, the implications of these findings for humans are unknown.

5.6.6 Lung cancer

Lung cancer is one of the most common causes of cancer-related deaths in the UK. Although the incidence of lung cancer appears to be declining amongst men, the incidence in women is still rising. Data from *in vivo* studies have shown that phytoestrogens can inhibit development of experimentally induced lung tumours and lung metastasis (Li *et al.*, 1999; Menon *et al.*, 1998). In addition, there is limited epidemiological data to suggest that consumption of soy products has a protective effect in lung cancer (Swanson *et al.*, 1992; Koo *et al.*, 1988). However, to date only a single study has investigated the role of phytoestrogens (Seow *et al.*, 2002). This study found a protective effect between non-smokers and a high intake of isoflavones.

5.7 The health effects of phytoestrogens: fertility, development and hormonal effects

5.7.1 Fertility and development

Phytoestrogens were first associated with adverse effects on mammalian development and fertility from observations of animals consuming phytoestrogen-rich plants. Ewes feeding on Australian clover developed abnormal plasma concentrations of endogenous hormones with subsequent loss of fertility (Bennett *et al.*, 1946; Moersch *et al.*, 1967; Obst and Seamark, 1975).

Subsequent investigations showed that when pregnant ewes were fed on yarloop clover, plasma progesterone and estrogen concentrations were lowered. This resulted in a substantial reduction in the proportion of mated ewes achieving successful conception, compared with ewes fed on grass. Similar but more extreme effects on fertility were observed in ewes administered large quantities of estradiol for periods of up to 26 months (Adams and Sanders, 1998). These effects on fertility by a known estrogen led to the hypothesis that estrogenic compounds in clover were responsible for the adverse effects on fertility. A number of phytoestrogens have subsequently been identified in several types of clover (Shutt, 1976).

It has been established that exposure to potent estrogens *in utero* can have adverse effects on human development and fertility as demonstrated by the synthetic estrogen diethylstilbestrol (Giusti *et al.*, 1995). These findings have raised concerns that *in utero* exposure to phytoestrogens may cause similar effects. Experiments to determine the effects of phytoestrogens on human development and fertility are limited. However, studies do indicate that phytoestrogens can produce weak estrogenic effects (See Section 5.7.2). A retrospective study found that soy formula produced no obvious adverse clinical effects on fertility and sexual development, although a small increase in the duration and discomfort of menstruation was reported (Strom *et al.*,

2001). In addition, it has been suggested that short-term exposure to phytoestrogens during adulthood does not affect male sex hormone concentrations or semen quality (Mitchell *et al.*, 2001).

Most of the published research on fertility and development has been conducted in laboratory animals. However, significant species differences in sexual development between animals and humans make the interpretation of any findings extremely difficult. Experiments in rodents suggest that phytoestrogens can produce estrogenic effects in both male and female animals which are most marked in the case of exposure during the perinatal, neonatal or prepubertal stages of development. However, the significance to humans of estrogenic effects observed in rodents is unclear, and experiments in rodents have often administered much higher doses than would be obtained from the diet.

In addition, a recent primate study reported that dietary consumption of soy infant formula reduced the neonatal testosterone surge and increased Leydig cell numbers in the testes of male marmosets. However, the long-term effects on the fertility of the animals has not been determined, and the health implications of these findings for humans are unclear (Sharpe *et al.*, 2002).

5.7.2 Hormonal effects

There is much interest in the possible hormonal effects of phytoestrogens in both men and women. The majority of studies conducted in women have examined the ability of phytoestrogens to alleviate menopausal symptoms. Whilst hormone replacement therapy is recommended for women experiencing menopausal symptoms, there remains some uncertainty as to whether HRT can increase the risk of breast cancer. As a result of these concerns, investigations into natural alternatives such as phytoestrogens have received considerable attention.

Peri- and postmenopausal women

Women from different countries report many different menopausal symptoms. However, in most Western countries almost 80% of women experience hot flushes whereas the incidence is much lower in Japan (Knight and Eden, 1995). The pathophysiology of the hot flush is uncertain, but it is thought that reduced (low) estrogen concentrations may be the primary trigger. It has been suggested that the difference in incidence may be due to cultural and dietary factors, particularly with regard to phytoestrogen intake (Eden, 1998). Studies investigating whether phytoestrogens provide relief from menopausal symptoms have produced inconsistent results. Although reductions in hot flushes have been demonstrated, similar responses have also been found in the placebo group (Brzezinski *et al.*, 1997; Upmalis *et al.*, 2000; Knight *et al.*, 1999).

Premenopausal women

Interest in the hormonal effects of soy in premenopausal women has centred on the potential of these compounds to alter menstrual cycle length. Increasing menstrual cycle length is predicted to lower the body's lifetime exposure to endogenous estrogen, which may have the potential to reduce the risk of hormone-dependent cancers such as breast cancer. Although the results of these studies have been inconsistent, a decrease in midcycle gonadotrophin concentration, a trend towards increased menstrual cycle length and decreased serum concentrations of estradiol and SHBG have been observed.

Hormonal effects in men

There has been some speculation that estrogenic compounds may adversely affect the fertility of men by reducing testosterone levels and sperm counts (Atanassova *et al.*, 2000). To date, however, the limited information available suggests that consumption of phytoestrogens does not affect reproductive hormones or semen quality (Nagata, 2000; Nagata *et al.*, 2001; Mitchell *et al.*, 2001).

5.8 Future trends and priorities for research

There is currently a substantial global research effort into the effects of phytoestrogens. However, there are still a number of gaps in knowledge with respect to the health implications of dietary phytoestrogens for humans. These gaps will form the basis of future research priorities.

Although phytoestrogens have been found in a wide range of different foodstuffs, information on dietary intakes is limited and often inconclusive. To date, most attention has been focussed on the isoflavones with little information being available on the levels of lignans in foods. In addition, the levels of isoflavones reported are highly variable and, as a result, large ranges of intakes have been reported both within and between foodstuffs. In order to determine whether ingestion of phytoestrogens will result in a biological effect, much better intake data is required on how much each consumer is exposed to, i.e., average and extreme consumers. Information on the intake of phytoestrogens from phytoestrogen-rich health food supplements is also needed.

In both animal and *in vitro* studies, phytoestrogens have been shown to cause a range of biological effects, and there is also evidence that phytoestrogens can result in a number of effects, both adverse and beneficial, in humans. However, the data is often inconsistent or inconclusive, and the health implications of either short- or long-term exposure remain to be established. Currently, our knowledge of the physiological processes involved following ingestion, such as absorption, is limited. In addition, there is only limited data on the metabolism of phytoestrogens. To identify products of phytoestrogen metabolism in humans, there is a need to develop new methods of analysis

and detection. Data on intra- and inter-individual differences with respect to metabolism and information on any factors that may influence the bioavailability or pharmacokinetic profile of phytoestrogens in humans is also required. In addition, the effect of food matrix on phytoestrogen metabolism remains to be established.

Most of the current epidemiological data has investigated the effect of soy rather than any specific health implications related to phytoestrogen intake. As such, more epidemiological studies investigating the clinical efficacy of phytoestrogens in the treatment of conditions like osteoporosis, cancer and menopausal symptoms are needed. In addition, data on the optimal concentration required to produce a specific effect needs to be generated. Studies investigating the adverse effects of phytoestrogens are also needed, including long-term trials to identify any potential *in utero* effects. In addition, any adverse effects for susceptible subgroups such as hypothyroid patients or individuals that consume a phytoestrogen-rich diet, such as infants fed soy infant formula or those consuming a vegetarian/vegan diet, need to be identified.

At the time of writing, Catherine Boyle worked for the Food Standards Agency but has since moved to SEAC.

5.9 Sources of further information and advice

Food Standards Agency, Room 507C, Aviation House, 125 Kingsway, London WC2B 6NH.

USDA & Iowa State University isoflavone database

<http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html>

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Phytoestrogens and bone health

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6.1 Introduction

Bone mass is influenced by many genetic, environmental, nutritional and life-style factors (Russell, 2001). In humans, optimal peak bone mass is achieved at around 20 years of age. With age, and especially following the withdrawal of estrogen at the menopause, bone loss exceeds bone formation leading to a gradual reduction in bone mass. Many cells are present in the bone marrow and bone matrix, and their tight interaction and regulation are essential to building and maintaining bone mass throughout life. Bone is formed and resorbed constantly in what are called bone remodelling units, involving the bone forming cells (osteoblasts) and bone resorbing cells (osteoclasts) (Ducy *et al.*, 2000; Teitelbaum, 2000). During growth, bone formation exceeds bone resorption whereas with age, and particularly after the menopause, bone resorption exceeds bone formation, rendering bones brittle and susceptible to fracture (Rodan and Martin, 2000).

Osteoporosis is a bone disease characterised by low bone density and microarchitectural deterioration of bone tissue leading to bone fragility and increased susceptibility to fractures. There are many factors which can lead to the development of osteoporosis, including excessive bone loss caused by estrogen depletion, ageing, immobilisation and nutritional inadequacy. While osteoporosis is more common in women after the menopause, its incidence is also rising in men. Estrogen plays a critical role in bone health. At menopause, women undergo an accelerated rate of bone loss. Over the following decade this accounts for cancellous bone losses of 20–30% and cortical bone losses of 5–10% (Riggs *et al.*, 1998). Indeed, withdrawal of estrogen increases bone turnover, with the rate of bone resorption exceeding that of formation, resulting

in a net loss of bone. Hormone replacement therapy (HRT) prevents accelerated bone loss and hip and vertebral fractures, especially when given in the early menopausal years (Cauley *et al.*, 1995; Komulainen *et al.*, 1999). Although HRT is clearly a successful therapeutic approach to osteoporosis, many women are willing to take it only for a few years to overcome the unpleasant symptoms associated with the menopause. However, long-term usage for protection against osteoporosis is less frequent. Therefore, alternative, nutritional approaches to protecting against bone loss in the longer term are of interest.

Nutrition plays an important role in bone growth and maintenance (Eastell and Lambert 2002). The nutrients which are important as constituents of bone or regulators of bone metabolism are minerals (calcium, phosphorous, zinc, magnesium, manganese), vitamins (D, K, C) and macronutrients such as proteins or fatty acids (Kruger and Horrobin, 1997; Heaney, 2000; Jasminka, 2000; Schaafsma, 2001; Booth, 2001; Rizzoli *et al.*, 2001; Watkins and Seifert 2001). The majority of nutritional studies have involved calcium and vitamin D. Calcium is the major component of the skeleton and is vital to ensuring optimal bone growth and maintenance. Adequate calcium intake contributes to a high bone mineral density (BMD) and influences bone strength and fragility (Heaney, 2000). High calcium intake (1300–1700 mg/day) helps prevent hip fractures in the elderly (Heaney, 2001). Vitamin D plays a key role in increasing calcium absorption in the intestine and its retention in the kidney. The optimal nutritional benefit comes from a combination of sufficient calcium intake and vitamin D together, especially in the elderly (Schaafsma, 2001).

6.2 Composition and metabolism of phytoestrogens

Phytoestrogens occur naturally in many fruits, vegetables and whole grain products. Soy and soy products are the most significant dietary source of isoflavones which, together with coumestans and lignans, are broadly defined as phytoestrogens due to the similarity in their structure to the natural estrogen, estradiol-17 β , and their weak estrogenicity (Reinli and Block, 1996; Kurzer and Xu, 1997). Soy diets are associated with many beneficial effects including lower risk of cardiovascular disease, cancer, osteoporosis and relief of menopausal symptoms (Messina *et al.*, 1994; Messina and Loprinzi, 2001; Anderson and Garner, 1997; Nagata *et al.*, 1998; Setchell and Cassidy, 1999; Vincent and Fitzpatrick, 2000; Arjmandi and Smith, 2002). These effects have been largely attributed to the isoflavones, either alone or as part of the soy protein isolate; however, the mechanisms of action are not yet elucidated. Interest is growing in the potential benefit of soy isoflavones as natural protectants of bone health, particularly in preventing bone loss during ageing.

The major isoflavones in soy are genistein, daidzein and glycitein (reviewed by Setchell, 1998; Setchell and Cassidy, 1999). Isoflavone content varies among soybean varieties, growing conditions and soil (Wang and Murphy,

1994a, b). Average concentrations are approximately 1–4 mg/g dry weight of bean depending on the year and location. Isoflavones exist mainly as glycosides (acetyl-glycoside or malonyl-glycoside) in the soybean and in non-fermented foods. The glycosides are converted to the aglycone form through the action of β -glycosidases via the gut microflora (Griffiths and Barrow, 1972; Xu *et al.*, 1995). The large inter-individual variation in gut microflora significantly affects the metabolism of isoflavones whether it be conversion from glycosides to aglycones or production of secondary metabolites such as equol and dihydrogenistein (Kelly *et al.*, 1993, 1995; Hutchins *et al.*, 1995; Slavin *et al.*, 1998; Watanabe *et al.*, 1998; Rowland *et al.*, 2000). More recent evidence suggests that glycosides can also be metabolized to aglycones via endogenous β -glucosidases present in the enterocyte (Ioku *et al.*, 1998; Day *et al.*, 1998, 2000), and a minor amount may even be absorbed directly from the small intestine without prior hydrolysis (Hollman *et al.*, 1995; Gee *et al.*, 1998; Andlauer *et al.*, 2000). The isoflavones are subsequently conjugated by glucuronidation or sulphation by the liver. They circulate in plasma and are excreted in urine in this conjugated form.

Traditional fermented soy foods such as tempeh, natto and soy sauce are rich in aglycones compared to the unfermented soy (Wang and Murphy, 1994a), and the isoflavones are more bioavailable (Hutchins *et al.*, 1995; Slavin *et al.*, 1998; Izumi *et al.*, 2000). Nevertheless, the efficiency of absorption of isoflavones is high, whether they be in the glycoside or aglycone form, and further processing of the glycosides is not essential to obtaining significant absorption and biological effects (Cassidy *et al.*, 1994; Setchell *et al.*, 2001; Richelle *et al.*, 2002; Tsunoda, 2002).

6.3 Human studies on soy isoflavones and bone maintenance

Only a few human studies have been reported on the effect on bone metabolism of soy diets or soy protein containing isoflavones (reviewed by Anderson and Garner, 1997; Messina *et al.*, 2001; Arjmandi and Smith, 2002). The results are not altogether consistent, but this is not surprising in view of the small number of studies carried out with different populations (Asian or American), age (pre-, peri- or postmenopausal), the type of diet or intervention involved (recording of dietary intake of soy products or intervention with soy protein containing varying levels of isoflavones), length of time of the intervention or follow-up (ranging from three months to three years).

Most of the studies are cross-sectional, population studies in Japanese or Chinese pre- or postmenopausal women, investigating the correlation between dietary intake of soy products and measurements of BMD or biomarkers of bone turnover (Table 6.1). Several studies showed a positive correlation between high intake of soy products and improved BMD, especially at the

Table 6.1 Cross-sectional, population studies based on dietary intake of soy products and bone mineral density (BMD) or biomarkers of bone turnover

Authors	Population	Correlation between isoflavone intake and bone measurements
Tsuchida <i>et al.</i> , 1999	Japanese premenopause n = 995	Dietary intake of soybeans(at least 2 servings/wk) correlated with higher BMD (4.5%) of the second metacarpal bone
Horiuchi <i>et al.</i> , 2000	Japanese postmenopause n = 85	Women with a high soy protein intake showed less bone loss and higher BMD at the lumbar spine Biomarkers: urinary deoxypyridinoline (bone resorption) decreased
Mei <i>et al.</i> , 2001	Chinese postmenopause n = 357 premenopause n = 293	Significant differences found in BMD of the lumbar spine and hip between highest and lowest tertile of isoflavone intake for postmenopausal but not for premenopausal women
Somekawa <i>et al.</i> , 2001	Japanese peri/post-menopause n = 478	The high isoflavone intake group (65 mg/d) had significantly higher BMD of the lumbar spine compared to the low intake group (35 mg/d). Some menopausal symptoms were better alleviated in the high vs low intake group
Ho <i>et al.</i> , 2001	Chinese premenopause n = 132	Estimated isoflavone intake range: 7.4–48.3 mg/d. Higher BMD of lumbar spine in the 4 th quartile
Nagata <i>et al.</i> , 2002	Japanese postmenopause n = 87	The estimated average intake of isoflavones was 32 mg/d and plasma concentrations around 0.6 μ M Soy product and isoflavone intake and serum isoflavones were not significantly correlated with BMD at the calcaneous site.

lumbar spine (Horiuchi *et al.*, 2000; Ho *et al.*, 2001; Mei *et al.*, 2001; Somekawa *et al.*, 2001). One study showed a positive correlation with soybean intake of at least two servings per week and an improved BMD of the metacarpal bone (Tsuchida *et al.*, 1999). The study of Nagata *et al.*, (2002) was unique in that the isoflavone content of the foods as well as the serum levels were actually measured. The mean intake was 32 mg isoflavones/day and the mean isoflavone serum levels were 0.6 μ M, but there was no effect on BMD at the calcaneous site. In the positive studies, the isoflavone intake was around 54 mg/day for postmenopausal women (Somekawa *et al.*, 2001) and a range of 7–50 mg for the premenopausal women (Ho *et al.*, 2001), but serum isoflavone levels were not measured. The improved BMD was at the lumbar spine.

Only a few short-term intervention studies have been carried out, principally in postmenopausal women (see Table 6.2). In the first study, the effect of soy

Table 6.2 Short-term intervention studies with soy protein containing isoflavones, measurement of BMD

Authors	Population	Correlation between isoflavone intake and bone measurements
Potter <i>et al.</i> , 1998	American postmenopause n = 66	40 g soy protein (containing 90 mg isoflavones/day) over 6 months results in 2.5% increase in BMD of the lumbar spine
Dalais <i>et al.</i> , 1998	Australian postmenopause n = 44	Women received 45 g of either soy grits containing 52 mg isoflavones, wheat flour or flaxseed daily for 3 months No change in BMD 5.2% increase in BMC in soy group
Alekel <i>et al.</i> , 2000	American perimenopause n = 69	40 g soy protein (containing 80 mg isoflavones/day) over 6 months attenuates bone loss in the lumbar spine
Hsu <i>et al.</i> , 2001	Chinese postmenopause n = 37	150 mg isoflavones daily for 6 months No change in calcaneus BMD
Morabito <i>et al.</i> , 2002	European postmenopause placebo, n = 30 genistein, n = 30 HRT, n = 30	First randomized, double-blind placebo-controlled study. Compared to placebo control, genistein (54 mg/day) consumed for 1 year significantly reduced urinary excretion of bone resorption markers and increased bone formation markers at 6 and 12 months; BMD was significantly increased at the femoral neck and lumbar spine; plasma genistein concentration was around 1.5 μ M. HRT showed similar effects to genistein for BMD.
Anderson <i>et al.</i> , 2002	mixed ethnic premenopause placebo, n = 13 supplement, n = 15	Controlled, double-blind intervention in young, adult females testing the effect of supplementation with soy protein providing 90 mg isoflavones/day compared to soy protein without isoflavones. No changes in BMD or BMC were observed in either group after 12 months.

protein (40 g/day) containing moderate (50 mg) or higher (90 mg) levels of isoflavones was tested on BMD and bone mineral content (BMC) (Potter *et al.*, 1998). After six months of soy protein/isoflavone intake, the group consuming 90 mg isoflavones (but not 50 mg) benefited from a significant increase in BMD and BMC of the lumbar spine. In a second study in perimenopausal women consuming 80 mg isoflavones over six months, attenuated bone loss was observed at the lumbar spine (Alekel *et al.*, 2000). In the study of Dalais *et al.*, (1998), isoflavones (52 mg/day) were given in the form of soy grits for a short period of three months, but no change in BMD was observed. This may be due to the very short duration period or to

the site at which BMD was measured. The Chinese study by Hsu *et al.*, (2001) showed no effect with 150 mg isoflavones given for six months.

Biomarker measurements with soy/isoflavone intake vary from study to study (some increased, some decreased, some no effect (e.g. Wangen *et al.*, 2000; Uesugi *et al.*, 2002)). However, such studies are usually of very short duration (4–12 weeks) with small numbers of subjects and there is frequently high variation in measurement of urinary biomarkers. Interestingly, a recent study in men investigated the effect of consuming 40 g soy protein supplementation compared to milk protein control for three months (Khalil *et al.*, 2002). Serum insulin-like growth factor 1 (IGF-1), which is associated with higher rates of bone formation, was greater in men consuming the soy protein compared to milk protein. However, there was no difference between the groups for markers of bone turnover.

In view of the limited number of human studies and several inconsistencies, only preliminary conclusions may be drawn at this stage. It would appear that the site at which BMD is measured is important, most of the positive effects having been observed at the lumbar spine. For Japanese women, the average daily intake of around 50 mg/day is sufficient to have a long-term positive effect on spinal BMD (Somekawa *et al.*, 2001). For occidental populations who do not have a history of soy intake, the effective dose is around 80–90 mg isoflavones/day, demonstrated over a six-month period (Potter *et al.*, 1998; Alekel *et al.*, 2000). With regard to which is the active component of soy, based on current studies, the dose of isoflavones is important, but it is not possible to distinguish between their effect and their effect in combination with soy protein. To prove an effect of isoflavones on bone metabolism, larger scale, randomized, controlled, intervention trials for longer time periods (1–3 years) are necessary along with a standardized source of soy protein/isoflavones and measurements of bioavailability and metabolism. In the first randomized double-blind placebo-controlled study reported recently (Morabito, 2002; Table 6.2), supplementation of postmenopausal women with genistein (54 mg/day) or HRT for one year led to a significant increase in BMD at both the femoral neck and the lumbar spine after 12 months. In the genistein group, a parallel reduction in biomarkers of bone resorption and stimulation of biomarkers of bone formation was observed at 6 and 12 months, whereas in the placebo group, the markers remained unchanged. This landmark clinical study clearly demonstrates that the pure isoflavone, genistein (in absence of soy protein) prevents bone loss in estrogen-deficient, postmenopausal women. On the other hand, in a controlled, double-blind, one year trial in estrogen-replete, premenopausal women, no change in BMD was observed following dietary supplementation with soy protein enriched with isoflavones (90 mg/day) or with the control soy protein diet without isoflavones (Anderson, 2002; Table 6.2).

6.4 Animal studies on soy isoflavones and bone maintenance

Prevention of bone loss has been tested in the ovariectomized (OVX) rat model of menopause using either isoflavones contained in soy protein or pure isoflavones (reviewed by Messina *et al.*, 2001; Arjmandi *et al.*, 2002) and summarized in Tables 6.3 and 6.4. In addition, a recent study has shown that soybean isoflavones also protect against bone loss induced by androgen deficiency in male mice (Ishima, 2002). Soy protein isolate/extract containing isoflavones attenuated femoral and vertebral bone loss when given at the time of ovariectomy but did not reverse bone loss once it was installed after ovariectomy (Arjmandi *et al.*, 1996, 1998a, 1998b; Harrison *et al.*, 1998; Picherit, 2001a). A biphasic dose response of a genistein-rich isolate was observed by Anderson *et al.*, (1998), the low isoflavone dose

Table 6.3 Effect of soy protein/extracts containing isoflavones on OVX-induced bone loss in rats

Authors	Soy protein/isoflavone treatments	Effect on bone measurements
Arjmandi <i>et al.</i> , 1996	95 d old OVX rats 30 d soy protein with isoflavones (8 mg/d) starting at OVX	Prevention of bone loss: soy protein/isoflavones prevented the BMD losses in the femur and 4 th lumbar vertebra after OVX
Arjmandi <i>et al.</i> , 1998a,b	95 d old OVX rats 35 d casein diet after OVX, then 65 d soy protein with isoflavones (9 mg/d)	No significant effect of soy protein in reversal of bone loss, assessed by BMD. Soy induced femoral IGF-I mRNA and some increases in bone turnover markers
Harrison <i>et al.</i> , 1998	10 week old OVX rats low calcium diet treatment starts 2 weeks after OVX: Soy protein with isoflavones (6 mg/d) for 4 weeks	Reduced bone loss (higher bone weight, higher calcium content) but no decrease of markers of bone turnover for the soy group
Anderson <i>et al.</i> , 1998	OVX, lactating rats low calcium diet genistein-rich protein source 3 doses isoflavones: (0.5, 1.6, 5.0 mg/d) for 2 weeks	A biphasic response to genistein was observed for bone weight (femur ash): low dose more protective than high dose
Picherit <i>et al.</i> , 2001	7 month old OVX adult rats 3 doses of isoflavone extract (20, 40, 80 mg/kg body wt/d) for 91 d	Isoflavones reversed femoral failure load, total femoral, diaphyseal or metaphyseal BMDs and normalized bone turnover markers induced by OVX. Optimal dose without uterotrophic effect was 40 mg/kg body wt

Table 6.4 Effect of pure isoflavones on OVX-induced bone loss in rodents

Authors	Isoflavone treatments	Effect on bone measurements
Ishida <i>et al.</i> , 1998	9 week old OVX rats calcium-deficient diet daidzin 10, 25, 50 mg/kg bw by gavage treatment for 28 d starting 7 d after OVX	High dose genistin or daidzin prevented bone loss. Daidzin retarded femoral bone loss in a dose-dependent manner
Fanti <i>et al.</i> , 1998	2 month old, OVX rats subcutaneous injection of genistein for 21 d (1, 5, 25 mg/kg body wt)	1 mg/kg genistein had no effect on BMD losses. 5 and 25 mg/kg genistein significantly and equivalently reduced BMD losses
Ishimi <i>et al.</i> , 1999	8 week old OVX mice low calcium diet genistein (0.7 mg/d) treatment for 4 weeks	Reduced bone loss by genistein although not as effective as estradiol
Picherit <i>et al.</i> , 2000	1 yr old OVX rats oral administration, 90 d genistein or daidzein at 10 mg/kg body weight	Overall daidzein > genistein in this study. Total femoral BMD losses and vertebral trabecular bone were protected by estradiol and daidzein, but genistein was not efficient. Bone strength (femoral failure stress) protected by estradiol, daidzein and genistein
Uesugi <i>et al.</i> , 2001	11 week old OVX rats tube feeding into the stomach isoflavone glycosides (25, 50 or 100 mg/kg/d) for 4 weeks	Daidzin, glycitin and genistin each significantly prevented OVX-induced bone loss at 50 mg/kg/d and reduced urinary excretion of pyridinoline and deoxypyridinoline

(0.5 mg/rat/day) being more efficient than the high dose (5 mg/rat/day). Studies with pure isoflavones differ in the form of isoflavone administered (aglycone vs glycoside), the dose and administration (ranging from 5 to 50 mg/kg/day, orally or subcutaneously) and the time of administration (1–3 months). Nevertheless, in all cases, the isoflavones reduced femur bone loss following ovariectomy (Fanti *et al.*, 1998; Ishida *et al.*, 1998; Picherit *et al.*, 2000; Uesugi *et al.*, 2001). The dose of 40 mg/kg body weight is proposed as optimal for a benefit on bone with no uterotrophic effect (Picherit *et al.*, 2001b).

Similar to the human studies, the animal studies are not entirely consistent, due to the different study designs (source and dose of soy protein/isoflavones; time, method and length of administration; age of rats, etc.). Nevertheless, a certain number of conclusions may be drawn. Overall, soy extracts or pure isoflavones show an osteoprotective effect in the ovariectomized rat model of menopausal bone loss. The time of administration is important and they must be given at the time of ovariectomy which allows prevention but not reversal of bone loss. Although the OVX-induced bone loss in the rat is a

recognized model of postmenopausal osteoporosis, there are a number of important differences between the rat and human studies with regard to isoflavones. In the rat studies, protection is seen at the femur and lumbar vertebrae whereas in the human studies, the spine was more consistently protected than the femur. The dose administered and the metabolism of isoflavones are not identical in rats and humans making it difficult to extrapolate an effective dose.

6.5 Mechanisms of action of isoflavones in bone health

The individual role of isoflavones contained in soy protein compared to isoflavones alone has not been systematically studied. In addition, it is possible that some of the minor components of soy also play a biological role. Nevertheless, in terms of bone health, the benefit of soy diets is largely attributed to the isoflavones due to their weak estrogenic activity and potential to alleviate the estrogen loss following the menopause.

6.5.1 Estrogenicity

Soy isoflavones are weak estrogens. They are 1000 times less potent than the natural estrogen, estradiol-17 β (Markiewicz *et al.*, 1993; Kuiper *et al.*, 1997). However, in women consuming a soy diet, circulating plasma levels of isoflavones are 1000 times higher than estradiol-17 β and result in physiological effects such as prolongation of the menstrual cycle in premenopausal women and vaginal changes in postmenopausal women (Cassidy *et al.*, 1994; Baird *et al.*, 1995). Daidzein is less estrogenic than genistein, but can, via metabolism by the gut microflora, give rise to equol, a compound as estrogenic as genistein and with other biological effects (Markiewicz *et al.*, 1993; Duncan *et al.*, 2000).

The mechanisms of action of estrogens and phytoestrogens are complex and differ at the cellular level. For example, there are several forms of estrogen receptor (ER) called ER α and ER β (Kuiper *et al.*, 1997; Dechering *et al.*, 2000). Estradiol has a higher affinity for ER α while the phytoestrogens have a higher affinity for ER β (Kuiper *et al.*, 1997). The tissue distribution and concentration of the different ERs, the affinity of binding of various ligands, as well as the interaction with cofactors in the cells determines the estrogenic/anti-estrogenic response (Diel, 2002). It is the basis of intensive research around the concept of selective estrogen responsive modulators (SERMs) to find compounds which have positive effects on bone while having no proliferative effect on the endometrium or breast (McDonnell, 1999; Diel, 2002). The classical estrogen-responsive tissues such as breast and endometrium mainly express ER α and are very sensitive to estradiol whereas bone cells have a higher level of ER β , making it an interesting target for phytoestrogens. However, the precise role of the ER in bone cells is not clearly understood.

It has been hypothesized that ER β is a modulator of ER α and attenuates its action (McDonnell, 1999).

6.5.2 Maintenance of calcium balance

Based on the knowledge of the effect of estrogens in improving calcium absorption, a second hypothesis for the mechanism of action of soy isoflavones on bone metabolism is that they maintain a positive calcium balance. Estrogen plays an important role in the maintenance of calcium balance, although the mechanism of action is poorly understood (Nordin, 1997). Depletion of estrogen at the menopause results in a decline in calcium absorption, increased urinary calcium losses and subsequent bone loss. Calcium absorption in postmenopausal osteoporosis is improved by HRT, particularly in combination with calcitriol (Prince *et al.*, 1991; Holzherr *et al.*, 2000). It is not clear whether the menopausal changes in calcium metabolism are the cause or the result of postmenopausal bone loss (Nordin, 1997; Prince and Dick, 1997). In the latter case, estrogen deficiency would lead to increased bone resorption and an indirect effect on calcium metabolism via calciotropic hormones. However, a number of animal studies give evidence for a direct positive action of estrogen on gastrointestinal absorption and renal tubular reabsorption of calcium (O'Loughlin and Morris, 1998; Bolscher *et al.*, 1999; Colin *et al.*, 1999; Draper *et al.*, 1999). Estrogen receptors are present in the intestine and kidney, and this could explain an estrogen regulation of vitamin D receptors and calbindin protein in these tissues (Arjmandi *et al.*, 1993; Salih *et al.*, 1996; Liel *et al.*, 1999).

Calcium is conserved in subjects consuming a soy protein rather than an animal protein diet (Anderson and Garner, 1997). This is probably due to the lower sulphur-containing amino acids content of soy protein compared with animal protein. An increased excretion of sulphate originating from sulphur-containing amino acids inhibits the tubular calcium reabsorption. Rats fed soybean milk or soybean milk peptide were shown to have improved calcium absorption (Omi *et al.*, 1992). The synthetic isoflavone, ipriflavone, has been shown to improve *in vitro* intestinal calcium transport in duodenal cells isolated from OVX rats fed ipriflavone at high doses (100 mg/kg daily for 35 days) (Arjmandi *et al.*, 2000). The same group recently showed that soy protein containing a normal level of isoflavones prevented the decreased rate of calcium absorption in duodenal or colonic cells isolated from OVX rats fed with either a casein- or a soy protein-based diet for 35 days (Arjmandi *et al.*, 2002). The mechanism of action is not yet clear.

6.5.3 Effect on bone cells and the remodelling process

Bone maintenance during adulthood is dependent on the equilibrium between bone formation by osteoblast cells and bone resorption by osteoclast cells. Bone loss with age is linked to increased osteoclast activity compared to

osteoblast activity. Osteoclasts are formed following differentiation of monocyte/macrophage cells under the influence of cytokines originating from osteoblast/stromal cells (Teitelbaum, 2000). Primary bone marrow cultures from rat, murine or rabbit origin treated with bone resorbing factors have been used to follow osteoclast formation (by staining for the enzyme tartrate resistant alkaline phosphatase (TRAP)) and osteoclast activity (by quantification of resorption lacunae on bone slices in the pit assay). The *in vitro* studies on isoflavones and bone resorption are summarized in Table 6.5. Most studies report that genistein or daidzein (10^{-5}M – 10^{-7}M) inhibit both osteoclast formation and bone resorbing activity in these models (Yamaguchi and Gao, 1998; Gao and Yamaguchi, 1999a,b, 2000; Kajiya *et al.*, 2000; Yamagishi *et al.*, 2001; Rassi *et al.*, 2002). The authors suggest that estrogen-dependent mechanisms may be involved as the effects can be reversed by estrogen antagonists. Other proposed mechanisms include induction of osteoclast apoptosis, cAMP or calcium signalling or inhibition of tyrosine kinase activity

Table 6.5 Effect of isoflavones in cellular models of bone resorption

Authors	Cell model of bone resorption	Effect of isoflavones
Yamaguchi and Gao, 1998	Rat femoral-metaphyseal tissues cultured for 48 h with bone resorbing factors PTH, PGE2 or LPS) +/- genistein: measured bone calcium content, acid and alkaline phosphatases	Genistein (10^{-5} – 10^{-7}M) inhibited bone resorption. Effect reversed by anti-estrogen, tamoxifen.
Gao and Yamaguchi, 1999b	Mouse bone marrow cells cultured for 7 d with bone resorbing factors (PTH, PGE2, LPS) +/- genistein: osteoclast formation assessed by TRAP enzyme	Genistein (10^{-7} – 10^{-5}M) inhibited osteoclast formation. Mechanism may involve cAMP signalling.
Gao and Yamaguchi, 1999c; Gao and Yamaguchi, 2000; Kajiya <i>et al.</i> , 2000; Yamagishi <i>et al.</i> , 2001	Bone marrow cells or isolated osteoclasts	Suppression of osteoclast formation by genistein is partly due to Ca^{2+} signalling mechanism and partly due to inhibition of tyrosine kinase activity.
Rassi <i>et al.</i> , 2002	Porcine bone marrow cells: measure osteoclast formation (TRAP staining) and activity (pit assay)	Daidzein, at the same concentration as estradiol, inhibits osteoclast formation and activity via caspase-3.
Tobe <i>et al.</i> , 1997	Pit assay with mouse bone marrow cells and dentine slices	Daidzein (10^{-8} – 10^{-10}M) stimulated pit formation while genistein had no effect at this concentration.

by genistein at high doses. Recently, daidzein, at the same concentration as estradiol (10^{-8}M), was reported to inhibit osteoclast formation and activity inducing the apoptosis of osteoclast pre-cursors via caspase-3 (Rassi *et al.*, 2002). At higher doses (20–50 μM) genistein can also affect cytokines with a crucial role in regulation of bone remodelling, such as inhibition of expression of receptor activator of nuclear factor κB (NF κB) ligand (RANKL) and stimulation of osteoprotegerin (OPG) gene expression in the murine stromal cell line ST2 (Yamagishi *et al.*, 2001). Interestingly, one group found that daidzein (10^{-8}M to 10^{-10}M) actually stimulated pit formation in murine bone marrow cells cultured on dentine slices whereas genistein had no effect at this concentration (Tobe *et al.*, 1997).

The effect of isoflavones on bone formation in osteoblast cells has been studied either in cortical bone cultures or in the murine osteoblast cell line, MC3T3 (Table 6.6). Daidzein or genistein (10^{-6}M , 10^{-5}M) stimulated osteoblastic function as shown by stimulation of osteoblast cell proliferation (by cell number or protein/DNA content), induction of alkaline phosphatase (ALP), collagen synthesis and calcium content (Gao and Yamaguchi, 1999c; Sugimoto and Yamaguchi, 2000 a,b; Choi *et al.*, 2001, Yamaguchi and Ma, 2001). Additionally, glycitein (10^{-7}M) inhibited the proliferation and stimulated the differentiation (induction of ALP activity and osteocalcin production) of MC3T3 cells (Yoshida *et al.*, 2001). Effects on both osteoblast proliferation and differentiation appear, at least in part, to be estrogen-receptor mediated

Table 6.6 Effect of isoflavones in cellular models of bone formation

Authors	Cell model of bone formation	Effect of isoflavones
Gao and Yamaguchi, 1999a; Yamaguchi and Ma, 2001	Femoral-diaphyseal tissues from elderly female rats cultured for 24 h	Daidzein or genistein (10^{-6}M , 10^{-5}M) induced calcium content and alkaline phosphatase (ALP) activity indicating stimulation of bone formation.
Sugimoto and Yamaguchi 2000a,b; Yamaguchi and Sugimoto 2000	Murine osteoblastic MC3T3 cells	Genistein or daidzein (10^{-6}M , 10^{-5}M) induced DNA and protein synthesis and ALP activity; effect reversed by estrogen antagonist, tamoxifen. Possible mechanisms via activation of aminoacyl-tRNA synthetase.
Yoshida <i>et al.</i> , 2001	Murine osteoblastic MC3T3 cells	Glycitein (10^{-7}M) suppresses the proliferation and stimulates the differentiation of osteoblasts.
Lee <i>et al.</i> , 2001	Murine osteoblastic MC3T3 cells	Genistein at physiological concentrations stimulates cell proliferation and prevents oxidative damage.
Choi <i>et al.</i> , 2001	Murine osteoblastic MC3T3 cells	Soybean ethanol extract (0.05 g/l) stimulated osteoblastic function (DNA synthesis, collagen synthesis).

as they were inhibited by the estrogen antagonist, tamoxifen. Recently, the possible role of genistein, at physiological concentrations, in preventing the free radical-induced oxidative damage of osteoblastic cells, has been proposed (Lee *et al.*, 2001).

6.6 Dietary recommendations

The mean dietary intake of soy isoflavones in Asian populations consuming soy-based diets ranges from 20–40 mg isoflavones/day, with upper percentile consumer intakes of 70 mg/day (corresponding to around 1 mg/kg body weight). In the six month intervention studies in Western postmenopausal women, the effective dose for improved BMD was around 80–90 mg/day, while in the one year, randomized, double-blind, placebo controlled clinical trial, the effective dose was 54 mg/day. Overall, the dietary recommendation is to consume 50 mg isoflavones/day in combination with standard nutritional requirements for calcium and vitamin D.

Some concerns have been raised as to the suitability of consuming isoflavones while on hormonal treatment for menopausal symptoms. Interactions between HRT and isoflavones are theoretically possible, i.e. influence on ER by isoflavones may enhance or reduce the HRT effect. A recent animal study investigated the interaction between genistein and tamoxifen on the growth of estrogen-dependent breast cancer cells (MCF-7) implanted in ovariectomized athymic mice and found that genistein negated the effects of tamoxifen (Young *et al.*, 2002). However, the doses were very high and outwith the normal dietary range; therefore it is difficult to extrapolate to the human situation. No human clinical studies have been reported; however, there is probably no need for concern on the part of women on HRT who are consuming isoflavones in the order of 'normal' dietary intake (1–2 servings of soy or around 50 mg isoflavones).

6.7 Conclusion and future trends

An overall osteoprotective effect is associated with soy diets, the major active component being the isoflavones although the contribution (if any) of soy protein has to be clarified. The spine, rather than the femur, appears to be the most consistently protected bone site. The average daily intake in Japanese women is around 50 mg/day and appears to be sufficient to have a long-term protective effect on the spine. In non-Asian, postmenopausal women, the demonstrated effective dose is 80–90 mg/day. In future clinical studies, investigating the effect of isoflavones on bone metabolism, larger scale, randomized, controlled, intervention trials for longer time periods (1–3 years) will be necessary with a standardized source of soy protein/isoflavones and

measurements of bioavailability and metabolism. Indeed, it has been recently proposed that the clinical effectiveness of soy isoflavones may be related to the subjects' ability to produce the secondary metabolite equol, from diadzein, via the glycosidase activity of intestinal microflora (Setchell, 2002).

Further research is also required into the mechanisms of action of phytoestrogens and whether there is no interaction with HRT.

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Carotenoids in food: bioavailability and functional benefits

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7.1 Introduction: the concept of bioavailability

When different individuals are given exactly the same oral dose of a drug it is well established that, although their physiological/biochemical response may be generally the same, its degree and duration may vary greatly. Exactly how an individual will respond is an integration of absorption and their whole body biochemistry at the time the dose was given. Exactly the same issues arise when considering the nutrients in foods, except that there is the added complication of the physical form of the food and the oral, gastric and intestinal processes that lead to the complete or partial absorption of the nutrient. Bioavailability of nutrients, as we shall see, tries to integrate absorption and the subsequent *in vivo* effects of transport, tissue distribution and metabolism, and to tie them to some relevant and measurable 'health outcome'.

The types and quantities of phytochemicals present in foods may have very little bearing on the nutritional quality of these foods and their contribution to human health. This is because:

- only a proportion (sometimes highly variable depending upon the food matrix, processing and storage) of these food components can be absorbed into, and utilised by, the body;
- the 'native' compound present in food before ingestion may not be the chemical form to which human tissues are exposed after digestion and pre- /post-absorptive metabolism;
- genetic heterogeneity in human populations may result in individual differences in the absorption, metabolism and/or the extent and rate of tissue targeting.

Thus, to explore the mechanisms of action of phytochemicals and their role in health promotion, an understanding of the factors that constrain their release from the foods in which they are contained, their extent of absorption and their fate in genetically diverse individuals is crucial.

The term bioavailability has various definitions. Previously, the authors of this chapter have defined bioavailability as the proportion of a nutrient (or other food component) that is digested, absorbed and utilised in normal metabolism – with the practical measurement of bioavailability usually relying upon estimates of amounts absorbed (Southon and Faulks, 2001). Biological activity, or ‘bioactivity’, has been viewed and described as a separate stage which follows on from bioavailability in the journey of a compound from food to function. However, here we present a new definition of ‘bioavailability’ that recognises the functional consequences of absorption.

Bioavailability is a term borrowed from the field of pharmacology, where it was originally a useful working concept of ‘rate and extent to which a drug reaches its site of action’. Such a concept, however useful, is generally physically and ethically unattainable in humans, with the result that the pharmacological definition has been redefined as ‘that fraction of an oral dose (parent compound or active metabolite) from a particular preparation that reaches the systemic circulation’ (Schumann *et al.*, 1997). However, despite the practical difficulty of quantifying delivery of food components at specific sites of biological activity, many nutritional scientists have deemed this element of bioavailability to be so important that they have retained it within the bioavailability concept. Irrespective of the practical difficulties associated with making appropriate measurements in humans, the authors of this chapter would now like to introduce the idea that there are also functional consequences associated with the ‘absorption’ of a compound into a body ‘pool’. Once a compound is absorbed it inevitably results in some biological activity, irrespective of whether or not it is chemically inert *in vivo*. The concept of ‘bioavailability’ is thus not separate from, but includes, an element of bioactivity. This proposal is built upon the following considerations.

- Any compound that enters the ‘system’ alters the concentration within the ‘pool’.
- Such a change in concentration of one ‘pool’ will have an impact on the concentration in other ‘pools’ and can therefore be considered bioactive in that it will have an impact on metabolism.
- Pool concentration of a substance that exceeds the ‘threshold’ – for example megadose vitamin C – or substances that are excreted unchanged because they cannot be metabolised, such as sugar alcohols, or compounds that are not biologically essential, such as carcinogens, bacterial toxins and some minor plant constituents, are also ‘bioavailable’ (and thus bioactive) in that they have a metabolic impact, even if this is only the stimulation of detoxification processes, or the use of energy for their excretion.

Thus, a compound that is absorbed will be ‘bioactive’ in that it has metabolic consequences, although this activity can vary enormously in its impact. Bioavailability incorporates, therefore:

1. absorption;
2. tissue ‘distribution’;
3. the functional consequences of absorption.

The absorption and transport processes of many of the phytochemicals present in food are complex and not fully understood, and prediction of their bioavailability is problematic. This is particularly true of the lipid-soluble phytochemicals. In this chapter the measurement of carotenoid bioavailability will be discussed. The carotenoids serve as an excellent example of where too little understanding of food structure, the complexity of their behaviour in foods and human tissues, and the nature and cause of widely different individual response to similar intakes, can lead to misinterpretation of study results and confusion in our understanding of the relevance of these (and other) compounds to human health.

The chapter will cover:

- the putative antioxidant and non-antioxidant functions of carotenoids;
- major food sources and intakes in human populations;
- major factors determining the extent of carotenoid availability from food sources;
- carotenoid absorption and tissue dispersal;
- experimental approaches for measuring absorption – their strengths and weaknesses;
- blood and tissue concentrations in different European populations;
- possible future trends;
- sources of further information and some relevant recently conducted projects.

7.2 Functional benefits of carotenoids: vision, cancer and cardiovascular disease

Most carotenoids have no pro-vitamin A activity with the notable exceptions of β -carotene, and to a lesser extent α -carotene and β -cryptoxanthin. They act as macular pigments (lutein and zeaxanthin) and they have antioxidant and biochemical properties other than pro-vitamin A activity.

7.2.1 Carotenoids and vision

Vitamin A is essential throughout life, including foetal development, but perhaps its most well researched role is that in vision where 11-cis retinaldehyde is the initial part of the photoreceptor complex in rods and cones. Retinoic acid induces differentiation in epithelial cells and deficiency leads to

mucus-producing cells become keratinised. One of the earliest signs of vitamin A deficiency is xerosis conjunctivae which, if left untreated, leads to xerophthalmia and blindness as the cornea becomes keratinised. The macula of the eye is also pigmented with lutein, which is believed to protect against photo-oxidative damage that may lead to loss of visual acuity in age-related macular degeneration (AMD), a major cause of loss of vision in the elderly. Although the progression of AMD may be slowed, and some visual acuity restored by supplementation with lutein, retinal damage is irreversible.

7.2.2 Carotenoids and cancer

One of the enduring problems for all life forms is the prevention of chemical reactions that are not under direct biochemical control and which can result in dysfunctional cellular processes. In the main such reactions are a result of living in an aggressively oxidative environment that contains not only oxygen (21%) but also a range of highly reactive oxygen species and oxygen-containing free radicals. Reactive oxygen species (ROS) derived from the environment, or as a result of normal or dysfunctional metabolism, have the capacity to react with vital systems within cells and to induce undesirable effects. Two such effects have been singled out as being particularly important:

1. genetic damage leading to mutation;
2. oxidation of lipids leading to atherosclerosis.

As a species that has evolved in such an aggressive environment we have developed mechanisms that both defend against oxidative damage and can undertake damage repair. Free radical generation *in vivo* has even been adopted as a mechanism to protect against physical, chemical and biological injury.

The carotenoids found in foods are generally linear all-trans (E) form C40 polyenes formed from eight isoprenoid units. They may be wholly linear (lycopene) or have undergone ring closure at one or both ends. The ring structure(s) may carry hydroxy or keto groups or may be epoxides. In all cases the molecules are predominantly hydrophobic and are usually found in lipid domains of plant and animal tissues. The linear conjugated polyene structure has the ability to delocalise an electron and hence the capacity to act to terminate free radical reactions with the production of resonance stabilised free radical structures.

Because the carotenoids favour hydrophobic domains they are generally localised in the membranes and lipoproteins of animal cells. In this location they can influence the oxidation of membrane lipids and prevent the passage of free radicals from one cellular compartment to another. Thus, DNA in the nucleus is protected from intracellularly generated ROS by (at least) the nuclear membrane and from extracellular ROS by a number of membranes. Should ROS reach the nucleus, base oxidation can occur. The base most susceptible to oxidation is guanine, although all other bases can also be affected. The cell has the ability to detect damaged bases, excise them,

replace them and re-ligate the ends of the molecule. However, some DNA damage does go undetected, or may be incorrectly repaired and replicated, leading to populations of 'transformed' cells that may develop into overt cancer. From this description it is evident that a reduction in cancer rates can be achieved by:

1. reducing the frequency of damage;
2. improving the efficiency (rate and accuracy) of repair.

There is some evidence that carotenoids (particularly β -carotene) may influence both arms of the DNA damage:repair balance (Astley *et al.*, 2002; Fillion *et al.*, 1998).

Other mechanisms proposed to explain the cancer-preventing activities of carotenoid include their impact on cell signalling processes, and particular attention has been given to the stimulatory effects exerted by carotenoids on gap junction communication (GJC) (Wolf, 1992). It has been demonstrated that carotenoids can up-regulate the expression of the connexin 43 gene, promoting the formation of gap junctions between neighbouring cells in a tissue and enabling more efficient exchange of signalling molecules of low molecular weight between those cells (Wolf, 1992). It has been proposed that this has the effect of suppressing cells that have undergone transformation because they are surrounded by, and in communication with, normal cells that suppress proliferation of the transformed cell or induce apoptosis. Indeed, it has been demonstrated that non-tumourous cells are contact-inhibited and have functional GJC, whilst tumour cells have dysfunctional GJC (Habermann *et al.*, 2001). Up-regulated GJC may be a mechanism by which oral doses of β -carotene reverse leucoplakia, a pre-malignant lesion of the buccal mucosa (Barth *et al.*, 1997), although β -carotene supplementation may also increase plasma concentration of tumour necrosis factor alpha (TNF- α) and hence tumour surveillance (Hughes *et al.*, 1996).

There is a growing body of evidence that in small animal models treated with chemical carcinogens carotenoids reduce cancer rates and severity may play a role in suppressing the growth of transplanted cancers (Smith, 1998). In animals, some carotenoids are capable of inducing xenobiotic metabolising enzymes, for example, P-450 dependent enzymes, p-nitro-phenol-UDP-glucuronosyl transferase and quinone reductase (Gradelet *et al.*, 1996). The relevance of laboratory animal studies to the human condition has, however, been called into question by studies such as The Alpha-tocopherol, Beta-carotene Cancer Prevention Group Study (1994) and the 1996 Carotenoid and Retinol Efficacy Trial (CARET) (Omenn *et al.*, 1996). These studies indicate that high dose β -carotene supplementation in 'at risk' groups may result in an increase in lung cancer mortality.

7.2.3 Carotenoids and cardiovascular disease

In lean athletic individuals the amount of fat in the body may be as low as 7% of body mass but this can rise to more than 40% in obesity. The greatest

proportion of fat is in the form of sub-cutaneous adipose tissue, the remainder being present around organs, or as membrane lipids in muscle and in nervous tissue. The plasma contains only a tiny fraction of the body lipids but all lipid transport (absorption from diet and transport to and from stores) is conducted via this pool. Unlike adipose tissue, circulating lipid and lipids in cell membranes are in very metabolically active environments with a high oxygen tension and are most subject to oxidative stress. The prevention of lipid oxidation in the plasma pool, particularly low-density lipoproteins (LDL), has been postulated as a major controlling factor in those disease states (for example, atherosclerosis) that have their genesis in oxidised plasma lipids.

As has already been stated, the carotenoids are lipophilic and are therefore absorbed and transported in association with the lipoprotein particles. In theory, this fortuitous juxtaposition of lipid and carotenoid should confer protection on the lipid through the antioxidant properties of the carotenoid. No doubt some antioxidant protection is afforded by the presence of the carotenoids derived from the diet. However, with one or two exceptions, human supplementation studies have not supported a role for higher dose carotenoid supplements in reducing the susceptibility of the low-density lipoproteins to oxidation, either *ex vivo* or *in vivo* (Wright *et al.*, 2002; Hininger *et al.*, 2001; Iwamoto *et al.*, 2000).

All the actual or putative functional benefits of carotenoids are dependent on their bioavailability: amounts consumed, amounts released from the food structure during digestion and extent of absorption and tissue distribution. The following three sections deal with each of these issues in turn.

7.3 Factors affecting carotenoid bioavailability: food sources and intakes

Of the wide range of animal and vegetable foods that comprise the human diet most contribute to the intake of the 600 identified carotenoids and related compounds. However, only a few carotenoids, primarily in fruits and vegetables, are ingested in sufficient quantity to be detected in human plasma (Khachik *et al.*, 1992), the most abundant being β -carotene, lutein, lycopene, α -carotene, β -cryptoxanthin and zeaxanthin, along with their more common *cis* isomers and some degradation products. In the plant, the carotenoids serve two essential functions: as accessory pigments in photosynthesis and in photoprotection. In most cases, the carotenoid (normally present as the all-trans form) is associated intimately with the light-harvesting complex in the thylakoid membranes of the chloroplast where it is found as an ordered structure in association with binding protein. In the case of the carrot root and tomato fruit the β -carotene and lycopene, respectively, occur as membrane bounded semi-crystalline structures derived from chromoplast or chloroplast structures. It might be expected that in plants the lipophilic carotenoids

Table 7.1 Common dietary sources of carotenoids in regular vegetable foods, µg/100 fresh weight. Data are means derived from literature sources. The normal range of values is the mean ± at least 85%, and depends upon variety, agronomic conditions, tissue sampled and maturity

Food	β-Carotene	α-Carotene	Lutein	Lycopene	β-Cryptoxanthin	Zeaxanthin	Capsanthin
Carrot	7975	2186	271	—	—	—	—
Spinach	4489	—	6265	—	—	—	—
Broccoli	1580	—	2560	—	—	—	—
Lettuce	890	—	1250	—	—	—	—
Green peas	548	—	1840	—	—	—	—
Watercress	5919	—	10713	—	—	—	—
Tomato	608	—	77	4375	—	—	—
Orange ^e	250	200	120	—	700	—	—
Orange juice ^e	375	—	1180	—	1980	—	—
Mandarin ^e	275	—	50	—	1775	140	—
Sweet corn	45	60	520	—	—	440	—
Red pepper ^e	1700	30	270	—	250	600	2520
Apricot ^e	3500	tr	70	tr	120	—	—
Mango ^e	3100	—	—	—	800	—	—
Papaya ^e	640	30	—	3400	770	—	—
Watermelon	180	tr	20	4750	300	—	—
Sweet potato	10–22600	Included because of breeding programmes to increase β-carotene					

e = carotenoid esters present; tr = trace.

would be found in association with sub-cellular lipid structures, but it is also known that there are associated binding proteins (Cogdell, 1988). Such a complex environment has implications for their extraction, analysis and behaviour during digestion.

Table 7.1 shows the common dietary sources of carotenoids in regular vegetable foods, $\mu\text{g}/100$ fresh weight. Although the greatest amount of the hydrocarbon carotenoids is present as the all-trans isomer, there is always a proportion of cis isomers present. This table represents only a small number of the fruits and vegetables that contribute carotenoids in the European diet. For more comprehensive information readers are directed to 'A European Carotenoid Database' O'Neill *et al.*, 2001, which lists the carotenoid composition of over 100 food items.

The range ($\mu\text{g}/100$ fresh weight) of lycopene and β -carotene in selected tomato cultivars can be 20–62000 and 35–2200 respectively, and of β -carotene and α -carotene in selected carrot cultivars 1100–64000 and 530–36000 respectively. Some of the carotenoids may be present as fatty acid esters (Breithaupt and Bamedi, 2001). More extensive listings can be found (O'Neill *et al.*, 2001; van den Berg *et al.*, 2000; Hart and Scott 1995).

Figure 7.1 shows the relative intakes of the carotenoids β -carotene, α -carotene, lutein (+zeaxanthin), lycopene and β -cryptoxanthin for five groups of individuals selected from five European countries (ca. 40 male, 40 females, 25–45 yrs, from each of the five countries: Spain, France, Northern Ireland, Eire, and The Netherlands) (O'Neill *et al.*, 2001). Not surprisingly the Spanish were shown to have the highest mean intake of β -cryptoxanthin (derived primarily from oranges) and lutein (derived primarily from spinach). However, in this study, the lycopene and β -carotene intakes in Spain were significantly lower compared to subjects from the other countries. This was ascribed to the lower intake of β -carotene-rich carrots and pizza (with its associated lycopene-rich tomato sauce) in the Spanish group.

7.4 Release from food structures: maximising availability for absorption

The physical properties of plant cells (rigidity, plasticity, elasticity, shape, function, etc.) are essential features in maintaining the architecture of the plant. Plant cell walls of edible tissues are essentially composed of cellulose fibrils laminated at different angles with pectic substances and other polysaccharides, many of which are x-linked to each other, either directly, by a variety of bonding, or indirectly, through intermediates, e.g. ferulic acid esters, divalent cationic bridges. The cell wall can be thin (1–2 μm) in some tissues, for example cereal grains or potato, or thick (10–15 μm) as in the case of mature legume seed. The cell wall is a living part of the cell, and it is subject to compositional and structural changes as the tissue grows, matures

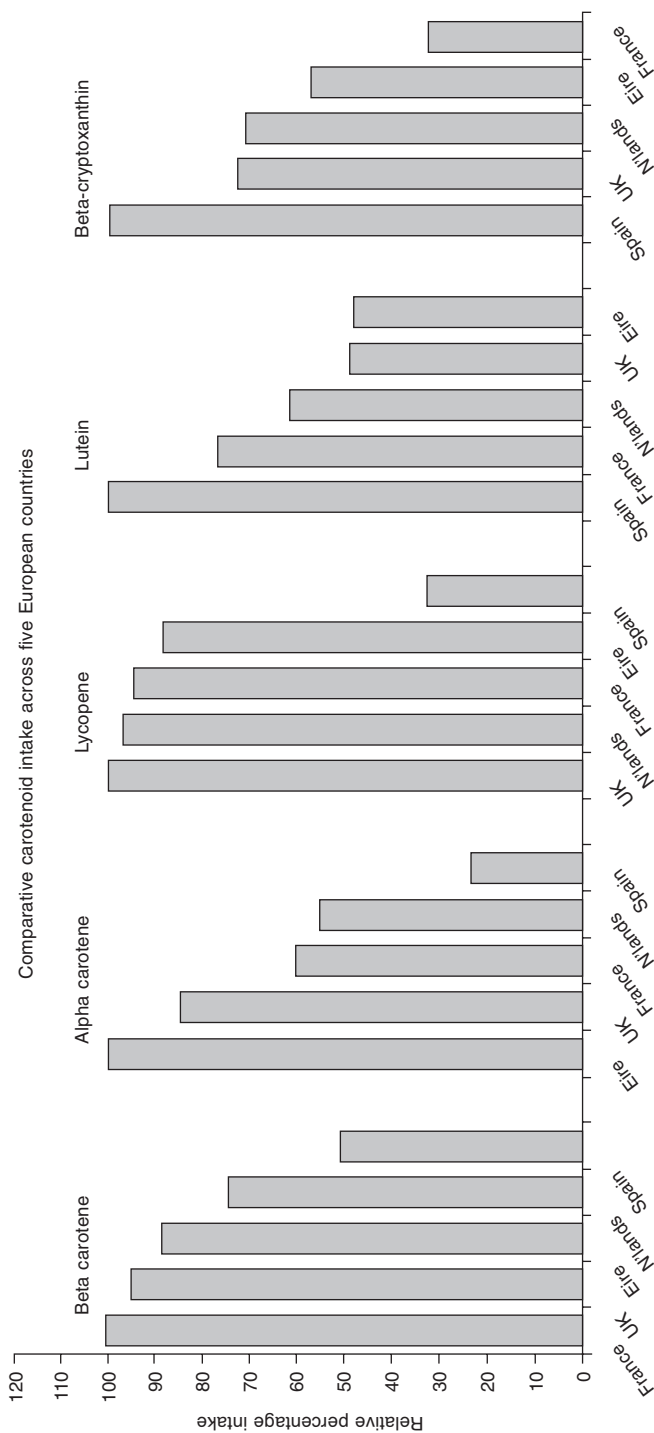


Fig. 7.1 Comparison of the relative carotenoid intakes in adults in five European countries. Data are mean values derived from O'Neill *et al.*, 2001.

and becomes senescent. Such compositional changes alter the physical characteristics of the tissue. Fruit softens during ripening, whilst soft meristematic tissues become stringy and tough as the tissues mature (runner beans, asparagus). Because of its chemical nature, the cell wall is resistant to the digestive enzymes of the gastrointestinal (GI) tract and is lost into the large intestine along with any other components that have escaped digestion and absorption. In the intestine they provide the substrates for the growth of the colonic microflora and contribute to faecal bulking.

From the above description it will be appreciated that the efficiency of release of nutrients from ingested plant material is dependent upon the ease with which the digestive enzymes can penetrate the cell wall to release the nutrients so that they can diffuse out of the structure to be absorbed. Thus tissue maturity, cooking, macerating, mastication and mode of tissue failure, all of which control particle size, cell wall softening or cell disruption, are key features which regulate nutrient release.

With few exceptions, small particles of vegetable foods are generally stripped of their more accessible nutrients during digestion in the GI tract. In this way starch, protein, fat and water-soluble small components (sugars, minerals) are usually well absorbed. This is not always the case, however, for larger food particles or for molecules that cannot diffuse out of the cell/tissue. Neither is it the case for the lipid-soluble components. These need to be dissolved in lipid before they can be physically removed from the cell to the absorptive surface, since the cell wall is unlikely to be permeable to lipid emulsions or micelles, and the presence of lipases will strip away the solvating lipid.

The analysed nutrient content of foods is therefore only a guide to what is actually digested and absorbed, particularly if the matrix in which the nutrient is embedded impedes extraction. The carotenoids are very hydrophobic and are normally associated with the lipid structures of the sub-cellular organelles. In green leafy vegetables, the main carotenoids, lutein and β -carotene, are bound to lipoproteins in the light-harvesting complex of the chloroplasts (organelles responsible for photosynthesis). In the carrot and tomato, the carotenoids may be present as membrane bounded semi-crystalline structures, or present in lipid droplets. In fruits, the carotenoids (and their fatty acid esters) are more frequently present in oil droplets, although the solubility of carotenoids in oil is low. The different types of plant tissue (leaf, root, fruit, seed) and the environment and physical nature of the cellular carotenoids have implications for the ease with which they are made available for absorption, as already discussed.

As a general rule, cooking and processing sterilises and softens the plant tissue through swelling of the cell wall, dissolution/depolymerisation of pectin and cell separation (the primary mechanism of tissue disintegration). In contrast, mastication of raw fruit and vegetables causes crushing and shearing of the tissue and tears the cells open (Fig. 7.2). Both mechanisms of particle size reduction will contribute to increased release, so it is not clear whether

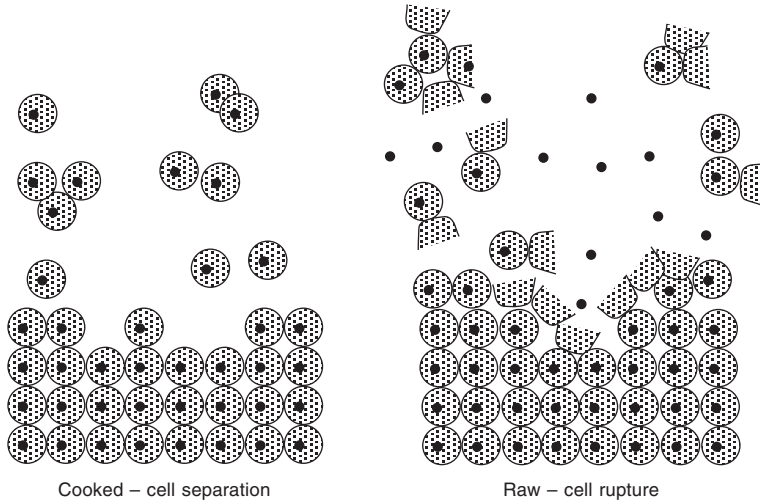


Fig. 7.2 The impact of cooking and mastication of raw food on plant cells.

the raw or cooked tissues will provide more bioaccessible carotenoid. This is clearly demonstrated by examination of grated carrot strips fed to ileostomy patients. Carrot tissue pieces recovered from the terminal ileum (having passed through the GI tract) show loss of the carotenoid from only the fractured surface cells. There is no evidence for loss of carotenoid from deeper plant tissue.

It will be appreciated that the delivery of nutrients from foods is attenuated by the structure of the food and the way in which it is digested. Thus, delivery from the food structure occurs over the same timescale as gastric emptying. Carotenoids, and other compounds, isolated from the food structure are generally emptied from the stomach and absorbed more rapidly. These different rates of delivery may have profound effects on subsequent metabolism.

There are proven health benefits from 'slow release' carbohydrate foods. They do not stimulate the over-secretion of insulin, cause undesirable large excursions in blood glucose or unnecessary glycosylation of proteins. By analogy, the slower delivery of other food components may maximise health benefits by not overloading transport systems or causing undesirable excursions in plasma concentration. The fact that a variable portion of ingested nutrients escapes absorption and is 'lost' to the colon should not automatically be interpreted negatively, since that part may contribute positively to colonic health and the production of beneficial products of colonic fermentation.

The complex nature of the mass transfer of carotenoids to absorbable lipid species, the diversity of raw and processed foods consumed, and individual variations in the degree of mastication, will lead to differences in the amount of carotenoid that becomes bioaccessible and potentially available for absorption. By understanding the underlying mechanisms of these processes, for a wider range of fruit and vegetable constituents, it will become possible

to recommend which 'five portions' a day are best to achieve a given health benefit. Such an understanding will also make it easier to suggest domestic and commercial processing practice to maximise potential health benefits by increasing the amount of bioaccessible carotenoid within the normal Northern European diet.

7.5 Absorption and metabolism

Irrespective of the physical form of the carotenoid in the plant tissue it needs to be dissolved directly into the bulk lipid phase (emulsion) and then into the mixed micelles formed from the emulsion droplets by the action of lipases and bile. Alternatively it can dissolve directly into the mixed micelles. The micelles then diffuse through the unstirred water layer covering the brush border of the enterocytes and dissociate, and the components are then absorbed. Although lipid absorption at this point is essentially complete, bile salts and sterols (cholesterol) may not be fully absorbed and are not wholly recovered more distally, some being lost into the large intestine. It is not known whether carotenoids incorporated into mixed micelles are fully or only partially absorbed.

Absorbed carotenoids pass through the enterocytes from the luminal to the serosal side where they are incorporated into chylomicrons along with triglyceride and exported in lymph that is carried by the thoracic duct to the inferior *vena cava*. The absorbed chylomicrons therefore bypass the liver and enter the main systemic circulation. On entering the extrahepatic capillary bed, the chylomicrons are acted upon by endothelial lipoprotein lipase and give up some of their lipid to the surrounding tissue to leave a lipid reduced chylomicrons remnant. It is not clear how much of the carotenoid and other lipid-soluble components are absorbed into the peripheral tissues. The liver clears the chylomicrons remnants with any associated carotenoid. It should be noted that chylomicrons only have a very short half-life (ca. 3–5 mins) as do chylomicrons remnants (ca. 11 min). Carotenoid sequestered by the liver is re-exported in very low-density lipoproteins (VLDL) which undergo a lipid reduction process similar to that of the chylomicrons to become intermediate-density lipoproteins and low-density lipoproteins (IDL, LDL). Ultimately circulating carotenoid is distributed between LDL and high-density lipoproteins (HDL) in roughly equal proportions, although LDL normally carries about 60% of the plasma β -carotene.

A small but variable proportion of the carotenoids with one or two β -ionone rings (mainly β -carotene) are cleaved in the enterocytes to produce retinol (vitamin A). This process is very tightly controlled, so that too much vitamin A is not produced, although the control mechanism is not clear. Some cleavage of β -carotene can also occur in the liver, but this does not account for the turnover of β -carotene in the body. Small amounts of carotenoids are subject to enterohepatic circulation, but this does not account for losses.

The mechanisms of the metabolism and excretion of β -carotene are not clear, other than the identification of a number of partially oxidised intermediates found in plasma (Khachik *et al.*, 1992). It is assumed that the carotenoids are metabolised in a manner analogous to the β -oxidation of fatty acids although there is no evidence for this.

It is well known that excessive intake of β -carotene may lead to carotenoderma (yellow skin), and it is undoubtedly the case that some carotenoid is directly lost via the skin or through photo-oxidation in the skin. As far as is known the carotenoids are not cytotoxic or genotoxic even at concentrations up to 10 times the normal plasma concentration which may cause carotenoderma. However, they are associated with amenorrhoea in girls who may be consuming bizarre diets and, in long-term supplementation studies, with an increase in lung cancer (The Alpha-tocopherol, Beta-carotene Cancer Prevention Study Group, 1994).

7.6 Methods for predicting absorption

The measurement of carotenoid absorption is fraught with difficulties and riddled with assumptions, and it is therefore a complex matter. Methods may rely on plasma concentration changes provoked by acute or chronic doses, oral-faecal mass balance method variants and compartmental modelling.

7.6.1 Chronic dosing

Chronic dosing studies are normally carried out within individuals in a group using a cross-over design with a washout period. With this design each volunteer acts as their own control. The regimen normally takes the form of supplementation to the normal diet, over a period of weeks, with isolated compounds (tablets/capsules) or carotenoid-containing foods. The concentration changes of carotenoids in the blood plasma or serum are tracked over time until a new plateau is reached. The relative absorption of the two or more supplements is then calculated against a 'standard', usually the isolated compound, because this is assumed to be most easily and thoroughly absorbed. With this design it should be noted that a new plasma concentration plateau must be reached, the same compound can be compared from different sources and that only relative absorption data can be acquired. With chronic dosing it is essential that the data not be compromised by daily fluctuations induced by the timing of the ingestion of the supplement and taking of the blood samples. The method is therefore useful for comparing carotenoid absorption from, for example, a food product that has been subjected to a number of different treatments (Castenmiller *et al.*, 1999).

7.6.2 Acute dosing

Classically, to measure absolute absorption the plasma area under the curve from an intravenous dose would be compared to that caused by the feeding of an oral dose. However, the carotenoids are lipid-soluble and are normally incorporated in chylomicrons synthesised in the enterocytes, a situation that cannot be replicated and applied to studies in humans because an intravenous preparation that would behave 'naturally' is not possible.

With acute dosing the objective is to measure either relative or absolute absorption by following the absorption and disposal of a single dose through monitoring the appearance and disappearance of the carotenoid in the plasma. The experimental design can be a cross-over where each volunteer acts as their own control, or can be based on a single dose if mathematical modelling is to be applied to the data. Relative absorption may be measured from the peak plasma excursion or by measuring the area under the complete curve. Absolute absorption may be measured from the complete area under the curve, if the clearance rate is known, and it is assumed that unit absorption provokes unit excursion, i.e. clearance rate is not plasma concentration dependent.

Because of the rapid clearance of the chylomicrons (half-life 3–5 min) that carry the newly absorbed carotenoid, it is not easy to provoke significant plasma response curves with a physiological dose (5–15 mg) of carotenoid. This has led to the belief that carotenoids are poorly absorbed by some individuals and that there are 'responders' and 'non-responders' as classified by plasma response to an acute oral dose of β -carotene. An added complication to using plasma is that the carotenoids which are carried by the chylomicrons are rapidly sequestered by the liver and re-exported in VLDL which in turn becomes LDL. Thus the carotenoid distribution among the lipoprotein carriers changes over the period of the study. There are two resolutions to this problem: (i) isolate the chylomicrons or; (ii) continue the study over several days until the carotenoid is cleared from the long-lived lipoprotein particles. The latter is impractical since it would mean maintaining the volunteers on carotenoid-free meals for several days and because, during this time, baseline carotenoid concentrations would start to fall. Analysis of the chylomicrons fraction is therefore to be preferred since the appearance of newly absorbed carotenoid in this plasma fraction is over within about 6 h and clearance is complete in 12–14 h. This is a tolerable time for volunteers to fast, or they can be given a low-fat carotenoid-free meal at 5 h when the test meal has been completely emptied from the stomach. This procedure also has the added advantage that, whereas the carotenoid concentration change may not be seen against the endogenous background in plasma, it will be seen in the chylomicrons which carry only about 10% of the total plasma carotenoid at peak absorption (van het Hof *et al.*, 2000, van den Berg and van Vliet, 1998, van Vliet *et al.*, 1995).

The single acute dose method can be applied to whole plasma studies if the dose can be discriminated from the endogenous background. Discrimination

can be achieved by using both stable and radioactively labelled isotopomers of the carotenoid and mass spectrometry. The application of compartmental analysis allows the calculation of absolute absorption and metabolic kinetics (Parker *et al.*, 1993; Novotny *et al.*, 1995).

7.7 Tissue concentrations

The concentration of carotenoids in various tissues is very uneven. With the exception of the retina those tissues with a large number of LDL receptors are those with the highest concentration of carotenoid. In more remote body tissues variable profiles and concentrations of carotenoids have been reported, but factors controlling carotenoid uptake by the tissues are still incompletely known (Kaplan *et al.*, 1990; Schmitz *et al.*, 1991; Stahl *et al.*, 1992; Furr and Clark, 1997). There is probably an association with lipoprotein (low- and high-density lipoprotein) uptake, which might explain the β -carotene accumulation in the liver, reproductive tissues and adrenals. However, the marked enrichment of the human macular pigment (MP) with xanthophylls (lutein and zeaxanthin), or β -carotene in the pineal gland and in the corpus luteum of cattle, suggests selective tissue uptake. Figures 7.3 and 7.4 provide examples of the concentrations of β -carotene and lycopene, respectively, found in a range of tissue samples.

An evaluation of the Health Professionals Follow-Up Study (Giovannucci *et al.*, 1995) has detected a lower prostate cancer risk associated with the greater consumption of tomatoes and related food products. Tomatoes are the primary dietary source of lycopene and lycopene concentrations are highest in testis and adrenal tissue (Clinton, 1998). In paired benign and malignant prostate tissue from 25 American men, 53–74 yrs, undergoing

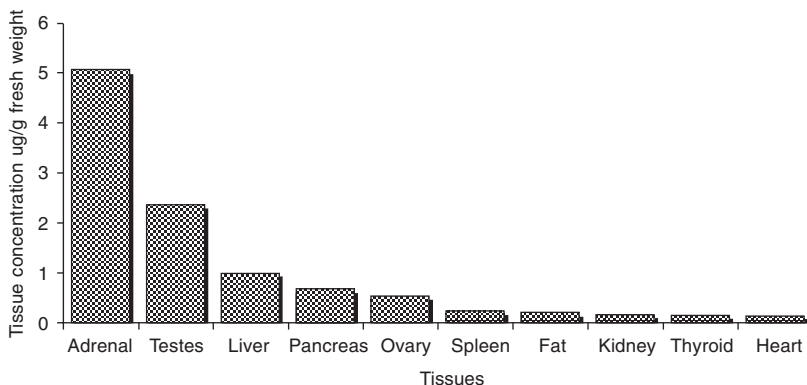


Fig. 7.3 Distribution of β -carotene in human tissues. After Kaplan *et al.*, 1990.

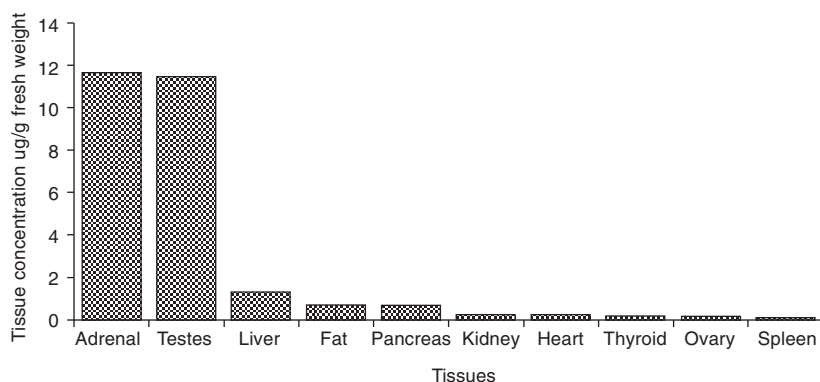


Fig. 7.4 Distribution of lycopene in human tissues. After Kaplan *et al.*, 1990.

prostatectomy for localised prostate cancer, lycopene and all-trans β -carotene were the predominant carotenoids present, with means \pm SE of $0.428 \pm 0.04 \mu\text{g/g}$ and $0.290 \pm 0.05 \mu\text{g/g}$, respectively. Lycopene concentrations ranged from $0\text{--}1.38 \mu\text{g/g}$, and all-trans β -carotene concentrations from $0.05\text{--}0.64 \mu\text{g/g}$. The 9-cis β -carotene isomer, α -carotene, lutein, α -cryptoxanthin, zeaxanthin and β -cryptoxanthin were consistently detectable in prostate tissue. The paper reporting these results claimed that the presence of lycopene in the prostate at concentrations that are biologically active in laboratory studies supports the hypothesis that lycopene may have direct effects within the prostate and may contribute to the reduced prostate cancer risk associated with the consumption of tomato-based foods (Clinton *et al.*, 1996).

The relationship between serum and tissue concentrations of lutein and zeaxanthin was recently studied by Johnson *et al.*, (2000). Dietary intake of xanthophyll-rich vegetables (for example, spinach and corn) resulted in significant increases in lutein concentration in serum, adipose tissue and buccal cells, and this correlated with changes in MP density. However, β -carotene and lycopene are normally the major carotenoids detected in buccal cells (Peng *et al.*, 1994).

The hydrocarbon carotenoids also accumulate in the skin (Ribaya-Mercado *et al.*, 1995; Stahl *et al.*, 2000), and were found to be decreased, especially lycopene, after UV exposure (Ribaya-Mercado *et al.*, 1995). Earlier data from Micozzi *et al.*, (1988) indicated that carotenoderma, i.e. yellowing of the skin, only occurs at high daily intake levels ($> 30 \text{ mg/d}$) of purified (synthetic) β -carotene. However, 3 out of 15 women given 15 mg/d of palm oil carotenoids showed carotenoderma after 21 days. Those exhibiting carotenoderma could not be distinguished from the group by BMI, weight, initial or final plasma concentration of β -carotene, indicating that these volunteers may disperse β -carotene differently between body tissues (Faulks *et al.*, 1998).

Although adipose tissue has a comparatively lower carotenoid concentration, it contains a significant portion of the total body carotenoid content, the proportion obviously depending on adiposity. Fat biopsies have been used as 'internal dose' biomarkers of long-term carotenoid 'exposure' (Su *et al.*, 1998). Adipose tissue carotenoid concentrations appear to be related to serum concentrations, with low, but significant, correlation coefficients between 0.24 and 0.39. Similarly, significant correlations have been reported between plasma or serum carotenoid concentrations and those in cervical tissue and normal breast adipose tissue (Gamboa-Pinto *et al.*, 1998; Yeum *et al.*, 1998). Erythrocytes, leucocytes, and cell membranes also contain carotenoids (Fotouhi *et al.*, 1996). Lymphocyte lycopene has been found to be related to plasma concentrations as well as to lymphocyte resistance to hydrogen peroxide induced oxidative stress (Porrini and Riso, 2000).

7.8 Future trends

Scope for improvement in dietary supply of carotenoids is an obvious area for future research, particularly since modern lifestyles require reduced energy intake, whilst research continues to indicate that increased micronutrient density of the diet is required for the maintenance of optimal health and the avoidance of chronic disease. The normal genetic diversity, and the spontaneous appearance of sports or mutants, in plant populations allows for selection of particular types with very different morphological and biochemical characteristics. Among these is the ability to express and accumulate carotenoids (and carotenoid pre-cursors), hence the huge ranges of carotenoid concentrations and diversity of carotenoid profiles that are possible within a plant species. The exception to this is the starchy seeds that provide most of the world's food energy. Some seeds contain low amounts of carotenoids, for example maize and chickpea, but most of the cereals, for example rice and wheat, are virtually carotenoid-free. Selective breeding programmes and genetic modification are currently being used to increase the β -carotene content of cereal crops (Golden Rice) and sweet potato (*Ipomea batatas*) with the intention of reducing the incidence of retinol deficiency in those populations who do not have ready access to pre-formed vitamin A in the diet. An increased concentration of a range of phytoprotectants, with proven efficacy, into staple foods also provides an important strategy for maintaining the micronutrient density of the diet in the face of reduced energy requirements.

Of course, the term 'proven efficacy' is central to any resource investment in this area. Basic information on time and dose responses in humans to complex foods rich in carotenoids (and other phytochemicals) is pitifully small. Much of our information is based upon inadequate databases derived from chemical analysis, *in vitro* models that have not been properly evaluated or validated, and short-term, high-dose human studies. Future research progress requires much more rigorous debate on the experimental systems employed

to determine efficacy of both isolated food components and the complex foods themselves.

7.9 Sources of further information and advice

Because of the large body of evidence that has accumulated on the biological activity of the carotenoids there are a growing number of reviews and papers relating to the epidemiology of disease and carotenoid intake, enhancement of carotenoid content of foods, effects of processing, bioavailability and the kinetics of absorption and distribution, effects on the initiation and progression of cancer and genetic effects in cell culture studies. At least two major reviews have been commissioned by the European Commission: they are FAIR CT 97-3233, The White Book on antioxidants in tomatoes and tomato products and their health benefits; and FAIR CT97-3100, Model systems, *in vitro* and *in vivo*, for predicting the bioavailability of lipid-soluble components of foods. Additionally, the European Commission has sponsored a concerted action, Nutritional Enhancement of Plant-based Food in European Trade (NEODIET), that has been published in *The Journal of Science of Food and Agriculture*, Vol 80, 2000. ISSN 0022-5142.

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The functional benefits of flavonoids: the case of tea

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8.1 Introduction: types of tea

Tea (*Camellia sinensis* L.) has been a traditional drink in China since 3000 BC (Balentine, 1997; Cheng and Chen, 1994) and has now become the most popular beverage after water throughout the world. In recent years, tea has attracted significant attention because of its reported health benefits, particularly as an antioxidant, but also as an anti-carcinogenic and anti-arteriosclerotic agent. It is generally believed that flavonoids are responsible for these actions (Balentine *et al.*, 1997; Dreostic *et al.*, 1997; Jankun *et al.*, 1997; Yang, 1997). The present review assesses recent developments in relation to former research, discussing the different functions of tea flavonoids and the preparation and utilisation of different tea extracts.

Many different teas are produced. These can be classified into three main types: green (unfermented), oolong (semi-fermented), and black (fully fermented) teas. Green tea is made by inactivating the enzymes in the fresh leaves, either by firing or by steaming, to prevent the enzymatic oxidation of tea catechins. Processed products are described as roasted green tea and steamed green tea. Black tea is made by a polyphenol oxidase catalysed oxidation of fresh leaf catechins, termed fermentation, which is initiated by withering and rolling processes. This fermentation process results in the oxidation of simple polyphenols, the tea catechins, to more complex condensed molecules which give black tea its typical colour and strong astringent flavour. Oolong tea is prepared by a variety of processes including firing, which is carried out shortly after rolling and arrests fermentation halfway through the process. Hence oolong tea is called semi-fermented tea. Its characteristics are between those of black and green tea.

In China some teas, such as Pu-er tea, are made through microbial fermentation and called post-fermented teas. The typical characteristic of these teas is a 'mouldy' or 'aged' flavour; the more intense this flavour, the better the quality. Other kinds of tea, such as white, yellow and dark, are produced in small quantities and consumed locally. Further processing of these teas produces scented and brick teas.

The differences in types of tea are due not to the variety of the tea, but rather to the tea processing. The same basic tea leaves can produce green, black or oolong teas, although some varieties of tea leaves are more suited to certain types of tea. So-called herbal teas are produced from mixtures of flowers, berries, peels, seeds, leaves and roots from many different plants. They are quite different from tea. However, combinations of these herbal materials and tea leaves are also present in the market.

8.2 Flavonoids and other components of tea

This section discusses the following components of tea:

- flavonoids: catechins, flavonols, theaflavins and thearubigins, and proanthocyanidins;
- other components: alkaloids, phenolic acids, vitamins and volatile compounds.

8.2.1 Tea flavonoids

Tea flavonols (catechins)

Structurally tea catechins are, primarily, flavonols and form 20–30% of the dry weight of green tea (Balentine *et al.*, 1997; Sanderson, 1972). These flavonoids are produced via the shikimic and acetate-malonate biosynthetic pathways, characterised by their C6–C3–C6 skeletal structure corresponding to 2-phenyl-substituted benzopyrans and pyrones. The major catechins in fresh tea leaves and green tea are (–)-epigallocatechin gallate (EGCG), (–)-epigallocatechin (EGC), (–)-epicatechin gallate (ECG) and (–)-epicatechin (EC) (Fig. 8.1). Catechins are colourless, water-soluble compounds which impart bitterness and astringency to green tea infusion. Almost all of the characteristics of manufactured tea, including its taste, colour and aroma, are associated directly or indirectly with modifications to the catechins. For example, a decrease in catechin content during black tea manufacture is associated with an increase in the content of monoterpene alcohols which can improve the aroma quality of the tea (Wang *et al.*, 1993); degalloation from ester catechins to non-ester catechins can result in a decrease in the bitterness and astringency of green tea (Nakagawa, 1975; Wang *et al.*, 1998a); and epimerisation, that is conversion of tea catechins to their corresponding

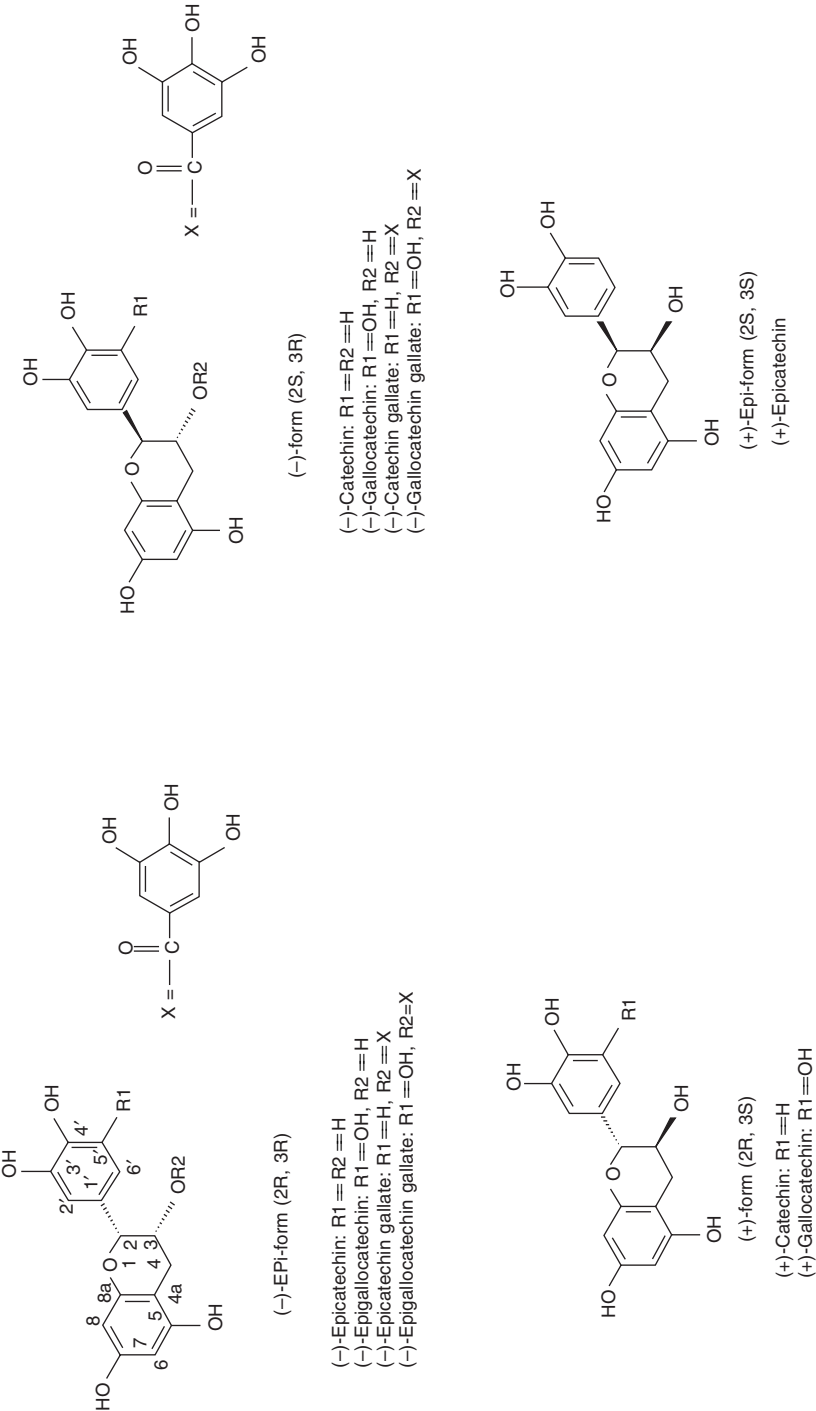


Fig. 8.1 Chemical structures of tea catechins and their epimers.

isomers, can occur during the production, brewing and storage of tea (Suematsu *et al.*, 1992; Wang *et al.*, 1998b; Wang and Helliwell, 2000).

Catechins are not unique to tea; they have been found in red wine, apples, grapes and chocolate. However, tea was found to be the only beverage that contained (+)-gallocatechin (GC), EGC, ECG, and EGCG in addition to (+)-catechin (C) and EC (Arts *et al.*, 2000). Tea catechins undergo extensive *O*-methylation, glucuronidation, and sulphation. These reactions may play important roles in determining the bioavailability of tea catechins (Yang *et al.*, 2001).

Flavonols

The main flavonols in tea leaves are quercetin, kaempferol and myricetin (Fig. 8.2). They compose up to 2–3 % of the water-soluble extractive in tea (Balentine *et al.*, 1997; Cheng and Chen, 1994). Flavonols are predominantly present as glycosides rather than as their non-glycosylated forms (aglycones). The presence of at least 14 glycosides of myricetin, quercetin and kaempferol in fresh tea shoots and green and black teas has been reported (Engelhardt *et al.*, 1992). The sugar moieties consist of glucose, rhamnose, galactose, arabinose and fructose. Mono-, di- and tri-glycosides have been identified (Finger *et al.*, 1991). The flavonol aglycones are not found in significant quantities in tea beverages due to their poor solubility in water. Variable values for the content of flavonol and flavonol glycosides in teas have been reported (Baily *et al.*, 1990; Biedrich *et al.*, 1989; Fieschi *et al.*, 1989; Finger, *et al.*, 1991; Hertog, *et al.*, 1993b; Price *et al.*, 1998; Wang and Helliwell, 2001). This is possibly due not only to the different tea samples used, but also to the different analytical methods utilised, for example, different quantities of tea samples extracted with different solvents at different temperatures for different periods of time, resulting in different extraction efficiencies.

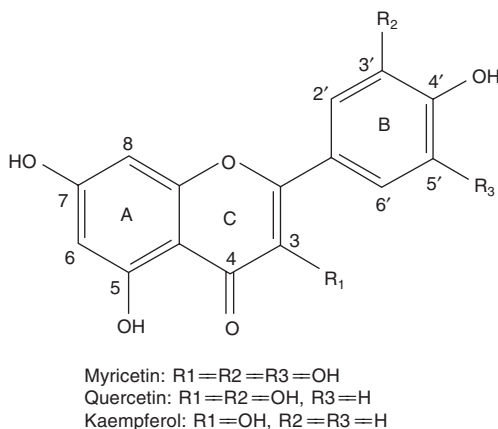
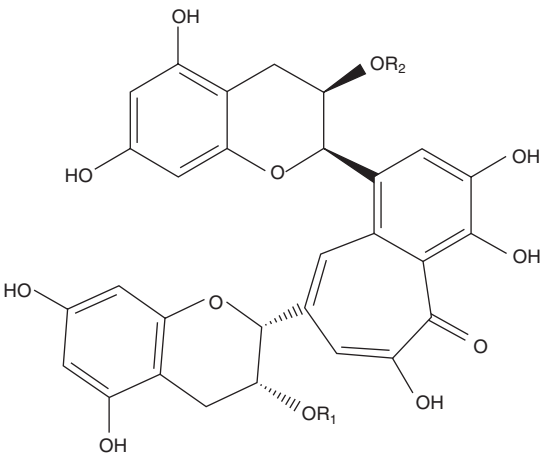


Fig. 8.2 Structures of flavonols in tea.

Theaflavins and thearubigins

In the manufacture of black tea, catechins can be oxidised to form the typical colour and flavour components of black tea. Classically, the pigments of black tea have been divided into orange-coloured theaflavins (TFs) and brownish thearubigins (TRs). As shown in Fig. 8.3, there are four main TFs in black tea: theaflavin, theaflavin 3-gallate, theaflavin 3'-gallate, and theaflavin 3,3'-digallate. These are formed through oxidative dimerisation between quinones derived from a simple catechin and a gallocatechin. Thearubigins are a heterogeneous group of phenolic pigments with relative molecular masses in the range 700–40,000 Da (Roberts, 1962; Sanderson *et al.*, 1972). They originate from further oxidative condensations via either C–O or C–C bond formation in oxidative polymerisation reactions. The content of TFs in black tea is 0.3–2.0% on a dry weight basis (Balentine *et al.*, 1997), while the TRs fraction comprises 10–20% of the dry matter of black teas (Sanderson, 1972). Both TRs and TFs contribute to tea brew characteristics such as colour, strength and body (Cheng and Chen, 1994; Roberts and Smith, 1963). Although the chemical structures of TRs have not yet been fully elucidated, many researchers have confirmed the presence of flavonoid structures (Bailey *et al.*, 1991, 1994; Ozawa *et al.*, 1996).



		R1	R2
Theaflavin	TF	H	H
Theaflavin 3-gallate	TF3G	Gallate	H
Theaflavin 3'-gallate	TF3'G	H	Gallate
Theaflavin 3,3'-digallate	TFDG	Gallate	Gallate

Fig. 8.3 Structures of theaflavins in black tea.

Proanthocyanidins

Proanthocyanidins are an important group of di- to oligomeric flavonoids in plants. Four proanthocyanidins (procyanidin B3, prodelphinidin B4, ECG-(4 → 8)-ECG and GC-(4 → 8)-EGCG) were determined quantitatively in tea. The amounts in fresh tea leaves were between 1 and 2 g/kg per compound (Nakabayashi, 1991). The occurrence of proanthocyanidins may serve as a criterion for the differentiation between fermented and non-fermented teas (Kiehne *et al.*, 1997).

8.2.2 Other components*Alkaloids*

Tea leaf contains 2.5–4.0% caffeine (1,3,7-trimethylxanthine) on a dry weight basis and smaller quantities of the related methylxanthines, theobromine (3,7-dimethylxanthine; 0.2–0.4%) and theophylline (1,3-dimethylxanthine; *ca.* 0.02%). Although it is said that *var. sinensis* is slightly lower in caffeine than *var. assamica*, black, green and oolong tea beverages all contain about the same levels of caffeine (Cheng and Chen, 1994).

Phenolic acids

Gallic acid is the predominant phenolic acid in tea. Gallic acid contents in brews obtained from Chinese tea samples ranged from 0.4–1.6 g/kg dry mass. It occurs at higher levels in black tea because of liberation from the gallated catechins during fermentation. Gallic acid also plays a key role in forming esters with various polyphenols (Cheng and Chen, 1994).

The content of theogallin in tea is around 1.0% on a dry weight basis. Theogallin was isolated and characterised as galloylquinic acid by Roberts and Myers (1958). Although theogallin is a particularly interesting compound, as it is specific to tea, little research has been published on it. Chlorogenic acid (3-caffeoylquinic acid) and isochlorogenic acids also occur (Li *et al.*, 1983).

Vitamins

Commercial green tea leaves contain ascorbic acid (vitamin C) about 280 mg per 100 g dried leaves. Vitamin E in tea leaves is around 24–80 mg/100g dry weight but, because of its lipophilicity, solubility is low in tea infusions. The content of B vitamins in tea is around 8–15 mg/100g (Cheng and Chen 1994).

Volatile compounds

The content of volatile compounds in tea ranges from 0.01–0.03% w/w. More than 630 volatile compounds have been reported in tea (Yamanishi, 1996). Aroma is one of the critical parameters of tea quality despite the very low levels of volatile components which produce the typical tea aroma.

8.3 Functional benefits

For centuries, there have been many records in China relating to the health benefits of drinking tea. People have believed that tea can stimulate thought processes and mental alertness; increase blood flow; clear the urine and facilitate its flow; prevent tooth decay; increase the body's power of resistance to a wide range of diseases; and prolong life expectancy. However, these claims were primarily anecdotal. It is only in the last few decades that the health benefits of tea are beginning to be demonstrated from a scientific perspective. Numerous recent reports on tea and human health have been examined and this chapter gives a brief review of certain aspects of current research.

8.3.1 Anti-carcinogenic activity

Cancer is a major cause of premature death in modern society. *In vitro* and animal research indicates that tea, mainly green tea, may be effective against a wide variety of cancers. Activity has been observed *in vitro* at all three levels of cancer progression, namely initiation, promotion and transformation (Mitscher *et al.*, 1997).

Lung cancer

It is reported that the Japanese have both the highest smoking rate and the lowest lung cancer rate in the industrialised world. This can be regarded as a kind of 'Japanese Paradox', similar to the so-called French Paradox where red wine consumption is associated with the low cardiovascular mortality rate observed in French populations despite their high saturated fat intake. One of the hypotheses accounting for the 'Japanese Paradox' is a substantial consumption of tea. EGCG, EGC and ECG have been shown to inhibit the growth of human lung cancer cell line PC-9. The mechanism for this has been related to cell cycle regulation by the catechins (Okabe *et al.*, 1997). In rat and mouse models, one component of tobacco smoke, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, can induce lung cancer, but the risk may be decreased by tea drinking (Chung, 1999).

Breast cancer

One study in Japan showed that early stage breast cancer spread less rapidly in women with a history of drinking five or more cups of green tea daily. In addition, there was also a lower recurrence rate and a longer disease-free period. It was found that premenopausal women in Japan who consumed green tea had a lower number of lymph node metastases; increased consumption of green tea prior to clinical cancer onset showed a significantly improved prognosis of stage I and II breast cancer (Nakachi *et al.*, 1998). One mechanism by which green tea helps protect against breast cancer is the enhancement of glucuronisation of estrogens in the liver. This process renders estrogens inactive

through conjugation with glucuronic acid, a form in which they are excreted from the body. In addition, estradiol levels can be lowered by drinking a significant amount of green tea.

Prostate cancer

Paschka *et al.* (1998) were the first to demonstrate evidence that EGCG was the most potent catechin at inhibiting the growth of prostate cancer cell lines LNCaP, PC-3 and DU145. Polyphenols from black tea were found to inhibit the insulin-like growth factor-I (IGF-I) signal transduction pathway, which has been linked to increased prostate cancer incidence in human populations (Klein and Fischer, 2002). Thearubigins from black tea showed significant synergistic inhibition effects with genistein on human prostate tumor cell (PC-3) growth via cell cycle arrest (Sakamoto, 2000). One study revealed that China, where large quantities of green tea are consumed, had the lowest incidence of prostate cancer in the world (Gupta *et al.*, 1999).

Stomach, pancreatic, oesophageal and colon cancer

Two separate population-based control studies in Shanghai, China found a negative correlation between green tea consumption and the rate of stomach, colon, rectum and pancreatic cancers (Ji *et al.*, 1997; Yu *et al.*, 1995). Residents of Shizuoka, Japan, where green tea is produced and consumed, were shown to have lower mortality rates from stomach, lung and liver cancers than comparable populations in non-green tea consuming areas (Oguni *et al.*, 1992). Green tea extract and EGCG were found to have a concentration and time-dependent growth inhibition and apoptosis (programmed cell death) in a line of human stomach cancer cells. Both green and black tea were found to protect against cancers of the oesophagus (Chen, 1992; Gao *et al.*, 1994; Ke *et al.*, 2002). The rates of oesophageal tumour formation were significantly reduced at 360 and 1200 ppm of TFs and EGCG respectively (Morse *et al.*, 1997).

Skin cancer

Ultraviolet (UV) radiation is, by convention, divided into three bands, each with a different energy level and with a different ecological impact. Of these, UV-B (280–315 nm) is the band of lowest wavelength and highest energy. UV-B is known to cause inflammation and immunosuppression, making the skin more susceptible to cancer. EGCG has the potential to block the UV-B-induced infiltration of leukocytes and the subsequent generation of reactive oxygen species in human skin (Katiyar *et al.*, 1999). In the mouse skin model of two-stage carcinogenesis, black tea polyphenol application markedly inhibited tumour growth (Javed *et al.*, 1998). In human skin, EGCG and green tea polyphenols were found to reduce UV-B-induced inflammatory responses and infiltration of leukocytes and the formation of cyclobutane pyrimidine dimers (Katiyar *et al.*, 1999; 2000b). Gensler *et al.* (1996) found that induction of skin tumours by UV radiation was significantly reduced by

topical, but not by oral, administration of purified EGCG. However, Lu *et al.* (1997) found that oral administration of black tea to skin-tumour-bearing mice inhibited proliferation and enhanced apoptosis in non-malignant and malignant skin tumours. In addition to protecting skin from UV radiation, green tea polyphenols were found to protect rabbit eye lens against photo-oxidative stress induced by UV-A wavelength radiation (Zigman *et al.*, 1999).

8.3.2 Anti-inflammatory and anti-microbial properties

Tea extracts have been demonstrated to inhibit a wide range of inflammatory responses and may be useful in treating chronic inflammatory states. For example, rheumatoid arthritis is an inflammatory disease that causes pain, swelling, stiffness and loss of function in the joints. The antioxidants in green tea may prevent or reduce the severity of these symptoms by reducing inflammation and slowing cartilage breakdown (Adcocks *et al.*, 2002; Haqqi *et al.*, 1999).

Dental caries is the commonest of all microbial related diseases. The polyphenols in green tea can prevent teeth from decaying by inhibiting the biological activities of the cariogenic species, *Streptococcus mutans* and *Streptococcus sobrinus* (Cao, 1995; Ishigami, 1991; Sakanaka, 1991, 1995). The minimum inhibitory concentration of tea polyphenols against these cariogenic bacteria was found to be 0.25–1.0 mg/ml. EGCG and ECG, the major components in green tea extract, showed an enzyme-inhibitory effect at, or below, 25–30 µg/ml. They also inhibit the adherence of streptococcal cells to teeth at concentrations of 50 µg/ml or less (Sakanaka *et al.*, 1990). Tea extracts not only prevent the growth of *S. mutans* but also deter its adhesion and inhibit glycosyl transferase activity (Kawamura and Takeo, 1989; Sakanaka *et al.*, 1989). Levels of maltose, the sugar released from starch, were found to be consistently lower after drinking tea in comparison to drinking water (Zhang and Kashket, 1998). It is believed that under normal circumstances tea will reduce the acid level on the tooth enamel for the normal length of time starch is trapped in the mouth, and this is the mechanism by which it exerts its anti-caries effect. Green tea extract in a concentration of 20 mg/ml reduced the number of *S. mutans* from 10^7 to 10^2 after three minutes. From these results, it is claimed that food or water containing 0.1% w/w of green tea extract is effective for dental hygiene (Takeo, 2001). Epidemiological studies also showed that school children from tea plantation areas in Japan had a lower incidence of caries than those from other districts (Onisi, 1993). Theaflavins and gallic acid in black tea also showed good halitosis-deodorising effect (Xiao *et al.*, 2000). It is worth noting that the tea bush is fluorine bioconcentrating. The anti-caries effect from tea drinking may also result from increased fluorine intake. In addition Suzuki (1983) and Ui (1991) have found that green tea polyphenols, especially EGCG, have a stronger deodorising activity than sodium copper chlorophyllin and so should also improve breath freshness.

The use of tea extracts has been reported for the treatment of cholera patients in epidemic areas and for the prevention of influenza virus infections (Shimamura, 1991; Tezuka *et al.*, 1997). Also, 50% inhibition of the AIDS virus using ECG and EGCG at concentrations of 0.01–0.02 µg/ml has been reported (Nakane and Ono, 1990). Theaflavins extracted from black tea have a similar effect (Nakane, 1991). Yamaguchi and co-workers (2002) found that the anti-HIV viral activity of EGCG might result from an interaction with several steps in the HIV-1 life cycle. It has been demonstrated that a component of tea extracts is able to reverse the methicillin resistance in methicillin-resistant *Staphylococcus aureus*, the so-called ‘superbug’ (Hamilton-Miller and Shah, 1999; Yam *et al.*, 1998). Green tea polyphenols were also used in treating inflammatory bowel disease (Varilek *et al.*, 2001).

8.3.3 Cardioprotective and neuroprotective benefits

Plasma low-density lipoprotein (LDL) oxidation has been recognised to be an important step in the formation of atherosclerotic plaques and subsequent cardiovascular disease. It has been reported that catechins, particularly EGCG, help to prevent the oxidation of LDL *in vitro*. It is known that lowering blood cholesterol is associated with preventing heart disease. The cholesterol-lowering (hypocholesterolemic) effects and the vasodilating effects of tea have been confirmed by animal and human epidemiological studies (Tijburg *et al.*, 1997). High consumption of green tea was found to be associated with higher HDLs (high-density lipoproteins) and lower LDL and VLDL (very low-density lipoprotein) cholesterol, resulting in improved HDL to LDL fraction ratios. The antioxidant effects of polyphenols from black tea were found to be capable of enhancing endothelial function and thereby reducing the risk of coronary events via improved vasodilator function of conduit arteries (Hodgson *et al.*, 2002). Ester type catechins have been found to inhibit the proliferation of smooth muscle cells lining blood vessels *in vitro*. One mechanism of this anti-proliferative action of catechins is apparently the inhibition of tyrosine kinase activity. Tea consumption has been associated with a lower risk of myocardial infarction and stroke (Uchida *et al.*, 1995). However, epidemiological evidence relating the regular consumption of tea or related polyphenols to cardiovascular disease is equivocal and thus more research and human trials are required (Peters *et al.*, 2001; Riemersma *et al.*, 2001).

Neurodegenerative diseases, for example Parkinson’s and Alzheimer’s diseases, are related to oxidative stress. EGCG was found to have protective effects against β-amyloid-induced neuronal apoptosis through scavenging reactive species. Such activity may be beneficial for the prevention of Alzheimer’s disease (Choi *et al.*, 2001). Eicosanoid accumulation and formation of oxygen free radicals have been implicated in the pathogenesis of ischaemia/reperfusion brain injury. A minimising effect of green tea extract on eicosanoid

accumulation and oxidative damage in addition to the reduction of neuronal cell death has been observed (Hong *et al.*, 2000).

Diabetic patients have reduced antioxidant defences and suffer from an increased risk of free radical-mediated diseases such as coronary heart disease. EC has a pronounced insulin-like effect on erythrocyte membrane-bound acetylcholinesterase in type II diabetic patients (Rizvi and Zaid, 2001). Tea polyphenols were shown to possess anti-diabetic activity and to be effective both in the prevention and treatment of diabetes (Choi *et al.*, 1998; Yang *et al.*, 1999). The main mechanism by which tea polyphenols appear to lower serum glucose levels is via the inhibition of the activity of the starch digesting enzyme, amylase. Tea inhibits both salivary and intestinal amylase, so that starch is broken down more slowly and the rise in serum glucose is thus reduced. In addition, tea may affect the intestinal absorption of glucose.

The catechin component of green tea has been shown to induce weight loss by increasing resting energy expenditure (Dulloo *et al.*, 1999) and to inhibit catechol *O*-methyltransferase, an enzyme that degrades norepinephrine. Green tea may also affect lipase activity, which itself may promote weight loss (Bell and Goodrick, 2002). The popularity of oolong tea in Japan is said to be because of its weight loss and beautifying properties.

8.4 Mechanisms of anticarcinogenic and other activity

The mechanisms for the anticarcinogenic activity of tea flavonoids at the molecular level are complex and not yet fully understood. However, the following may be postulated.

1. As strong metal ion chelators due to their catechol structure, tea flavonoids are able to bind and thus decrease the level of free cellular ferric and ferrous ions, which are required for the generation of reactive oxygen radicals via the Fenton reaction (Yang and Wang, 1993).
2. As strong antioxidants and scavengers of superoxide, hydroxyl and peroxyl radicals, tea flavonoids can suppress radical chain reactions and terminate lipid peroxidation (Kumamoto and Sonda, 1998, Yang and Wang, 1993).
3. As inhibitors of certain enzyme reactions and apoptosis related to the development of cancer (Naasani *et al.*, 1998; Yang *et al.*, 2001), specifically by selective induction or modification of phase I and phase II metabolic enzymes so as to increase the formation and excretion of detoxified metabolites of carcinogens.
4. As inhibitors of carcinogenesis by affecting changes at the molecular level in the initiation, promotion, and progression stages, including lowering the rate of cell duplication and thus the growth and development of neoplasms (Weisburger, 1999), and by the induction of apoptosis (Hibasami *et al.*, 1996).
5. As modifiers of the intestinal microflora by favouring the growth of health promoting bacteria (Weisburger, 1999).

8.4.1 Relative activity of tea catechins

Individual green tea catechins are not equally chemically or biologically active. The measured antioxidant activity of tea catechins varies according to the medium and the method used. Activity in descending order is: EGCG > EGC > ECG > EC as determined by the active oxygen method at 97.8°C (Matsuzaki and Hara, 1985); ECG > EGCG > EGC > EC determined using marine oils at 60°C (Wanasundara and Shahidi, 1996); EGC > EGCG > EC > ECG determined using canola oil (Chen and Chan, 1996); ECG > EGCG > EGC > EC \equiv C determined using artificial water-soluble phenothiazine radical cations (Salah *et al.*, 1995) and EGCG > EGC > ECG > C determined in a mixture of LDL and VLDL. However, in the oxidation of unilamellar liposomes of phosphatidylcholine initiated with a water-soluble azo compound at 37°C, the antioxidant activities of EGCG and EGC were lower than those of EC and ECG at pH 7.4, and their depletion of EGCG and EGC was faster than that of EC and ECG (Terao *et al.*, 1994).

It is generally accepted that galloyl esters of catechins are more active than non-galloylated catechins because they have lower redox potentials (Balentine *et al.*, 1997) and that they have very strong antioxidant activities against the peroxyl radical because of the multiple OH substitutions in the structures of the flavonoids (Cao *et al.*, 1997). Some catechins, for example EC, were found to be less effective against particular cancer cell lines (Okabe *et al.*, 1997); however, they showed synergistic effects with other catechins (Suganuma, *et al.*, 1999) and caffeine (Kajimoto, 1963). Thus, it is believed that an unfractionated green tea extract has synergistic and therefore stronger effects than single individual tea components (Fujiki, 1999; Han and Chen, 1995; Tang *et al.*, 2002). Synergistic effects of tea components with non-tea components are also worthy of note. It was found that the growth inhibition of oral cancer in an *in vitro* model using a combination of EGCG and curcumin (a powerful anti-carcinogenic compound from the spice turmeric) was enhanced (Khafif *et al.*, 1998). Likewise, the effects producing apoptosis were synergistically increased when catechins were combined with other anti-cancer agents such as tamoxifen (a protein kinase antagonist) (Suganuma *et al.*, 1999). Male/female differences were found in the antioxidant effects of tea catechins against aortic atherosclerosis, the protective effect being more pronounced in women than in men (Geleijnse, *et al.*, 1999).

Therefore depending upon the conditions used to simulate either *in vitro* or *in vivo* oxidation, catechins or other phenolic compounds display differences in their antioxidant properties. Catechins also limited the consumption of α -tocopherol, allowing it to act as a scavenger within cell membranes whilst the catechins scavenged aqueous peroxyl radicals near the membrane surface (Pietta and Simonetti, 1998).

8.4.2 Antioxidant properties of tea flavonols

Quercetin, kaempferol and myricetin were found to inhibit carcinogen-induced

tumours in rats and mice (Deschner *et al.*, 1991; Wei *et al.*, 1990). The former two flavonols were effective in protecting cells from both superoxide and peroxide at concentrations of 5 μM and 20 μM , while catechin was effective at concentrations of 500 μM and 1000 μM (Nakayama *et al.*, 1993). Quercetin is by far the most important flavonol because of its powerful antioxidant activity and metal-ion binding properties, as well as its radical scavenging abilities (Hertog *et al.*, 1993a). However, it is noteworthy that the content of myricetin is much higher in tea than in many other plants, and thus should not be ignored when discussing tea extracts and their activity.

8.4.3 Comparison of black tea and green tea

Most of the previously reported results were derived from research using green tea; the research on TFs and TRs in black tea is still at an early stage. However, black tea extracts also demonstrate strong antioxidant activity (Sohn *et al.*, 1994; Wang *et al.*, 1992), indicating that the TFs and TRs are biologically active. It has also been found that polyphenols with an intermediate oxidation state can exhibit higher radical scavenging efficiency than non-oxidised forms (Kikugawa *et al.*, 1990). The higher antioxidant properties of the partially oxidised polyphenols could be attributed to their increased ability to donate a hydrogen atom from the aromatic hydroxyl group to a free radical and/or to the capacity of their aromatic structures to support the unpaired electron through delocalisation around the π -electron system. Wiseman *et al.*, (1997) have reported that black and green teas have equally strong antioxidant activity, whereas Han and Chen (1995) found that it was difficult to accurately compare the relative potency of TFs with either polyphenols or individual catechins. However, some studies comparing these two kinds of tea *in vivo* showed that the increase in plasma antioxidant activity was about 1.5 times more after drinking green tea when compared to black tea (Leenen *et al.*, 2000; Serafini *et al.*, 1996; van het Hof *et al.*, 1997). Heijnen and co-workers (2000) found that green tea powder was approximately five times more potent than black tea powder for nitrogen monoxide (NO) scavenging. However, it has been found that tea discriminates between the good and bad effects of NO. Oolong tea extract also exhibited marked antioxidant activity and reduction potency (Yen and Chen, 1995).

Green tea consists of a wealth of simple phenolics (monomers), whereas black tea provides more complex polyphenols (dimers and polymers). It was found that with lipids the simple compounds were more effective antioxidants, while under aqueous conditions, polymers tended to have more activity. Weisburger (2001) suggested that polymers formed from a 2–5 unit polymerisation state seemed to be optimal, probably because the monomer is metabolised and excreted too rapidly, whereas the higher 6–10 unit polymers may suffer from difficulty in penetrating cellular membranes and be poorly absorbed.

8.5 Potential side-effects of tea constituents

There has been some concern about the inhibition of iron absorption through tea polyphenols forming insoluble complexes in the gastrointestinal tract thus reducing iron bioavailability when consuming tea (Brune *et al.*, 1989; Disler *et al.*, 1975b). Tea was found to inhibit the absorption of non-haem iron to a significant extent, but had no inhibitory effect on iron derived from cooked haemoglobin (Disler *et al.*, 1975a). It was reported that the addition of ascorbic acid or milk could counteract the inhibitory effect of tea on iron absorption (Christian and Seshadri, 1989). Record *et al.*, (1996) also found that both black and green teas and a green tea extract did not represent a risk of reduced iron availability to animals consuming tea beverages as their sole fluid intake. Other work has shown that when tea and iron were provided separately no influence could be observed on iron absorption (South *et al.*, 1997). Therefore, the recommendation is that tea should be consumed between meals rather than during the meal (Zijp *et al.*, 2000). An epidemiological investigation also indicated that no significant anaemia occurred after drinking an average of 3.7 cups of tea per day (Mehta *et al.*, 1992). Nevertheless it is recommended that oral iron drugs should never be taken together with a cup of tea (Gabrielli and de Sandre, 1995).

Flavonols are easily oxidised to their corresponding *O*-quinones. These flavonols and quinones can function as either hydrogen acceptors or hydrogen donors. In biological systems, catechin and EC were found to be able to accelerate damage to DNA in the presence of a bleomycin-iron complex (Scott *et al.*, 1993). Some commercial flavonoids can demonstrate pro-oxidant activity when a transition metal, for example copper, is available (Cao *et al.*, 1997). It was reported that at 10 μM , quercetin and EGCG protected Jurkat T-lymphocyte DNA against 25 μM H_2O_2 ; however, when present at 100 μM they damaged the DNA (Johnson and Loo, 2000). It was noted that from 100 μM of EGCG or EGC, around 70 and 100 μM H_2O_2 respectively was generated in cell culture media, while in solutions prepared in distilled water, no H_2O_2 was found (Long *et al.*, 2000). Therefore, whether tea flavonoids act as antioxidants or pro-oxidants appears to be dependent on the concentration used, the media and the method used to evaluate oxidation potential.

8.6 Tea drinking and flavonoid intake

Although the utilisation of tea extracts is of increasing interest to many industries, making a cup of tea by brewing tea leaves is still the most common way to consume tea. Differences in tea drinking habits vary between populations and countries. In China and in some other Southeast Asian countries, where green tea is mostly consumed, people customarily brew the same tea leaves two or three times to produce a first, second and third infusion. In these countries, unlike their Western counterparts, even when drinking black tea, milk and sugar are not added.

It is important to quantify the content of tea infusions when evaluating the intake of flavonoids from consuming tea. However, the daily consumption of flavonoids from tea is difficult to estimate because values depend on accurate assessment of drinking habits and flavonoid content in teas.

The highest content for both catechins and flavonols was found in the first infusion. The content of catechins substantially decreased with the later infusions; however, for the flavonols, even in the third infusion the decrease was less marked, indicating that the infusing rate for the flavonols was relatively slow (Wang *et al.*, 2000b). According to the conventional tea brewing method, about 70 mg of total catechins and 4 mg of flavonols as aglycones can be found in the first infusion from 1 g of common gunpowder tea using 100 ml of boiling water (Wang *et al.*, 2000b). Fujiki *et al.* (1992) suggested drinking green tea in large amounts, such as 10 cups per day, as a possible form of cancer prevention for the general population.

Kühnau (1976) estimated that the total intake of flavonoids in the United States was 1 g/day expressed as glycosides or 170 mg/day expressed as aglycones. However, Hertog *et al.* (1993a) found that the average intake of flavonols and flavones in the Netherlands was 23 mg/day (expressed as aglycones), and tea was reported to be the major source (48% of total intake) of these compounds. Lakenbrink *et al.* (2000) have found that the typical UK black tea consumer brewing time of 40–60 seconds, for cup/mug brewing, delivers 137–141 mg total flavonoids (including the TRs) per average serving (235 ml mug). Of those, the structurally identified flavonoids (catechins, TFs and flavanol/flavone glycosides) together constitute 34.0–40.6 mg, whereas the TRs are the dominant contributors to flavonoid delivery (100–102 mg). These values are only indicative because they vary widely according to tea varieties and measuring methods. As stated by Aherne and O'Brien (2002), total flavonoid intake and content in foods can be assumed to be greater than the values that have been reported. This is because dietary flavonoid content and intakes have been based mostly on the content of selected major flavonoids only.

When considering tea flavonoid intake, several parameters should be taken into account; amongst these are tea concentration (strength), brewing time and temperature, and drinking time after tea has been brewed. The quality of water also affects the stability of catechins (Wang and Helliwell, 2000). Milk has long been added to black tea by almost all tea drinkers in Britain and other Western countries. It was found that the content of individual catechins decreased following the addition of milk. For EGCG, GCG and ECG the decreases were as high as 70, 65, and 70.4% respectively (Wang *et al.*, 2001). There is some controversy as to whether the absorption of flavonoids from tea is reduced after the addition of milk, due to a binding of milk proteins with catechins rendering the catechins unavailable for absorption. Serafini *et al.* (1996) reported that adding milk to tea may eliminate tea's antioxidant properties *in vivo*. However, the effect of tea ingestion, with or without milk, on plasma catechin concentrations was not assessed in the study. Other studies

have shown that the increase in plasma antioxidant capacity and catechin concentrations after tea consumption are not affected by the addition of milk to tea (Hollman *et al.*, 2001; Leenen *et al.*, 2000; van het Hof *et al.*, 1998).

8.7 Tea extracts and their applications

8.7.1 Types of tea extract

Tea can be consumed directly either by brewing loose leaves or tea bags or in a ready-to-drink form. In addition, extracts of tea (primarily green tea) may be prepared in a variety of physical forms in order to cover most application requirements.

Strong infusions

Strong infusions are made by soaking tea leaves in alcohol/water mixtures (the catechin content is about 2% w/v).

Soft extracts

Soft extracts are made by concentrating the strong infusion to a water content of 20–25% (the catechin content is about 20% w/w).

Dry extracts

Dry extracts or powders are made by spray drying the strong infusions after they have been concentrated to 40–50% solids (the catechin content is above 25% w/w). The residual water content is less than 5% w/w and the extract is usually processed as a powder containing inert processing aids to render it suitable for a variety of uses (tablets, capsules, dry mixes, etc.).

Partly purified extracts

Purified extracts contain higher contents of tea catechins obtained by further purification processes, for example solvent extraction or column chromatography techniques (Takeo, 2001). New techniques, such as membrane extraction and separation, may be beneficial in producing such extracts (Nwuha, 2000, Wang *et al.*, 1995).

Purified catechins

Formerly, only column chromatography techniques were available; however, in recent years, high-speed counter-current chromatography (HSCCC) has become a very useful tool for the fractionation of both crude extracts and semi-purified fractions. This technique has been successfully used to produce purified catechins, flavonol glycosides and proanthocyanidins from crude green and black tea extracts (Baumann *et al.*, 2001; Degenhardt *et al.*, 2000; Du *et al.*, 1997, 1998). As the sample load capacity in HSCCC is superior to that of preparative high-pressure liquid chromatography (HPLC), it is suitable for scale-up to significantly larger quantities on a preparative scale. EGCG

is regarded as the most important of the tea catechins because of its high content in tea and the fact that its activity is mirrored by green tea extracts. Therefore methods for producing tea extracts with high EGCG ratios have been developed (Copland *et al.*, 1998). A low-speed counter-current chromatographic technique was also introduced to produce purified EGCG (Du *et al.*, 2000).

8.7.2 Applications for tea extracts

Health care supplements

Many solid dosage forms made with green tea extracts can be seen in the market, and several are standardised to a content of polyphenols or catechins.

Foods and beverages

Antioxidants are not important only to the health conscious; food manufacturers also rely on these chemicals to maintain the shelf life of their products. Synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tert-butyl hydroquinone were widely used in food processing to control oxidation and maintain food quality. However, as these synthetic antioxidants are suspected to be carcinogenic they now have restricted use in food (Madahavi and Salunkhe, 1995). Therefore, natural antioxidant sources, especially of plant origin, are of great interest to the food industry.

The natural antioxidant properties of catechins in green tea extracts can improve the marketing potential of cereals, cakes and biscuits as well as traditional health food products and dietary supplements. Green tea extracts have been reported to be potent antioxidants in pork (Shahidi *et al.*, 1992), chicken meat (Tang *et al.*, 2001, 2002), vegetable oil (Chen and Chan, 1996), fish oil (Wanasundara and Shahidi, 1996) and fish flesh (Lin and Lin, 2000), food emulsions (Huang and Frankel, 1997) and animal fat (Wang and Zhao, 1997). They can also be employed to give dairy products, instant noodles (Yang *et al.*, 1995), confectionery, ice-cream (Jiang *et al.*, 1995) and fried snacks a healthier appeal to the consumer. A successful example has been their application in Mooncake, a traditional cake consumed during the Chinese Middle Autumn Festival, into which the incorporation of green tea extract both increased the shelf life and improved the flavour. Furthermore, many antioxidant-rich health drinks containing extracts from green and black tea, in combination with other natural extracts, can be found on the market. It has been proposed that tea extracts could be used to protect apple juice and other foods against microbial contamination (Friedman and Jürgens, 2000). There is also an application for tea extract in a tobacco-control, health care drink which has been claimed to be able to help smokers stop smoking (Chen and Shi, 1995). Green tea polyphenols were reported to have colour protection effects on β -carotene (Unten *et al.*, 1997). A new application of tea extract

in meat cooking has been reported recently (Weisburger *et al.*, 2002), where the application of tea extracts either from green tea or black tea to both surfaces of ground beef before cooking inhibited the formation of mutagens which might be produced during the broiling or frying process.

Toiletries and cosmetics

The anti-bacterial and deodorising effect of catechins slows tooth decay (anti-caries) and improves breath freshness, so providing natural added value for toothpastes, mouthwashes, chewing gums (Miki *et al.*, 1991; Yoshinori *et al.*, 1987) and breath-fresheners (Yasuda, 1992), with the potential for other body, skin and hair product applications. For example, many shampoos, moisturising creams, perfumes and sunscreens contain tea extracts as they are believed to have a soothing effect on the skin as well as acting as antioxidants to protect the skin from free radicals (Alexis *et al.*, 1999; Masao *et al.*, 1994). Some research suggested that green tea constituents might help protect the skin from sun damage and sunburn and even skin cancer (Elmets *et al.*, 2001; Katiyar *et al.*, 1995, 1999, 2000a,b). Therefore tea extracts might offer synergistic benefits if combined with standard sunscreens. A bath liquid made from oolong tea extract has been reported to depress the swelling, inflammation and itchiness of the skin in patients suffering from atopic dermatitis (Takeo, 2001).

Other applications

Japanese scientists found that tea extract could be used as a dye. Wool, silk and synthetic yarn were dyed in an aqueous solution of green tea in combination with different metals. This resulted in colours from pale-green through yellow-green and yellow to brown. Even after repeated washing the dyed yarn retained its colour for at least one year (Takeo, 2001). It has been shown that cloths or garments such as T-shirts, blouses and socks made of yarn dyed with green tea extract are not susceptible to microbial contamination and possess strong deodorant properties against smells caused by perspiration. Furthermore, green tea extract dyed clothes were prepared as underwear or over-wear garments for patients in Japanese hospitals.

Catechins are water-soluble; however, they can be rendered insoluble by chemical reaction (Yayabe, 2001). Insoluble catechins do not lose their phenol hydroxyl groups, and their anti-bacterial and deodorising actions remain almost unaffected. In this form they are useful as natural anti-bacterial and deodorising materials for application to fibres and plastics.

8.8 Analytical methods for detecting flavonoids

It is necessary to offer consumers a consistent level of quality in their products. To achieve this, tea leaves, their extracts and the consumer products themselves need to be standardised and monitored throughout their shelf life. Furthermore,

health care applications require data on flavonoid content in order to estimate the dietary intake of flavonoids and thus enable epidemiological and human intervention studies to be interpreted. Therefore many different types of analytical method have been developed.

8.8.1 Spectrophotometric method

As the amount of individual flavonoids in tea is usually complex, they have often been described unspecifically as 'total polyphenols'. Total polyphenols in general are determined using the Folin–Ciocalteu assay (Singleton and Rossi, 1965). This method reports results in gallic acid equivalents. Because this assay is non-specific, any interfering reductants must be removed prior to assay. Furthermore, due to their lack of selectivity, this spectrophotometric method tends to over-estimate the polyphenol content. As the structure of TRs is uncertain, their content is difficult to determine directly. To solve this problem, there was a proposal to derive the content of TRs by subtracting the content of catechins, TFs, flavanols and non-flavonoids (such as gallic acid and chlorogenic acids) from the content of total polyphenols as determined by the Folin–Ciocalteu assay (Wiseman, *et al.*, 2001).

8.8.2 HPLC method

HPLC combines the advantages of simultaneous separation and quantification of flavonoids without, in most cases, preliminary derivatisation. Therefore many HPLC methods for analysing catechins in tea leaves have been published (Bronner and Beecher 1998; Dalluge *et al.*, 1998; Goto *et al.*, 1996; Price and Spitzer, 1993; Umegaki, *et al.*, 1996). In most of these methods reversed phase columns (C18) are used, although some comparisons have been made using different columns (Dalluge *et al.*, 1998; Wang *et al.*, 2000a). Methanol or acetonitrile in aqueous solution is often used as the eluting mobile phase, and it has been found that a small amount of acetic acid, phosphate buffer or formic acid incorporated in the mobile phase markedly improved separations (Merken and Beecher, 2000; Wang *et al.*, 2000a). UV or photodiode array detection is most commonly used, with typical detection wavelengths of 210 nm (Bronner and Beecher, 1998; Dalluge *et al.*, 1998; Wang *et al.*, 2000a), 278 nm (Khokhar *et al.*, 1997), and 280 nm (Kumamoto and Sonda, 1998). Other detectors, for example, electrochemical (Umegaki *et al.*, 1996; Unno *et al.*, 1996), fluorimetric (Kayali-Sayadi *et al.*, 1998), electrospray ionization mass spectrometric and chemiluminescence (Wu *et al.*, 1998; Nakagawa and Miyazawa 1997) have been used for the determination of tea compounds. It has been reported that the sensitivity of the electrochemical detector to tea catechins was about 1000 times higher than that of the UV detector (Umegaki *et al.*, 1996). Post-column derivatisation has received little attention but offers a number of advantages, including enhanced selectivity. An HPLC method with detection at 640 nm after post-column chemical

reaction with *p*-dimethylaminocinnamaldehyde has been applied to determine catechins in beverages, including tea (de Pascual-Teresa *et al.*, 1998). In recent years, the use of solid phase extraction systems seems to have become popular for the clean-up of tea samples before HPLC analysis (Engelhardt *et al.*, 1992; Kayali-Sayadi *et al.*, 1998; Naik and Nagalakshmi, 1997). As tea catechins can be absorbed on membrane filters, centrifugation instead of filtration was used prior to injection for HPLC analysis (Goto *et al.*, 1996; Lee and Ong, 2000; Wang and Helliwell, 2000).

8.8.3 Capillary electrophoresis techniques

Capillary electrophoresis (CE) techniques, including capillary electrochromatography and micellar electrokinetic chromatography, have been attracting much attention as the preferred methods for separating a wide range of neutral compounds because of the rapid run-times, high separation efficiency and low sample requirements. Horie and Kohata (1998) reported an application of capillary electrophoresis in tea quality assessment. With this method not only catechins but theanine, caffeine and ascorbic acid can be analysed simultaneously. A micellar electrokinetic chromatographic method was developed for analysing catechins and caffeine in tea in only 20 min without extensive sample preparation (Worth *et al.*, 2000). Wright *et al.* (2001) reported a new non-aqueous CE method to quantify the four major TFs occurring in black tea, by which the problems of poor selectivity and considerable band broadening with aqueous-based CE methods could be solved. Lee and Ong (2000) compared HPLC and CE methods for the analysis of catechins and TFs. They found that both methods were reliable and comparable. The analysis time of CE was three times faster, but five times less sensitive, than HPLC.

8.8.4 Other methods

The on-line coupling of methods has enormous potential because the selectivity can then be optimised, which in turn can be translated to either a faster analysis or an improvement in the limits of detection. Zeeb and co-workers (2000) have developed a method of liquid chromatography/atmospheric pressure chemical ionization-mass spectrometry for the direct microscale determination of 12 catechins in green and black tea infusions. With this method the identities of eight major catechins and caffeine in tea were established based on liquid chromatographic LC retention times and simultaneously recorded mass spectra. In addition, monitoring of the catechin-specific retro-Diels-Alder fragment ion at m/z 139 throughout the chromatogram provided a unique fingerprint for catechins in the samples and led to the identification of four minor chemically modified catechin derivatives, (–)-epiafzelechin, (–)-epiafzelechin gallate, (–)-epicatechin methylgallate and (–)-epigallocatechin methylgallate. In addition to analytical methods for beverages, published methods also covered biological fluids, such as saliva (Tsuchiya *et al.*, 1997), plasma

(Dalluge *et al.*, 1997; Lee *et al.*, 1995; Maiani *et al.*, 1997), and urine (Lee *et al.*, 1995).

As relatively few standard compounds are available from commercial or other sources, identification of flavonol glycosides has to be achieved by alternative means, for example UV-, ^1H - and ^{13}C -NMR spectroscopy. Therefore hydrolysing all glycosides to aglycones followed by HPLC determination offers a practical method for the quantitative determination of flavonoids in tea (Hertog *et al.*, 1993a; Wang and Helliwell, 2001).

8.9 Future trends

Tea has been consumed for many thousands of years, but it is only in the last few decades that we are beginning to understand the full potential of this widely enjoyed beverage. The studies under review have increased understanding about the positive health benefits of tea consumption. Increasing consumer demand for a healthier lifestyle has led to a need to develop more products containing functional components, and this consumer trend is likely to be maintained in the future. Tea beverages and tea extracts, particularly those based on green tea, have several applications which can meet some of these consumer aspirations. Therefore, traditional manufacturers will also participate in tea extraction and application in major health markets like cancer and heart disease prevention or just simply in general functional use. However, the following are thought to be some research requirements.

8.9.1 Epidemiological studies and basic research

Tea flavonoids, or tea extracts, have been linked to benefits in reducing the risk of certain cancers and cardiovascular diseases in experimental animals. However, epidemiological studies have produced inconsistent evidence in the relationship between tea drinking and cancer (Blot *et al.*, 1997; Goldbohm *et al.*, 1996; Hertog *et al.*, 1997; Yang *et al.*, 1996). Therefore, further research is needed before definitive conclusions on the impact of tea consumption upon the cancer risk in humans can be reached. The metabolites of catechins and flavonols after consumption of tea infusions have scarcely been investigated, and thus more research is needed as to the role of those compounds in the reported health benefits of tea consumption.

8.9.2 Interactions

Tea flavonoids, or tea extracts, are increasingly being added to foods. However, interactions with food and drink components remain unclear, and thus need to be carefully assessed in order that the full potential benefits from consuming tea, in whatever form, can be achieved. Meanwhile, tea catechins themselves undergo extensive *O*-methylation, glucuronidation, sulphation and ring fission

(caused by intestinal microflora) (Yang *et al.*, 2001; Pietta *et al.*, 1998). These reactions may play key roles in determining the bioavailability of tea catechins and remain to be studied in detail.

Although the majority of tea functionality appears to be due to flavonoids, some other factors, such as the presence of volatile compounds, were suggested as being responsible for the anti-mutagenic effect of tea extract (Ohara *et al.*, 2001). It is not necessarily true that any plant possessing clinical effectiveness must contain an active principle which can completely replace the plant extract. The use of extracts as opposed to isolated single entities may have some advantages because of internal synergy. Therefore, the usefulness of tea flavonoids, or tea extracts, may be extended by combining them with other components such as food ingredients and vitamin supplements. This 'designer-item' approach may have significant potential, but requires further investigation.

8.9.3 Further analytical work

Tea flavonoids can be analysed by various state-of-the-art analytical techniques; however, as the structures of TRs have not yet been fully elucidated, quantitative analysis of TRs in black tea remains elusive. This is possibly one of the major challenges in tea analysis. Methods for analysing flavonoids in tea and tea extracts vary greatly between laboratories and between commercial sources. Uniform analytical methods are needed for clinical studies, stability studies, active ingredient analysis and manufacturing control and to allow data from different sources to be compared. For packaging declarations of flavonoid content an agreed method for quantifying flavonoids is also essential.

In conclusion, a multi-disciplinary approach to the research into, and applications of, tea flavonoids as functional compounds would yield significant benefits.

8.10 Sources of further information and advice

Tea and health

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Phytochemicals and gastrointestinal health

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9.1 Introduction

The interactions between the characteristics of the gastrointestinal tract (GIT) and diet composition are well known. For example, the ability to rapidly and reversibly alter GIT characteristics allows animals to adapt to daily or seasonal changes in diet composition. During the evolution of species these interactions eventually led to genetic determinants of GIT structure and functions that are matched to the natural diet. The use of plants for medicinal purposes highlights how the interactions between phytochemicals and GIT characteristics have been recognized for millenia. For this review, phytochemicals are considered to be secondary plant metabolites that do not directly provide energy or nutrients, but which can influence the nutritional and health status of consumers. The influences of phytochemicals can be direct or indirect, and the responses can range from:

- the good – beneficial, enhancing structures and functions; to
- the bad – reducing functions; to
- the ugly – compromising health.

As could be predicted, the magnitude and patterns of responses to phytochemicals vary among individuals and between animals with different evolutionary diets.

No other organ system has the same level of exposure and diversity of responses to phytochemicals. Because virtually all characteristics of the GIT are influenced by phytochemicals, there are many opportunities to improve health and nutritional status. Our objective in preparing this contribution was to provide readers with insights into the known and potential interactions

between phytochemicals and the GIT. We did not set out to provide a comprehensive listing of the various phytochemicals and their associated influences on the GIT. Instead, we have selected examples to illustrate the variety of interactions so that readers can explore topics of interest in greater depth. We first provide a brief background about the basic structural and functional characteristics of the GIT and the resident populations of microorganisms before presenting different interactions between the GIT and phytochemicals. A concluding section describes opportunities and future possibilities for using phytochemicals to manage the GIT in order to improve health and nutrition.

9.2 The gastrointestinal tract

The GIT is basically a tube that courses through the body. Its principle functions are digestion, osmoregulation, immunity, elimination of toxins and metabolites, and endocrine regulation of GIT and systemic functions. Corresponding with the wide diversity of feeding habits and functional demands placed on the GIT, it is the most variable organ system of the vertebrate body, with the size, shape, mucosal architecture, and functional features of the various GIT regions highly variable among animals (Stevens and Hume, 1996). This is obvious from a comparison of the complex GITs of animals that are dependent on plants as their principal source of nutrition (e.g. ruminants) with the relatively short and simple GIT of most carnivores.

The mucosa of the GIT represents an interface between the external and internal environments. The expansive surface area is necessary for the efficient hydrolysis of foodstuffs and the absorption of energy and nutrients. The mucosa also influences the systemic availability of non-nutrient compounds in the diet, both beneficial and detrimental. Digestion and absorption of glucosinolates are critical determinants of health benefits (see Chapter 4). Similarly, the bioavailability and health benefits of phytoestrogens, such as genistein (see Chapters 5 and 10) are at least partly dependent on the carrier-mediated processes of absorption associated with the GIT (Oitate *et al.*, 2001). Moreover, the metabolic activities of the mucosa can influence the systemic concentrations and forms of dietary phytochemicals, as exemplified by research with soy isoflavones (Andlauer *et al.*, 2000).

The different regions of the GIT harbor diverse assemblages of bacteria that have numerous interactions with the host and responses to dietary inputs. Collectively, the GIT and the resident bacteria represent a small, but complex, ecosystem that influences the health, disease resistance and nutritional status of the host. Food infections and the GIT responses to various pathogenic and putrefactive bacteria provide evidence for the role of the resident bacteria. Less obvious, but of great importance, are the influences of the commensal bacteria that have evolved with hosts. These groups provide resistance against potential pathogens and, by fermenting undigested food materials, are sources

of energy and nutrients; in addition, they are critical for normal mucosal functions. The best understood commensals are the lactic acid producing bacteria (e.g. members of the *Lactobacilli* and *Bifidobacteria*), which are associated with several nutritional and health benefits (Teitelbaum and Walker, 2002; Van den Driessche and Veereman-Wauters, 2002).

9.3 The influence of phytochemicals on gastrointestinal function

People are being encouraged to increase the proportion of the diet which is derived from plants in order to obtain health benefits (Messina and Messina, 1996). However, plants have developed three basic strategies to counter herbivorous and casual consumption and to protect against disease. The first strategy to inhibit consumption is by reducing nutritional quality. This is exemplified by plant proteins which are less digestible than those of animal tissues and often provide insufficient quantities of essential amino acids (e.g. lysine, methionine). Second, most food plants and spices contain secondary metabolites that can compromise GIT functions (Bland, 1996), further limiting nutrient availability. Similar bioactive compounds are present in mushrooms used for food or medicinal purposes, though less is known (Chang, 1996). Many of these secondary metabolites, known as 'anti-nutrients', can be considered as allelochemicals (Aletor, 1993). The various types of legumes, which are important nutrient sources for many populations, contain numerous and often high concentrations of anti-nutrients which further decrease nutritional score. As a consequence, if legumes are not processed appropriately, they have only limited usefulness as foodstuffs (Siddhuraju and Becker, 2001; Friedman and Brandon, 2001; Grant, 1989; Gupta, 1987). Corresponding with this, substituting unprocessed legumes in animal feeds adversely impacts some, but not all, GIT characteristics and decreases growth performance (Erlwanger *et al.*, 1999). Third, many plants produce toxic metabolites to discourage consumption, such as toxic amino acids or cyanogens (Jones, 1998).

The different types or classes of phytochemicals can have multiple influences on the GIT (Kitts, 1994). For example, caffeine stimulates the motor, hormonal and secretory functions (Boekma *et al.*, 1999). Although many phytochemicals reduce nutrient quality or availability, traditional herbal medicines have exploited some of these characteristics to improve health. Moreover, some of the systemic responses attributed to certain phytochemicals (beneficial or detrimental) can be explained by their influences on the GIT (Carbonaro *et al.*, 2001). To date, the most of the information about phytochemicals is focused on the reductions in the functional capacities of the GIT (the bad), or the toxic properties (the ugly). There are also numerous examples of phytochemicals that can be used to obtain desirable GIT characteristics (the

good). The various types of non-starch polysaccharides that are now promoted as food supplements to improve the health of the colon and the host are noteworthy (Bruce *et al.*, 2000). Many such phytochemicals are considered to be 'functional foods', in that they provide benefits other than serving as sources of energy and nutrients (Marriott, 2000). Some are even considered for therapeutic purposes, such as the derivatives of lignin that are used for treatment of diarrhea (Mitjans *et al.*, 2001).

By necessity, herbivores have evolved GIT and systemic compensatory mechanisms that allow them to subsist on plant-based diets that have limited nutrient quality and include phytochemicals. Still, herbivores remain susceptible to some of the anti-nutrient and toxic phytochemicals. For example, several herbivores are sensitive to the phytotoxins associated with autumn crocus, which include colchicine (Yamada *et al.*, 2000). As a consequence, herbivores tend to select species and portions of plants based on a combination of nutrient quality and concentrations of phytochemicals (Yeager *et al.*, 1997), and this has an impact on habitat selection and plant ecology (Duncan and Gordon, 1999). Carnivorous species have not been under selective pressure to develop similar compensatory mechanisms, generally have only limited abilities to subsist on plant-based diets, and in many cases are less tolerant of phytochemicals.

The following sections use selected examples to illustrate the major mechanisms by which phytochemicals influence some of the characteristics and functions of the GIT. Although much more is known about their influences on the processes associated with the digestion of feedstuffs, there is increasing awareness of the impact on the other functions of the GIT.

9.4 Phytochemicals and digestion

Feedstuffs consist largely of complex polymers (e.g. proteins, starches, fats) that must be hydrolyzed to the constituent building blocks before they can be absorbed and made available to the host. The digestibility of many plant proteins is inherently lower compared to proteins from animal tissues. This is particularly true for the structural proteins (Carbonaro *et al.*, 2000; Mariotti *et al.*, 1999). As a consequence, amino acid scores for many plant proteins often do not reflect true availability to the host (Mariotti *et al.*, 2001).

The digestibility of plant proteins is further reduced by the presence of various phytochemicals that inhibit the hydrolytic and absorptive processes (Shimizu, 1999). This has two consequences. First, the lower digestive efficiency when animals are fed plants must be compensated for by increasing dietary loads (Aletor, 1993). Paradoxically, the reduced digestion caused by some phytochemicals actually reduces feed intake (Ramirez, 1992). The unpredictable changes in feed intake associated with diets containing phytochemicals complicate attempts to understand the mechanisms by which anti-nutrients decrease digestion and influence GIT characteristics (Kataoka and DiMagno,

1998). Second, the presence of anti-nutrients requires heightened stimulation of digestive secretions (Santoro *et al.*, 1997). This increases the costs of digestion, which are already appreciable (Tamminga *et al.*, 1995).

9.4.1 Inhibiting hydrolysis of macromolecules

The complex polymers in feedstuffs are broken down to the constituent building blocks by a sequential process. Hydrolysis of the polymers is initiated in the lumen of the GIT by enzymes and other secretions produced by the pancreas, stomach, intestine, liver and gall bladder, and other GIT tissues, and completed by another suite of enzymes associated with the brush border membrane (BBM) or intracellular organelles. Anti-nutrient phytochemicals can decrease the hydrolysis of feedstuffs, and thereby reduce nutrient availability, either by increasing the inherent resistance of the polymers to hydrolysis or by decreasing the activities or amounts of enzymes and other secretions produced by the GIT.

One class of anti-nutrients is the polyphenols, which include tannins, catechin, chlorogenic acid and others. The tannins are the best understood and have been further characterized as condensed or hydrolyzable (Reed 1995; Butler 1992). These two types differ in composition, in the GIT responses they elicit, and in how they influence the bacteria in the GIT. The influences of tannins and other polyphenols can be considered as non-specific (Carmona, 1996) in that they bind to proteins (Charlton *et al.*, 2002), and the resulting complexes are insoluble and precipitate (Salunkhe *et al.*, 1982). Apparently, the changes in protein conformation caused by the binding of polyphenols reduce access by proteolytic enzymes, causing lower protein digestibility (Velez *et al.*, 1985). In addition to food proteins, polyphenols can bind to and inhibit digestive enzymes secreted by the pancreas (Griffiths, 1986) and proteins associated with the BBM of enterocytes (Carbonaro *et al.*, 2001). This is a likely mechanism of action for the reduced activity of folate deconjugase activity of the BBM caused by phytochemicals (Bhandari and Gregory, 1990). Despite the adverse influence on protein digestion, the binding characteristics of polyphenols partly explain their efficacy in some herbal medicines (Haslam *et al.*, 1989).

Lectins are a heterogeneous group of proteins synthesized by most organisms and include phytohemagglutinins, which are lectin homotetramers (Carvalho and Sgarbieri, 1998). The highest concentrations of lectins are commonly found in seeds (up to 50% of seed protein; Sparvoli *et al.*, 1998) and other storage tissues of plants (more than 100 lectins have been identified in legumes alone). Although lectins play important roles in cell recognition (Sharon and Lis, 1989), some plant lectins have probably evolved for defense purposes, including toxic forms (e.g. ricin and those from elderberry, camphor tree and mistletoe). Despite being proteinaceous, lectins are resistant to degradation by heat or digestive processes and often survive GIT transit intact and retain biological activity (Hara *et al.*, 1984). Lectins bind to specific

sugar residues, including those of glycoproteins associated with the glycocalyx and BBM, and can thereby exert multiple GIT influences. These include inhibition of digestive processes and stimulation of the endocrine and immune functions of the GIT; and they elicit several systemic responses (Pusztai and Bardocz, 1996). For example, some lectins reduce the activities of targeted enzymes, and when the target is enterokinase (Rouanet *et al.*, 1983), this has an impact on the activation of the proteases in pancreatic juice. Low doses of specific plant lectins have been used to regulate digestion and thereby control systemic concentrations of nutrients (Bardocz *et al.*, 1996).

Phytates, oxalic acid and other chelators reduce the availability of several minerals (Urbano *et al.*, 2000), and can also reduce protein digestion (Ravindran *et al.*, 1999). Dietary levels of phytate can be reduced by supplementing the diet with phytases (Rapp *et al.*, 2001; Boling-Frankenbach *et al.*, 2001) or by selective breeding of plants used as feedstuffs. Both strategies improve mineral bioavailability and can increase protein digestion (Douglas *et al.*, 2000).

9.4.2 Enzyme inhibitors

Some of the best investigated anti-nutrients are the enzyme inhibitors present in legumes and other plants. The Bowman–Birk and the Kunitz inhibitors of trypsin and other proteases are among the best characterized. In contrast to the non-specific and widespread influences of tannins and lectins (Carmona, 1996), the Bowman–Birk, Kunitz and other such inhibitors target specific enzymes. Corresponding with this, proteases and other digestive enzymes vary in sensitivity to the different inhibitors.

Many of the enzyme inhibitors are proteins (Zahnley 1984) whose molecular weights range from about 4 to 21 kD, with most in the region of 8–12 kD (Rackis *et al.*, 1986). The various protease inhibitors bind to target enzymes (Mirkov *et al.*, 1995) as if they are substrates with a stoichiometry of 1:1 (Laskowski, 1986). Although enzyme inhibitors are not toxic *per se*, they reduce survival by compromising digestive functions (Garthoff *et al.*, 2002), conferring insecticidal properties (Fabre *et al.*, 1998). Many of the inhibitors are resistant to digestion by vertebrate proteases (Gupta, 1987) and are not degraded during passage through the rumen (Bainter *et al.*, 1993) or gastric regions (Krogdahl and Holm, 1981). Based on immunoreactivity, the majority of the Kunitz inhibitors survive transit through the stomach and small intestine intact (Hajós *et al.*, 1995), whereas the Bowman–Birk inhibitor is largely degraded.

In addition to proteases, other inhibitors reduce the activity of amylase and other digestive enzymes (Ishimoto *et al.*, 1999). Many varieties of beans produce a glycoprotein that complexes with and inhibits α -amylase (Mirkov *et al.*, 1995). The amylase inhibitors are non-competitive and thermostable (Gallaher and Schneeman, 1986) and, unlike protease inhibitors, do not elicit heightened secretion of amylase (Toskes, 1986). Although over-expression

of the α -amylase inhibitor confers insecticidal properties, it has minimal influences on rats (Pusztai *et al.*, 1999).

Amylase inhibitors have been used for production of diet supplements (e.g. 'starch blockers'). However, the use of plant extracts purported to contain amylase inhibitors has resulted in inconsistent products that contain varying proportions of both the desired and other anti-nutrients (Liener *et al.*, 1984), have limited potential to affect body mass (Koike *et al.*, 1995), and can adversely affect mineral availability (Umoren and Kies, 1992). Despite these limitations and precautions, since glycemic responses are related to carbohydrate digestibility (Thorne *et al.*, 1983), amylase inhibitors have some potential for control of post-prandial blood glucose levels in diabetic patients (Layer *et al.*, 1986). Additional inhibitors in plant tissues target the α -glucosidase activity associated with the BBM and also reduce peak height of post-prandial serum glucose concentrations in humans (Fujita *et al.*, 2001a) and laboratory rodents (Fujita and Yamagami, 2001; Fujita *et al.*, 2001b).

There are few, if any, specific inhibitors of lipase activity. Instead, reported inhibition of lipase by saponins (Han *et al.*, 2002), polysaccharides and tannins (Longstaff and McNab, 1991) is probably due to the non-specific interactions these phytochemicals have with proteins.

9.4.3 Influence digestive secretions

Pancreatic secretion for many, if not most, species is regulated in order to insure adequate protein digestion. Correspondingly, protease inhibitors have a greater impact on pancreatic secretion than do inhibitors of amylase and lipase (Toskes, 1986). The secretory response of the exocrine pancreas to protease inhibitors can be rapid (< 10 min), does not involve parallel increases in the secretion of all enzymes (Holm *et al.*, 1992), and is probably mediated by a signaling pathway (see below).

There is evidence that protease inhibitors selectively regulate the activity of specific digestive enzymes at the level of gene expression (Rosewicz *et al.*, 1989). Specifically, soybean trypsin inhibitor increases secretion of proteases, including a form of trypsin that is resistant to inhibition but does not cause an increase in amylase secretion. Although the relationships between protease inhibitors and exocrine pancreatic secretion have received the most attention, pancreatic secretion is increased when potato fiber is added to the diet (Jacob *et al.*, 2000), although the mechanism and signaling pathway have not been elucidated.

Phytochemicals influence other digestive secretions. Several traditional herbal medicines stimulate gastric mucous secretion, providing protection (Sairam *et al.*, 2001). The secretion and recycling of bile are also responsive to phytochemicals. The way in which certain polysaccharides increase fecal concentrations of bile acids (Dall' Angelo and Lino van Poser, 2000) and thereby influence recycling and synthesis is particularly noteworthy.

9.4.4 Reducing absorption of nutrients

Although a portion of the nutrients released from feedstuffs is absorbed by diffusing across the apical membrane of enterocytes or through the junctional complexes of adjacent enterocytes (paracellular absorption), the majority of nutrients are absorbed from the lumen of the GIT by carrier proteins that are inserted into the apical membrane of enterocytes and colonocytes.

The responses of the carriers to phytochemicals are best known for the apical membrane, sodium-dependent glucose transporter SGLT-1, with phloridzin commonly used in research as a selective inhibitor. The influences of soy isoflavones on post-prandial glucose homeostasis (Kreydiyyeh *et al.*, 1994) can be partly explained by decreased activity of SGLT-1 (Vedavanam *et al.*, 1999). The lower rates of glucose transport caused by tea polyphenols (Shimizu *et al.*, 2000; Kobayashi *et al.*, 2000) are probably due to non-specific binding to proteins, with differences among stereo isomers (Tsuchiya, 2001). Corresponding with this, tea polyphenols also decrease rates of carrier-mediated amino acid absorption and the activity of sodium–potassium ATPase (King *et al.*, 2000; Kreydiyyeh, 1996). A similar mechanism would explain the non-specific reductions in absorption of nutrients and electrolytes caused by tannins (Silverstein *et al.*, 1996). Alternatively, changes in BBM fluidity caused by catechins and other phytochemicals would influence the functions of various transporters (Tsuchiya, 1999). However, the amounts of catechins normally consumed may not be sufficiently high to alter BBM fluidity *in vivo*. It is not known if the decreased transport associated with peppermint oil (Beesley *et al.*, 1996) is specific for SGLT-1, or is non-specific and shared by other transporters.

Historically, the absorption of lipid-soluble nutrients has been considered to be carrier-independent, with solutes diffusing into enterocytes down concentration gradients. This is true for some lipid-soluble components of plants (e.g. the hydroxytyrosol in olive oil; Manna *et al.*, 2000). However, transporters have been reported for several lipid-soluble nutrients. For example, absorption of cholesterol is partly dependent on a carrier-mediated process that is inhibited by tea polyphenols (Dawson and Rudel, 1999) and other phytochemicals (Park *et al.*, 2002). A portion of the decreased absorption caused by tea polyphenols may be due to precipitation of the cholesterol associated with micelles (Ikeda *et al.*, 1992). Alternatively, plant stanols and other phytochemicals may compete with cholesterol for transporter sites (Plat and Mensink, 2002). It is likely that transporters for other lipid-soluble nutrients are also affected by phytochemicals, although this has not been adequately investigated.

The lower rates of nutrient absorption associated with diets high in non-starch polysaccharides are probably due to the increased viscosity of digesta (Vaugelade *et al.*, 2000), which increases the thickness of the unstirred layer overlying the enterocytes and causes a non-specific decline in solute absorption. This explains why diets high in β -glucans, which are structural carbohydrates and which increase viscosity of digesta, reduce absorption of nutrients and

may be useful for reducing the post-prandial plasma concentrations of glucose in diabetic subjects (Lifschitz *et al.*, 2002). However, the availability of other nutrients, including minerals, may be lowered (Morais *et al.*, 1996). In contrast, gum arabic causes an acute increase in small intestine absorption of nutrients, electrolytes and water, but the mechanism is uncertain (Wingertzahn *et al.*, 2001).

Several classes of phytochemicals are absorbed by the GIT and transferred to the systemic circulation. For example, some lectins are absorbed intact, appear in the blood at rates that exceed those for other proteins, and elicit systemic responses (Carreno-Gomez *et al.*, 1999).

9.4.5 Altering GIT motility and food transit

Phytochemicals can be used to either stimulate or inhibit motility of the GIT. For example, caffeine and other phytochemicals stimulate motility (Lis-Balchimi *et al.*, 2001; Boekema *et al.*, 1999), whereas motility is slowed by peppermint oil (Beesley *et al.*, 1996), protease inhibitors (Schwartz *et al.*, 1994) and several other phytochemicals (Abdullahi *et al.*, 2001; Odetola and Acojenu, 2000; Rojas *et al.*, 1999; Amos *et al.*, 1998). Many of the traditional herbal medicines used for treatment of diarrhea are based on aqueous extracts that slow small intestine transit and increase residence time for digesta (Lin *et al.*, 2002). The opiates and derivatives are particularly noteworthy (Williams *et al.*, 1997).

Phytochemicals that influence motility can do so by altering the signaling pathways that regulate contraction of the GIT smooth muscle. For example, the spasmolytic responses to essential oils and other inhibitors of motility appear to be post-synaptic and may involve changing intracellular concentrations of cAMP and the activity of calcium channels (Lis-Balchimi *et al.*, 2001; Amos *et al.*, 1998). Oryzatensin, a peptide isolated from rice, stimulates intestinal motility, apparently by interacting with smooth muscle receptors and altering responses to neural inputs (Takahashi *et al.*, 1994, 1996). Food transit is also influenced by the viscosity of luminal contents. For example, complex polysaccharides that form gels slow gastric emptying (Dall'Angelo and Lino von Poser, 2000).

9.5 Phytochemicals, waste and toxin elimination and other functions

The GIT supplements the kidney in the elimination of wastes and toxins. The P-glycoprotein of enterocytes, which is implicated in multi-drug resistance, plays a critical role. This export carrier exhibits varying responses to the different polyphenols present in green tea (Wang *et al.*, 2002), and is inhibited by one or more components of grapefruit juice (Wagner *et al.*, 2001). It is

likely that other non-specific inhibitors of BBM proteins will decrease the activities of P-glycoprotein and other export carriers, which include the organic cation and anion transporters. There is a need to better understand how phytochemicals can be used to modulate the export carriers and thereby improve the systemic availability of administered drugs or increase the elimination of toxins and environmental contaminants.

9.5.1 Mucosal metabolism and intracellular responses

To date, there is very little known about if and how phytochemicals modulate the metabolism of GIT tissues other than the liver. Of particular interest are the xenobiotic metabolizing enzymes of the GIT, which are involved with transformation of drugs and toxins. Whereas the metabolic activities of the resident microflora dominate in the large intestine, mucosal enzyme activities are more important in the small intestine where bacterial densities are lower and the villi and microvilli increase the area of exposure.

Small intestine transglutaminase activity and the production of polyamines are responsive to phytohemagglutinin (Sessa *et al.*, 1996). Moreover, the high glucosinolate content of cruciferous vegetables modulates the activities of enzyme systems involved in biotransformation of molecules (Lampe and Peterson, 2002), which would include the Phase I and Phase II enzymes associated with the small intestine mucosa. There is some research directed at identifying specific phytochemicals that modulate CYP3A4 and other xenobiotic metabolizing enzymes of the mucosa to improve the oral availability of therapeutic drugs subject to biotransformation (Edwards *et al.*, 1999; Lown *et al.*, 1997). The decreased expression of heat shock proteins by enterocytes exposed to lectins indicates that other intracellular functions are responsive (Ovelgonne *et al.*, 2000).

9.5.2 Osmoregulation

The several liters of fluid that are secreted each day by the GIT mucosa, pancreas and gall bladder, and other associated glands are necessary for the digestion of feedstuffs. Due to efficient reabsorption, less than 100 ml of fluid and only a small percentage of the secreted electrolytes are lost in the feces. The disturbances of mucosal secretion and reabsorption of water and electrolytes caused by various bacterial toxins, such as cholera, are well established.

Opiates and various derivatives are commonly used to treat diarrhea, partly because they inhibit electrolyte secretion (Suzuki *et al.*, 2000; Turnberg, 1983). Other phytochemicals counter the secretory responses to cholera toxin (Oi *et al.*, 2002). An example would be the decreased chloride secretion caused by proanthocyanide and the ability to inhibit the secretory diarrhea caused by cholera toxin, but only if administered first (Hor *et al.*, 1995). In the light of the co-transport of water and electrolytes by carriers of glucose

and other nutrients, oral rehydration solutions should not be made with components that have tannins or other inhibitors of the nutrient carriers (Silverstein *et al.*, 1996).

9.5.3 Endocrine function

The great diversity and amounts of hormones and regulatory molecules secreted by the GIT influence local and systemic functions. As a consequence, phytochemicals that modulate the endocrine functions of the GIT can alter its characteristics and those of other organ systems.

Several phytochemicals alter the secretion of GIT hormones. Protease inhibitors stimulate secretion of gastrin (Temler and Mettraux, 1986) and cholecystokinin (CCK) (Herzig *et al.*, 1997; Jordinson *et al.*, 1996). The higher CCK concentrations increase pancreatic secretion (Furuse *et al.*, 1990; Rosewicz *et al.*, 1989), and the satiety influences of CCK (Beglinger, 2002) may partly explain the decreased food consumption associated with diets containing protease inhibitors (Garthoff *et al.*, 2002). Caffeine also stimulates secretion of CCK (Boekma *et al.*, 1999). Gastrin secretion is increased in response to protease inhibitors. The patterns of secretion for several GIT regulatory peptides are responsive to the saponins in a number of herbal medicines (Arai *et al.*, 1997) and to lectins, but the responses and magnitude vary among the lectins (Jordinson *et al.*, 1997).

Several phytochemicals act as hormone mimics. An example would be the phytoestrogens which are present in many feedstuffs (Ibarreta *et al.*, 2001) and which elicit multiple influences (Liggins *et al.*, 2000). Although phytoestrogens and synthetic derivatives increase calcium absorption and status in females (Arjmandi *et al.*, 2000; Mei *et al.*, 2001), the specific responses of enterocytes have not been adequately investigated. However, estradiol increases rates of small intestine absorption for some nutrients (Nielsen *et al.*, 2002) and reverses calcium malabsorption after ovariectomy in animal models (Ten Bolscher *et al.*, 1999). Other phytochemicals bind to receptors for various regulatory peptides and neurotransmitters (Misra, 1998) and can thereby influence enterocyte functions by activating signaling pathways.

The interactions between the endocrine and neural systems of the GIT are complex, include shared signaling molecules, and can be hard to separate. Therefore, it is likely that the influence(s) of some phytochemicals will cross over between neural and non-neural pathways. Corresponding with this, luminal administration of capsaicin interacts with visceral neurons to increase GIT motility (Zittel *et al.*, 2001; Topcu *et al.*, 2002) and abrogates the decreased electrolyte and fluid secretion caused by piperine (Capasso *et al.*, 2002).

9.5.4 GIT defense functions

The GIT has several defense functions that protect against invasion by pathogens and exposure to compounds that pose health risks. The mucosal immune

system includes innate and adaptive components that respond to organisms or harmful compounds in the lumen of the GIT or those that transcend the epithelial barrier.

A wide diversity of herbal remedies have purported abilities to stimulate defense functions. Complexes of carbohydrate and lignin, which are present in some herbs, modulate enteric immune functions (Kiyohara *et al.*, 2000), and the changes in cytokine secretion (Matsumoto and Yamada, 2000) can trigger systemic responses. The polysaccharides present in other herbal medicines augment production of immunoglobulin (Ig) A by the Peyer's patches in the small intestine (Sakushima *et al.*, 1997; Yu *et al.*, 1998). The responses of the enteric immune system to lectins are variable (Pusztai 1993), and can elicit systemic responses (Lavelle *et al.*, 2000). Other phytochemicals provide protection by inducing detoxification pathways in mucosal cells (Williamson *et al.*, 1998).

Proteinaceous phytochemicals can contain toxic epitopes that elicit defense responses; for example gliaden and gluten peptides which cause celiac disease and other mucosal disorders (Tighe and Ciclitira, 1995; Van de Wal *et al.*, 1999). The mucosal inflammation caused by feeding carnivorous Atlantic salmon diets with soybean meal decreases rates of nutrient absorption (Nordrum *et al.*, 2000), whereas the detrimental influence of such diets is much less pronounced when fed to omnivorous fish, such as catfish and tilapia.

The mucosal responses to antigens (i.e. food allergies) may be subject to 'programming'. Specifically, exposure during early development can have lifelong consequences for both the enteric and the systemic immune functions (Scott *et al.*, 2002). In the light of these findings, there is some research into understanding whether early exposure to antigens can induce, or repress, oral tolerance by altering the numbers, proportions and responses of mucosal lymphocytes and the profiles of secreted cytokines (Kaneko *et al.*, 2001). It is relevant that components of soy appear to sensitize pre-ruminant calves and increase secretion of some, but not all, classes of immunoglobulins (Lalles *et al.*, 1995).

Certain phytochemicals provide protection by several mechanisms against the various forms of cancers that occur along the length of the GIT. For example, some protease inhibitors have been associated with a lower incidence of esophageal cancer (Sammon, 1998), whereas cruciferous vegetables are purported to provide protection against colorectal cancers (Barrett *et al.*, 1998). The organosulfur compounds isolated from garlic and other plants are reported to reduce the risk of certain GIT cancers (Sparnins *et al.*, 1988). Tumor growth has also been reduced by lectins (Pryme *et al.*, 1998).

9.5.5 Structural features of the GIT

The quantity and organization of the mucosa are critical determinants of GIT functions. Feeding chicks and rats diets with tannins causes mucosal atrophy and villus shortening, with liver damage, and decreases growth and survival

(Ortiz *et al.*, 1994). Within hours after feeding certain plant lectins there is noticeable damage to the microvilli of enterocytes and villus length is reduced, with corresponding decreases in the activities of enzymes associated with the BBM (Kik *et al.*, 1991; Kimura *et al.*, 1986). Apparently, the lectins trigger the release of trophic hormones (Jordinson *et al.*, 1999). This causes a hyperplastic response by the small intestine, increasing the proliferation and turnover of enterocytes and altering the patterns of enterocyte glycosylation of BBM proteins (Pusztai *et al.*, 1995). Although the concurrent changes in the cytokinetics, characteristics and functions of the enterocytes (Pryme *et al.*, 1998) are not considered advantageous for healthy individuals, the ability to induce proliferative responses has been considered as a therapeutic approach to addressing the atrophy caused by parenteral nutrition (Jordinson *et al.*, 1999). Other phytochemicals, such as capsianoside from sweet peppers, alter the cytoskeleton of enterocytes, thereby disrupting tight junctions and increasing epithelial permeability (Hashimoto *et al.*, 1997).

The responses are not limited to the mucosa. There is a direct relationship between pancreatic hypertrophy and dietary levels of protease inhibitors (Grant *et al.*, 1993). The speculation is that a chronic decrease in protein digestion up-regulates synthesis and secretion of proteases, thereby stimulating compensatory growth of the pancreas. There is concern that the pancreatic hypertrophy may increase the risk of adenomas. In contrast, long-term exposure to amylase inhibitors selectively decreases amylase secretion, lowers food intake, and reduces the pancreas mass of growing rats (Kataoka and Dimagno, 1998). The growth responses of the pancreas to lectins are not universal and vary among animals (Myer *et al.*, 1982). Protease inhibitors stimulate pancreatic hypertrophy in rats and mice, but not in hamsters, rabbits and guinea pigs. There is a notable lack of evidence from histology for lectin-induced pancreatic hypertrophy in a non-human primate (*Cebus* monkeys; Harwood *et al.*, 1986).

9.6 Phytochemicals, gastrointestinal bacteria and gut health

The bacteria resident in the GIT are important determinants of GIT health and of host health and nutrition. This is obvious from comparisons of the GIT of germ-free and conventional animals, and from elegant studies showing how bacteria modulate patterns of enterocyte gene expression (Hooper *et al.*, 2001; Sharma and Schumacher, 2001). The metabolic activities of the GIT bacteria provide the host with short-chain fatty acids as energy substrates and vitamins, can convert phytoestrogens and other phytochemicals to other forms (Rowland *et al.*, 2000), and activate or deactivate carcinogens. The ability of the resident bacteria to release phytoestrogens and other phytochemicals from complexes (Liggins *et al.*, 2000) has been exploited for the release of therapeutic compounds, natural or synthesized, from pro-drugs

(Kim *et al.*, 2000). The specific metabolic activities depend on the composition of the resident assemblages and dietary inputs. Collectively, these findings highlight how changes in the populations and metabolic characteristics of the resident bacteria can affect the GIT and the host.

Antibiotics are commonly used to manage the GIT bacteria. However, the growing concern about antibiotic resistance is increasing interest in identifying alternatives. Several classes of phytochemicals protect plants against infectious agents and can be considered as anti-microbials (Naidu, 2000). It is not surprising that the same compounds influence the composition and metabolic activities of the bacteria resident in the GIT. An example would be the anti-microbial compounds associated with garlic (Ross *et al.*, 2001; Shashikanth *et al.*, 1994), notably the thiosulfinates which exhibit activity against *Candida* (Lemar *et al.*, 2002) and *Shigella* (Iwalokun *et al.*, 2001). Oils extracted from a wide diversity of plants exhibit anti-microbial properties against most gram-positive and gram-negative bacteria (Lis-Balchin *et al.*, 2001), as do alkaloids (Ata *et al.*, 2002), flavones (Tsuchiya and Iinuma, 2000) and polyphenolic compounds (Naidu, 2000). It is now recognized that many of the traditional herbal remedies for diarrhea and the spices used for flavoring have anti-bacterial properties (Rojas *et al.*, 1999; Longanga Otshudi *et al.*, 2000a,b; Otshudi *et al.*, 2000). Moreover, phytochemicals have been proved to be useful for food preservation (Naidu, 2000).

Saponins and phenolics also have anti-microbial properties (Chung *et al.*, 1998) and have been associated with reduced rumen functions (Klita *et al.*, 1996; Reed, 1995), thereby limiting the nutrient quality of forages. Also, carbohydrate complexes with lignins and other compounds reduce carbohydrate utilization by rumen bacteria (Cornu *et al.*, 1994). The influence of such anti-microbials on rumen functions can affect small intestine characteristics by altering nutrient concentrations (Barry and McNabb, 1999).

Whereas the above phytochemicals can be considered as bacteriostatic or bacteriocidal, other phytochemicals, notably some of the complex, non-digestible oligosaccharides present in plants, can be metabolized by specific members of the resident bacteria (Gibson *et al.*, 1995). Such compounds have been called prebiotics (Roberfroid 2001; Roberfroid, 1996; Gibson and Roberfroid, 1995), although other compounds that escape digestion and are metabolized by selective groups of the resident bacteria can also be considered as prebiotics. The fructooligosaccharides (FOS) and inulin are the best understood prebiotics. Other prebiotics that are available, or are being considered, include the transgalactosylated oligosaccharides, soybean oligosaccharides, xylooligosaccharides, lactosucrose, verbascose, polydextrose, lactulose and palatinose.

The selective utilization of prebiotics by some, but not all, of the resident species alters the assemblages, densities and metabolic activities of the GIT bacteria. Of importance is the ability of prebiotics to increase the proportion of the resident bacteria represented by the lactic acid producing bacteria (LAB), resulting in changes of GIT and systemic functions (Swanson *et al.*,

2002a; Floch and Hong-Curtiss, 2002). These include improved lactose digestion (Sanders, 1993), increased mineral availability (Greger, 1999), and a laxative effect due to fecal bulking. The responses of the GIT to prebiotics have led to the concept of 'functional foods', which do not directly provide energy and nutrients, but which do improve host health (Marriott 2000; Diplock *et al.*, 1998).

Diets supplemented with prebiotics derived from plants provide other GIT benefits. The short-chain fatty acids produced by metabolism of prebiotics by LAB are available as energy sources for both the GIT (particularly butyrate) and the entire host (Cummings *et al.* 2001). Moreover, the provision of a fermentable source of carbon decreases bacterial production of harmful protein catabolites (Swanson *et al.*, 2002b). The increased densities of LAB after feeding prebiotics reduce the densities of potential pathogens by altering the GIT chemical environment (Gibson and Roberfroid, 1995) and by enhancing the enteric and systemic immune functions (Buddington *et al.*, 2002b). As a consequence, feeding diets with prebiotics increases host resistance to enteric and systemic infections (Sekine *et al.*, 1995; Schley and Field, 2002; Buddington *et al.*, 2002a), and provides relief to inflammatory bowel disease (Jacobasch *et al.*, 1999).

Prebiotics also reduce the incidence of colorectal cancers in laboratory rodents (Reddy *et al.*, 1997; Perrin *et al.*, 1997; Roland *et al.*, 1998; Verghese *et al.*, 2002; Buddington *et al.*, 2002a,b). The protection against cancer and tumor growth provided by prebiotics has been partly attributed to the increased concentrations of butyrate (McIntyre *et al.*, 1993; Avivi-Green *et al.*, 2000), which increases colonic mucosal growth, suppresses the proliferation and differentiation of tumor cells in the colon (Gamet *et al.*, 1992), induces apoptosis of cells derived from colonic adenomas and carcinomas (Hague *et al.*, 1993; Avivi-Green *et al.*, 2002), and changes patterns of gene expression in carcinoma and metastatic cell lines (Hague *et al.*, 1997; Menzel *et al.*, 2002).

Although as yet poorly understood, prebiotics may reduce absorption of or accelerate the elimination of environmental contaminants by the GIT (Kimura *et al.*, 2002). This may be partly mediated by changes in the activities of bacterial enzymes. Providing prebiotics reduces the activities of α -glucuronidase and glycocholic acid hydroxylase (Buddington *et al.*, 1996) and other enzymes that have been implicated in the activation of procarcinogens (McBain and MacFarlane, 2001). The magnitude and types of health benefits vary among prebiotics. There is a need to identify which forms, alone or in combination, are most effective.

9.7 Future trends

Phytochemicals have been used for millennia because of their systemic influences. This review summarizes the way in which they can also modulate

GIT characteristics, either to improve or limit specific function, and thereby influence host health. However, the complex, multiple and varied nature of the combinations of phytochemicals present in plants and traditional herbal medicines has complicated efforts to better understand the specific interactions between phytochemicals and the GIT (Yuan and Lin, 2000). Phytochemicals have other applications, such as the use of guar gum as a 'vehicle' to deliver therapeutics (Krishnaiah *et al.*, 2001).

There is a need to identify, isolate and characterize specific phytochemicals that influence the GIT, and then understand the mechanisms of action, the dose-response relations, and the possible interactions with other feedstuffs. Of critical importance will be differentiating between the good, the bad, and the ugly, and understanding how they can be used to manage GIT characteristics to improve nutritional and health status. A long-term goal is to gain a better understanding of the structure–function relations of phytochemicals. This information will prove useful for the development of synthetic compounds that elicit specific responses.

Efforts to reduce the detrimental influences of phytochemicals have historically focused on processing prior to consumption (Reyes-Moreno and Paredes-Lopez, 1993). Alternatively, supplements have been added to the diet to compensate for the presence of certain phytochemicals. An example would be the addition of phytase in order to increase the nutrient values of feedstuffs (Rapp *et al.*, 2001; Ravindran *et al.*, 1999). Nutritional genomics and proteomics will take on greater importance in the future through the development of plants with desired combinations of phytochemicals (DellaPenna 1999). Accomplishments to date include plants with reduced phytic acid and improved digestion (Douglas *et al.*, 2000; Spencer *et al.*, 2000), production of edible vaccines (Bonn, 2002; Mercenier *et al.*, 2001), and inducing expression of phytochemicals that provide insect resistance (Down *et al.*, 2001; Sudhakar *et al.*, 1998). Further accomplishments will be aided by a better understanding of how phytochemicals interact with the GIT. Although animal models will play an important role in the research, caution must be used when extrapolating results from one species to humans and other species (Liener, 1983).

9.8 References

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Part II

Developing phytochemical functional products

10

Assessing the intake of phytoestrogens: isoflavones

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10.1 Introduction

Phytoestrogens are naturally occurring plant-derived phytochemicals whose role is to protect plants from stress or to act as part of a plant's defence mechanisms. Although composed of a wide group of non-steroidal compounds of diverse structure, phytoestrogens have been shown to bind to estrogen receptors and to behave as weak agonists or antagonists in both animals and humans. Among several classes of phytoestrogens, lignans and isoflavones are the most interesting as potentially beneficial in a broad range of hormone-dependent conditions. Isoflavones are the most widely studied, but lignans and coumestans will be also considered in this chapter in view of their presence in the European diet(s). Although most research has been done on isoflavones, phytoestrogens as a whole are implicated in cancer prevention as well as in protection against several other degenerative diseases such as coronary heart disease and stroke, osteoporosis, chronic renal disease, neurological disorders, diabetes and obesity. The objectives of this chapter are to highlight sources of phytoestrogens in currently available European foods and in specially designed 'novel' foods, to review the studies on bioavailability of phytoestrogens in humans and to examine the evidence for their health effects.

10.2 Assessing the dietary intake of isoflavones

This section looks first at the various sources of phytoestrogens in the diet, including:

- plant sources of phytoestrogens;
- soy-protein products used as ingredients;
- the use of phytoestrogens in supplements.

It then addresses the ways in which phytoestrogen intake from these sources is measured and some of the problems associated with such measurement.

10.2.1 Dietary sources of phytoestrogens

Isoflavones are present in legumes and particularly in soybeans. Total isoflavone (IF) content in soybeans ranges between 0.4 and 1.4 mg per gram of dry weight, while in soy foods it reaches 0.2–1.6 mg IFs per gram of dry weight (Mazur and Adlercreutz, 1998). It has been previously estimated that soy foods contain 0.1–3.0 mg IFs per gram of dry weight (Kurzer and Zu, 1997). More typical European legumes contain about 150 times less than this amount (Liggins *et al.*, 2000). Chickpeas have the highest concentration reaching 1.24 µg IFs per gram of dry weight, i.e. 100 times less than soybeans. Isoflavone content in raw materials is further influenced by plant variety and by pedological and climatic conditions (Aussenac *et al.*, 1998).

In Europe, lignans are potentially a more relevant source of phytoestrogens because of their presence in most fiber-rich foods. They are found at high levels mainly in oil seeds, up to 3.7 mg per gram in flaxseeds (Mazur *et al.*, 1996), and at lower levels in cereals (Carcea and Schiavoni, 2001; Liggins *et al.*, 2002). No values are known for whole grains, but foods derived from them, such as whole grain and rye breads, have been estimated to contain 0.55–1.2 mg lignans per 100 g of dry weight (Mazur *et al.*, 1996; Boker *et al.*, 2002). Generally speaking, our limited knowledge of the phytoestrogen content in foods, and the wide variability of content even within the same category of products, is a major limitation in the use of food composition tables to estimate levels of intake.

10.2.2 Soy protein products as ingredients in processed foods

The presence of soy is obvious in products such as tofu, soymilk, whole soybeans, soy flour, tempeh (fermented soybeans), miso (fermented soybean paste), or roasted soy nuts. However, soy can also be used as an ingredient to prepare a whole range of vegetarian products as well as food bars and powdered soy protein beverages. Full-fat soyflour comes from whole, dehulled soybeans that have been ground into a fine powder. Although soyflour is not commonly used in home baking, it is used extensively by the food industry. Soyflour is approximatively 50% protein on a dry weight basis. Soy proteins are commercially available ingredients used in the preparation of other foodstuffs since they lower costs and improve product manufacturing and handling. Functional properties of soy proteins that make them important ingredients in many processed foods include their ability to: absorb water and fat; act as an emulsifier; aid in whipping; improve food texture; and increase the total

protein content and essential amino acids profile of foods to which they are added (Lusas and Riaz, 1995). Such manufacturer advantages are often 'offered' to the consumers as claimed nutritional improvements.

In order to produce soy protein, soybeans are first dehulled, flaked, and defatted to make 'white flakes'. Soy protein concentrates are obtained by removing a portion of the carbohydrates from defatted and dehulled soybeans. Alcohol extraction is the method most commonly used to manufacture soy protein concentrates even though it results in the loss of isoflavones. Soy protein concentrates retain most of the fiber in the original soybean and must contain at least 65% protein on a moisture-free basis to meet quality standards. The most concentrated source of soy protein is soy protein isolates (or isolated soy protein, ISP), which is required to be at least 90% protein on a moisture-free basis. It is heat-treated during processing to insure inactivation of trypsin inhibitors. Most isolated soy protein is manufactured by water extraction from defatted and dehulled soybeans and it retains the natural isoflavones.

10.2.3 Phytoestrogens in supplements

Since 1999, when the Food and Drug Administration allowed the first health claim for soy-fortified foods in the USA, there has been a large increase in the sales of food products claiming to contain soy isoflavones. At the same time, over-the-counter supplements have become widely available. However, concerns have been raised about the real health benefits of such supplements in the absence of adequate information about bioavailability, pharmacokinetics and safety. To fill this gap, an extensive study on pure isoflavones and commercial soy isoflavone supplements has recently been carried out (Setchell *et al.*, 2001). A selection of 31 commercially available supplements showed a wide variation in isoflavone composition and in the amount provided by one tablet. Furthermore, a lower isoflavone content, with respect to the claimed levels, has been observed in almost 50% of the analysed products. In one case, no isoflavones at all could be detected (Setchell *et al.*, 2001).

10.2.4 Estimation of phytoestrogen intakes

In most studies, phytoestrogen intake has been estimated by direct methods that evaluate food intake either by recall (food-frequency questionnaires – FFQs) or by record (food diary), and subsequently by composition databases based on information of this kind. Food-frequency questionnaires are widely administered to subjects involved in epidemiological studies. Their validity and reproducibility is considered sufficient when statistically correlated to data obtained from dietary records (a properly-completed and comprehensive food diary) and from analysis of blood and urine samples (Kirk *et al.*, 1999; Huang *et al.*, 2000; Yamamoto *et al.*, 2001; Verkasalo *et al.*, 2001). FFQs can be repeated several times a year and may be administered to large populations. Such an approach provides an easy and low-cost method of assessing the

habitual intake of foods containing phytoestrogens, but the results are of limited value on their own in any detailed quantitative analysis (Kirk *et al.*, 1999; Huang *et al.*, 2000).

Dietary records (usually 3–5 days) are used to evaluate intake of most macro- and micro-nutrients in the context of large surveys. Depending on the survey design, detailed information on phytoestrogen-containing foods may or may not be available. In particular, soy is present as an ingredient in many manufactured products, with a content that varies according to the brand. Only surveys with a detailed identification of products, followed by inclusion of the industrial recipe in composition databases, allow an accurate estimate of intake. Although applicable to a limited number of individuals, duplicate diet collection should be the most accurate method of estimating phytoestrogen intake. Duplicate diet analysis is based on the collection, by participants, of an exact duplicate of everything they eat or drink over a certain period. The samples are then analyzed for the presence of target compounds such as phytoestrogens. Dietary exposures are calculated from the concentrations of phytoestrogens in the duplicate diet samples and from the weights of the samples. This information is particularly useful in creating databases (see below and also Section 10.7: Sources of further information and advice) about the concentration of chemicals in food in order to estimate dietary exposures and assess health risks. On the other hand, duplicate diet studies are time consuming and intrusive, and it is known that participants can change their diets for the duration of the study (e.g., eat more ready prepared foods than usual), fail to collect exact duplicate portions or under-report the amounts of foods eaten. Furthermore, this method does not take into account seasonal differences.

Biomarkers of diet may provide a more accurate and objective measure of dietary intake than estimates of current or usual intake since this approach is not dependent on the reliability of the subject's memory (Wild *et al.*, 2001). However, the role and relevance of a biomarker have to be assessed on a case by case basis, keeping in mind that it should allow the measurement of exposure to a certain compound or category of compounds that are involved in the genesis of an adverse or of a beneficial health effect (Branca *et al.*, 2001). Further, a biomarker has to be highly sensitive and specific, but minimally invasive and inexpensive. Above all it needs to be validated before it can be used in a nutritional study (Weber, 2001). For isoflavones, exposure biomarkers can be the serum levels of genistein and daidzein, whose fasting levels can be considered indicative of habitual intake. For lignans, matairesinol and secoisolariciresinol could theoretically be used, but this is not currently done because of the complex analytical problems created by these compounds. Urinary metabolites may be even more convenient biomarkers of exposure. The presence of urinary metabolites, however, is not only influenced by dietary intake, but also by the gut metabolism, pharmacokinetics and host metabolism. For isoflavones, genistein and daidzein are also suitable as biomarkers, as well as the metabolites O-desmethyl-angolensin

(O-DMA) and equol, while for lignans enterodiol and enterolactone should be used.

To evaluate the potential of phytoestrogens as health-enhancing dietary compounds, the amount available through food consumption has to be measured at national levels and not only in small cohorts of subjects. At the population level, comparison of intake data from an individual country with that from different countries should facilitate an epidemiological surveillance on diet-related diseases and should improve the quality of nutritional surveys, as well as checking food quality. On the other hand, the reliability of a database is reduced by the limited amount of data with respect to the number of foods consumed; by the incomplete information on soy ingredients present in manufactured foods (such as in meat products); by the different analytical methods used to determine phytoestrogen contents; and by the influence of locations, seasons, crops and varieties on the food content values (Kiely *et al.*, 2003). However, continuous updating with new food consumption data and the development of new analytical techniques should reduce some of these gaps in information and, in the longer term, lead to better national and international databases for the estimation of exposure to phytoestrogens.

High levels of isoflavone consumption are reached only in typical Asian diets (Chen *et al.*, 1999; Nagata *et al.*, 1997; Adlercreutz *et al.*, 1991), with a peak at approximately 100 mg/day in the Hong Kong Chinese population (Ho *et al.*, 2000). In Western countries, isoflavone intake is much lower. The most recent evaluations in the USA (de Kleijn *et al.*, 2001), as well as in different European countries, indicate a mean intake of isoflavones of less of 1 mg/day. Even amongst EU consumers eating mainly soy foods it does not exceed 6–10 mg/day (van Erp-Baart *et al.*, 2003).

10.3 Factors affecting phytoestrogen absorption and metabolism

In plants and, consequently, in soy food products, isoflavones occur in three main biologically active forms:

- genistein;
- daidzein;
- glycitein.

These are present either as:

- aglycones;
- sugar-conjugated glycosides – genistin, daidzin and glycitin.

Glycosides are the predominant forms. Although in humans flavonoids have been shown to be absorbed in their naturally occurring glycosidic forms (Hollman and Katan, 1998), isoflavones are not. It is generally accepted that to be adsorbed by enterocytes across the intestinal wall, isoflavone glycosides

need to be hydrolysed to aglycones. Their bioavailability seems to require an initial hydrolysis of the sugar moiety by intestinal B-glucosidases to allow the subsequent uptake by enterocytes and the flow through the peripheral circulation. Clearly, the extent of intestinal bacterial metabolism influences the bioavailability of phytoestrogens and, therefore, their potential for physiologic effects, although the degree of influence varies among individuals.

The absorption of isoflavones appears to occur by diffusion since aglycones have an appropriate molecular weight to permit diffusion and since no evidence for facilitated or active transport of isoflavones has been demonstrated. Such a requirement has been demonstrated by intestinal perfusion and by cell culture studies showing how the enterocytes are readily permeable for an efficient uptake of the aglycone genistein (Day *et al.*, 1998; Andlauer *et al.*, 2000a), whereas the uptake of the glycoside genistin was found not to exceed 1.3% in isolated rat small intestine (Andlauer *et al.*, 2000b). Although absorption of isoflavone glycosides is low, because they are more water-soluble than aglycones and require enzymatic cleavage of the sugar moiety by mammalian or bacterial glucosidases before absorption, isoflavones become bioavailable and reach high plasma concentrations irrespective of whether they are ingested as aglycones or glycosides.

Two recent studies reached apparently different conclusions on this issue. In one approach (Setchell *et al.*, 2002), a specific and sensitive electrospray mass spectrometry was used to detect glycosides in human plasma. No intact glycosides, even in traces, were detected. On the other hand, Liu and Hu (2002) used both a Caco-2 cell culture model and a perfused rat intestinal model and reached the opposite conclusion: in both intestinal models, genistein and the corresponding glycoside, genistin, are well absorbed. This study also showed that aglycones underwent extensive phase II metabolism via glucuronidation and sulfation in the upper small intestine. Based on genistein studies, it has been extrapolated that glucuronidation of isoflavones occur rapidly by UDP-glucuronosyltransferases and/or sulfotransferases in the gastrointestinal mucosa and that a further conjugation happens in the liver (Zhang *et al.*, 1999a).

The rate and the extent of absorption of isoflavones is usually determined in adults by measuring plasma and urinary isoflavone concentrations following a single dose ingestion of a soy product. Data from different human studies (Rowland *et al.*, 2003, and refs therein) have shown a rapid absorption of phytoestrogens with a significant increase in plasma isoflavones already at 15–30 minutes post-ingestion (Rowland *et al.*, 1999; Morton *et al.*, 1997) and reaching a peak between three and seven hours post-ingestion (Setchell *et al.*, 2001; Busby *et al.*, 2002). On the contrary, lignans possess a slower rate of absorption since their metabolites appear in plasma only at around 8.5 hours post-ingestion. Urinary excretion of isoflavones indicates that the apparent fractional absorption of daidzein is similar to glycitein and that both are greater than genistein (Zhang *et al.*, 1999b). Isoflavones as well as lignans show a dose response effect in their apparent fractional absorption (Nesbitt

et al., 1999). The differences in urinary excretion may be a consequence of the greater gut microbial degradation of genistein (Xu *et al.*, 1995). However, isoflavone excretion in urine accounts for only about 30% of the ingested dose. Considering the low faecal excretion of such compounds, most of the ingested dose is probably metabolised along the intestinal tract. Both plasma and urinary isoflavones measurements indicate that their levels return to baseline at approximately 48 hours post-ingestion (Setchell *et al.*, 2001; Kiely *et al.*, 2003, and refs therein).

Metabolism of isoflavones and lignans is complex and requires both microbial processes in the gut and enzymatic processes in the liver. The gut metabolism of dietary phytoestrogens produces various compounds having estrogenic activity different from that of their corresponding precursors. In humans, these metabolites appear in plasma several hours after soy product consumption, probably reflecting the time necessary for the unabsorbed precursors to reach the colon (King and Bursill, 1998). Their appearance in urine occurs within 24 hours after consumption. A large body of evidence indicates that the isoflavonoid aglycones undergo further fermentation by the intestinal and colonic microflora to produce various metabolites prior to absorption (Axelson *et al.*, 1984; Setchell, 1998; Wiseman, 1999; Rowland *et al.*, 1999; Rowland *et al.*, 2000). Incubation *in vitro* of soy protein or isolated isoflavones with human faecal organisms resulted in the conversion of genistein and daidzein to their metabolites (Chang and Nair, 1995).

Recently, some of the specific faecal bacteria involved in the metabolism of dietary isoflavonoids were isolated (Hur *et al.*, 2000). They have been shown to selectively convert genistin and daidzin to their respective aglycones. One of the isolated bacteria, under anoxic conditions, was further shown to metabolise genistein and daidzein to their respective dihydroxy-genistein and dihydroxy-daidzein. In the case of lignans, enterodiols and enterolactone were shown to be excreted *in vivo* only in rats harbouring a gut microflora (Rowland *et al.*, 1999).

Research suggests that isoflavone and lignan metabolism has an extensive inter-individual variation. In a human cross-over study (Rowland *et al.*, 2000), it was recently demonstrated that equol (a daidzein metabolite having a stronger estrogen-like and antioxidant activities) is not produced with the same efficiency by all individuals. Analysis of urinary equol excretion identified a group of good equol excretors corresponding to about 35% of the healthy subjects studied. The urinary excretion of O-desmethyl-angolensin (O-DMA, another daidzein metabolite) also showed an apparent but less sensitive inter-individual variation, even if no relationship between the excretion of the two daidzein metabolites was found. The major lignan metabolite, enterolactone, has also been found in urine but showed a lower inter-individual variation than daidzein metabolites (Rowland *et al.*, 2000). In the same study, it was found that the dietary fat intake decreases the ability of gut microflora to synthesize equol, stressing the importance of dietary habits in influencing the bioavailability of phyto-estrogens.

Following absorption, isoflavones are then reconstituted mainly to glucuronic acid and, to a lesser degree, to sulphuric acid. Proof that the rate of conjugation is high comes from the low levels of free aglycones detected in blood. In humans, however, the most recent reports (Setchell *et al.*, 2001; Busby *et al.*, 2002) on the pharmacokinetics of pure isoflavones suggest that for 50 mg of isoflavone intake, the expected plasma concentrations will be about 1 μ M, whereas the tissue absorbable form, the unconjugated one, will correspond to about 1–2%. Even considering inter-individual variations (Urban *et al.*, 2001), it is reasonable to consider that ‘plasma concentrations of unconjugated isoflavones will not reach more than 50 nM, a value that has to be taken into account in the cell culture models’ set up to unravel isoflavones activities and their mechanisms of action (Barnes, 2003).

10.4 Isoflavone intake and health

This section gives an overview of the known or suggested physiological effects of phytoestrogens as well as their relevance in pathologies affecting different human tissues and organs. Topics covered are:

- effects in young children;
- cancer prevention;
- immune response;
- coronary heart disease and lipid metabolism;
- osteoporosis and bone diseases;
- diabetes and obesity;
- chronic renal disease;
- cystic fibrosis;
- fertility and post-menopausal symptoms;
- cognitive function and neurological disorders;
- age-related macular degeneration;
- thyroid function.

Other chapters in this book cover some of these individual topics in more detail.

10.4.1 Effects in young children

Due to a greater susceptibility of young children to modification of the sex steroid homeostasis, compared to adults, concerns have been raised about the possible effects of soy isoflavones in infants (Irvine *et al.*, 1998). In countries with a high level of soy consumption there are no reports of negative effects on growth and development, although the amount of isoflavones present in mother’s breast milk is very low (Setchell *et al.*, 1998). The isoflavone content in different soy formulas is much higher, giving an estimated isoflavone intake between 32 and 47 mg/day (Setchell *et al.*, 1997), or 4.6–9.6 mg/kg

body weight/day (Irvine *et al.*, 1998). The circulating plasma concentrations of isoflavones in infants are an order of magnitude higher than in adults with similar isoflavone intake (Setchell *et al.*, 1998). Despite this, only conjugated isoflavones, with negligible biological activity and rapid excretion, were detected in the plasma of infants (Huggett *et al.*, 1997). Up to now, there are no reports describing adverse effects of soy formula-based diets on infant endocrine function. Indeed, protection from cancer and hormone-dependent diseases in adulthood have been proposed as a consequence of early and even prenatal exposure to phytoestrogens (Franke *et al.*, 1998; Setchell *et al.*, 1998).

Another aspect to be considered is soy allergenicity. A few cases of soy anaphylaxis have recently been reported in Sweden and an underestimation of soy involvement in fatal over-reactions to food has been suggested (Foucard and Malmheden-Yman, 1999). Previous studies have failed to show such allergic reactions. A study assessing the risk of atopic diseases in infants fed soy protein formula until six months of age reported no adverse effects in a five-year follow-up (Bruno *et al.*, 1993). A two-year prospective study of infants with confirmed cow's milk allergy (CMA) demonstrated that soy formula is well tolerated (Klemola *et al.*, 2002). In a double-blind, placebo-controlled food challenge study, allergy to soy formula occurred only in 14% of the young children with CMA (Zeiger *et al.*, 1999). Finally, the incidence of allergy to soy in an Italian epidemiological analysis was about 3–4% (Cantani, 1999). Caution should be taken given the recent introduction of genetically modified (GM) soybeans, in which the transfer of a known allergen into a non-allergenic target crop may occur. However, so far no allergic threats have been raised by GM foods (Lack, 2002). Indeed, among the GM soybeans already introduced into the market, 'Round-up Ready'TM (RR) soy flour 'is no different from natural soy flour in terms of its allergenic potential' (Wuthrich, 1999).

10.4.2 Cancer prevention

Soy isoflavones were first supposed to be potential cancer chemopreventive agents on the basis of epidemiological studies showing an inverse correlation between high soy consumption and risk of certain cancers such as prostate, breast, endometrial and colon cancer (Messina *et al.*, 1994; Goodman *et al.*, 1997; Adlercreutz, 1998; Messina and Bennink, 1998; Messina, 1999). Genistein has been identified as a novel anti-cancer compound in screenings for anti-proliferative factors and for tyrosine kinase inhibitors (Polkowski and Mazurek, 2000 and refs therein). Several studies using experimental animal models and cell cultures have confirmed the anti-proliferative role of genistein as well as of other phytoestrogens, suggesting that cell cycle arrest and induction of apoptosis are two main targets of isoflavonoid action (Birt *et al.*, 2001 and refs therein).

However, the estrogenic and anti-estrogenic activity of isoflavonoids could

mean that these compounds might be responsible for promotion of hormone-dependent cancers. A possible explanation of this paradoxical behaviour might be provided by long-term studies and by exposure timing since it has been shown that chronic exposure of target tissues to genistein before tissue maturation reduces the possibility of such tissue producing tumours (Hsieh *et al.*, 1998; Polkowski and Mazurek, 2000 and refs therein). The most recent suggested isoflavone mechanism of action in cancer prevention is in the regulation of DNA methylation (Day *et al.*, 2002). DNA methylation is thought to inhibit transcription of genes by regulating alterations in chromatin structure. The possible involvement of genistein in preventing the development of certain prostate and mammary cancers by maintaining a protective DNA methylation profile has been investigated. Mice fed with a genistein-enriched diet showed a positive correlation with changes in prostate DNA methylation pattern of specific mouse genes (Day *et al.*, 2002). Indeed, other research has shown a positive correlation in premenopausal women between equol excretion and lowered breast cancer risk (Duncan *et al.*, 2000). Such an association was not merely due to an increased isoflavone intake, since a hormonal pattern profile consistent with a lowered breast cancer risk was obtained only in good equol excretors independently of the different levels of isoflavone intake.

10.4.3 Immune response

The lower incidence of certain cancers in the South-East Asia, where soy consumption is higher, also suggests a possible estrogenic effect of soy isoflavones in enhancing immune defenses. A recent investigation in hypercholesterolemic men and postmenopausal women (Jenkins *et al.*, 2002), given one-month diets with different isoflavone content, showed no difference in acute-phase proteins or pro-inflammatory cytokines. Effects may be gender specific: following isoflavone intake (73 mg/d), serum concentrations of interleukin-6 (IL-6) rose in women more than in men (Jenkins *et al.*, 2002). The enhancement of host immune functions by phytoestrogens might also be due to their antioxidant properties via inhibition of the oxidative damage to the immune cells and to their ability to stimulate humoral immunity and cell-mediated immunity (Birt *et al.*, 2001).

10.4.4 Coronary heart disease and lipid metabolism

Observational studies have suggested possible favourable effects of estrogen replacement therapy (ERT) on the risk of coronary heart disease in postmenopausal women. Since elevated plasma cholesterol has been identified as the primary risk factor for cardiovascular disease, investigations have focused on the inverse association between plasma cholesterol concentration and soy protein consumption. The cholesterol-lowering properties of soy have been demonstrated, and a good correlation has been found in

hypercholesterolemic human subjects (Anderson *et al.*, 1995), whereas it is reduced or absent in normocholesterolemic ones (Gardner *et al.*, 2001; Baum *et al.*, 1998; Nestel *et al.*, 1997; Sirtori *et al.*, 1997). However, a recent randomised cross-over trial has shown that, even if to a lesser extent, normocholesterolemic and mildly cholesterolemic postmenopausal women given isolated soy protein (ISP) have a small but significant reduction in low density lipoprotein (LDL) cholesterol plasma concentrations and in the ratio of LDL to HDL (high-density lipoprotein) cholesterol (Wangen *et al.*, 2001).

The cholesterol-lowering effect should be the result of the synergy of soy proteins and soy isoflavones (Potter, 1995). A three-month, double-blind randomised controlled trial found that ISP decreased plasma concentration of total cholesterol and the LDL fraction, but only when the ISP contained at least 37 mg of isoflavones and no ethanol-extracted ISP was used (Crouse *et al.*, 1999). A study of premenopausal women showed a significant inverse association between consumption of isoflavone-rich soy protein and plasma concentration of LDL cholesterol as well as with the ratio of total to HDL cholesterol and of LDL to HDL cholesterol (Merz-Demlow *et al.*, 2000). On the other hand, it was also demonstrated that cow's milk proteins have the same effect as soy isoflavones on LDL cholesterol concentrations (Gardner *et al.*, 2001) and that soy milk supplemented with high doses of glycitein does not reduce total and LDL cholesterol concentration even in hypercholesterolemic subjects (Sirtori *et al.*, 2002). In a double-blind randomised study performed on postmenopausal women (Lucas *et al.*, 2002), flaxseed supplementation (providing a high level of lignans) caused an improvement of the lipid profile, decreasing the serum level of both LDL and HDL cholesterol and, more significantly, reducing the serum level of both apolipoproteins A-1 and B.

In addition to the effects on blood lipids, it has been suggested that soy consumption has a beneficial action on arterial function and improves antioxidant status (Lichtenstein, 1998 and refs therein). Genistein and daidzein were shown to have antioxidant properties *in vitro* (Kerry and Abbey, 1998), to enhance endothelium-dependent vasodilation and to reduce the development of atherosclerosis in monkeys (Honore *et al.*, 1997; Wagner *et al.*, 1997).

Some studies have investigated hypertension, another key risk factor in coronary heart disease and stroke. The observation that individuals with high systolic and diastolic blood pressure have lower levels of circulating isoflavones (Moline *et al.*, 2000) has led to the hypothesis that consumption of flavonoid-rich foods such as fruits, vegetables, red wine and green tea, might decrease blood pressure. Indeed, a decreased diastolic blood pressure was observed in women given isoflavones, provided as ISP (Crouse *et al.*, 1999), and was observed also in postmenopausal women whose diet was supplemented with soy proteins containing at least 34 mg phytoestrogens per dose (Washburn *et al.*, 1999).

Recently, another plausible mechanistic basis for isoflavone-mediated cardiovascular disease prevention has been proposed. In a study to find the

molecular mechanisms leading to greater cardiovascular risk in men, a significant gender difference was demonstrated in the myocardial activation of a protein kinase, Akt/PKB, involved in a broad range of physiological responses (Camper-Kirby *et al.*, 2001). In young women the activated kinase has a greater nuclear localization than in men of similar age and than in postmenopausal women. Such an effect might be responsible for cell protection in heart diseases: the transcription factor forkhead, a nuclear target of Akt known to increase cell death by apoptosis, was found to be inactivated in all cases in which the nuclear localization of Akt was enhanced (Camper-Kirby *et al.*, 2001). The same study has shown that 10 nmol/L 17 β -estradiol and 250 μ mol/L genistein, both *in vitro* and *in vivo*, could also enhance Akt-nuclear localization.

10.4.5 Osteoporosis and bone diseases

Osteoporosis is a typical disease of Western society determined by an imbalance in skeletal turnover due to a high rate of bone resorption in excess of bone formation. Enhanced bone resorption is due to an increased number of osteoclasts, bone cells whose unique function is to resorb bone, and to their enhanced activity (Manolagas, 2000 and refs therein; Teitelbaum, 2000 and refs therein). Estrogen deficiency causes both the early and late forms of osteoporosis in postmenopausal women and contributes to the development of osteoporosis in elderly men (Riggs *et al.*, 1998). Estrogen appears to inhibit bone resorption through a number of factors such as the osteoclast differentiation factor, ODF/RANKL/TRANCE, and several growth factors and cytokines, whose dysfunctional regulation modulates osteoclast activity and causes bone inflammatory lesions (Riggs, 2000; Teitelbaum, 2000). Besides osteoporosis, several other bone diseases, such as Paget's disease, paraneoplastic bone diseases (mainly breast, lung and prostate cancers), and inflammatory bone diseases (rheumatoid arthritis, for instance), are due to increased bone resorption, making its modulation a primary therapeutic target. To date, therapeutic inhibition of bone resorption (Rodan and Martin, 2000) has been mainly achieved by hormone replacement therapy (HRT), by treatment with bisphosphonates (BPs, for instance pamidronate and alendronate), selective estrogen receptor modulators (SERMs, for instance tamoxifene and raloxifene) and by calcitonin.

It has been shown that in postmenopausal women habitually high intakes of dietary isoflavones are associated with higher bone mineral density (BMD) values at both the spine and hip region (Mei *et al.*, 2001). It is conceivable that an isoflavone-rich diet may help to reverse the state of secondary hyperparathyroidism associated with estrogen withdrawal and hence lower the rate of bone turnover in postmenopausal women, thus reducing the risk of osteoporosis (Valtueña *et al.*, 2003). Phytoestrogens could be used as 'natural' SERMs (Brzezinski and Debi, 1999) and some studies (Setchell, 2001 and refs therein) support such an idea since the molecular targets of

phytoestrogens appear to be similar to those of drugs used in therapy. Indeed, there is a similarity in the mechanism of action of phytoestrogens to BPs. BPs are a class of pyrophosphate analogues able to prevent bone loss in osteoporosis and Paget's disease, to block metastatic bone diseases, to reduce tumor-induced hypercalcemia and to alleviate bone pain (a consequence of bone breakdown). Among the known mechanisms of action of BPs, there is the inhibition of matrix metalloproteinases (MMPs) and also of the vascular endothelial growth factor (VEGF), as well as of an enzyme of the cholesterol synthesis pathway, the farnesyl diphosphate synthase (Teronen *et al.*, 1999; Goto *et al.*, 2002; Rodan and Reszka, 2002; Wood *et al.*, 2002). Phytoestrogens such as lignans have been shown to be active regulators of VEGF in breast cancer angiogenesis (Dabrosin *et al.*, 2002). However, isoflavones and coumestans were reported to interfere with the cytoskeletal organization of rat endometrial carcinoma (Dopp *et al.*, 1999). Finally, the secretion of MMP-9 appears to be regulated by isoflavones in primary cultures of human isolated osteoclasts (Lorenzetti *et al.*, 2001).

10.4.6 Diabetes and obesity

Soybeans and legumes may have a protective role in the development of diabetes because of their fiber and carbohydrate content. There is an inverse association between total dietary fiber, or a food's low glycaemic index, and the risk of non-insulin-dependent diabetes mellitus (NIDDM) in both men and women (Marshall *et al.*, 1991; Salmeron *et al.*, 1997a,b). A specific role for isoflavones is not well established, even if several cardiovascular risk markers in type 2 diabetic subjects were positively affected by the ingestion of 50 g/day of ISP containing more than 165 mg/day of phytoestrogens (Hermansen *et al.*, 2001). Specific effects have only been described in experimental models. A diabetogenic role would be suggested by the experiments conducted in a pre-adipocyte cell line (3T3-L1), in which insulin-mediated glucose uptake was inhibited by genistein (Kamei *et al.*, 2002). On the other hand, genistein showed the capacity to protect against vasculopathy, one of the consequences of diabetes. In an experimental diabetic rat model, it was demonstrated that long-term oral administration of genistein significantly inhibited retinal vascular leakage compared with non-diabetic control rats (Nakajima *et al.*, 2001). It might be speculated that such an effect depends on genistein effects on the neovascularization process similar to what has recently been demonstrated in another study of the degeneration of ocular tissues by Luty *et al.* (1999). See Section 10.4.11 below.

In addition to fiber and carbohydrate content, protein intake from legumes may have weight-loss benefits for obese individuals just because proteins enhance post-meal satiety (Rolls, 1995). However, a possible specific role for phytoestrogens in obesity has been postulated through the modulation of the satiety response, a neuroendocrine mechanism controlled by leptin (a hormone secreted by adipose tissue and already known to be regulated by

estrogens) and insulin signalling. Such a mechanism could be hypothesised because of genistein inhibition of protein tyrosine kinases (Polkowski and Mazurek, 2000). It has been suggested that genistein mimics the activity of the protein tyrosine phosphatase (PTP1B) known to regulate both leptin and insulin signalling, modulating food intake and body weight (McMinn *et al.*, 2000; Johnson *et al.*, 2002). Resistance to both leptin and insulin signalling are hallmarks of type 2 diabetes mellitus and obesity. To date, no studies have fully demonstrated such a putative interaction, although a randomized, cross-over study on pre- and postmenopausal women showed that no effects on leptin concentrations are associated with even high levels (130 mg/day over a three-month period) of isoflavone consumption (Phipps *et al.*, 2001).

10.4.7 Chronic renal disease

Soybeans and flaxseeds are thought to have a beneficial role in chronic renal disease, since nutrition intervention studies have shown reduced proteinuria and attenuated functional and structural damages of kidneys in both animals and humans (Ranich *et al.*, 2001). Nonetheless, it is not evident which components of soy and flaxseed are responsible for the beneficial effects. Indeed, human studies on diabetic nephropathy suggest that an excessive protein intake is harmful to kidneys. Neither a dietary protein restriction nor a soy protein substitution has been shown to protect or to slow down the progression of renal disease. Excessive protein intake in subjects at risk of diabetic nephropathy causes hyperfiltration and glomerular hypertension, leading to the progressive deterioration of the renal activity and subsequently to nephropathy (Anderson *et al.*, 1999). The series of events leading to nephropathy has not been observed in long-term studies to confirm whether soy-protein based diets, compared to animal protein diets, show a lower renal blood flow, a decreased glomerular filtration rate and a reduced fractional clearance of albumin as they occur in short periods (Kontessis *et al.*, 1990).

10.4.8 Cystic fibrosis

Cystic fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR), a chloride (Cl^-) channel characterised by chloride permeability and secretion, and also by the regulation of other epithelial ion channels (Eidelman *et al.*, 2001). Mutations in the CFTR gene lead to an impaired or absent Cl^- conductance in the epithelial apical membrane, which leads to defective Cl^- secretion and absorption across the epithelium. Genistein (Illek *et al.*, 1995; Weinreich *et al.*, 1997) and other flavonoids (Illek and Fisher, 1998) have been shown, in different animal and tissue models, to activate wild-type CFTR and CFTR mutants by (Eidelman *et al.*, 2001; Roomans, 2001; Suaud *et al.*, 2002):

- restoring chloride permeability and secretion;
- decreasing pro-inflammatory cytokines in CF airways;
- restoring regulatory interactions on CFTR mutants with other ion channels.

The mechanism of action proposed is based on a direct binding to the channel and the following partial block of the ATP-binding pocket of CFTR (French *et al.*, 1997), a mechanism similar to that used by genistein to inhibit the activity of other ATP-utilizing enzymes such as protein kinases and topoisomerase II (Polkowski and Mazurek, 2000 and refs therein). The selection of flavonoid compounds or the development of synthetic drugs reasonably selective for CFTR activation might be an area for future clinical trials.

One of the complications of CF is osteoporosis which is characterised by low bone mineral density (BMD) (Haworth *et al.*, 1999). Low BMD is clinically important in CF, since it results in an increased rate of fracture. The predominant abnormalities in CF patients seem to be a marked trabecular and cortical osteopenia with an increased perforation and decreased thickness (Haworth *et al.*, 2000). This condition does not occur in osteoporosis which is characterised by either perforated trabeculae of relatively normal thickness (postmenopausal, type 1, osteoporosis) or thin trabeculae with some perforations (senile, type 2, osteoporosis; Rehman *et al.*, 1995). Since lung transplantation has become a treatment for end stage lung diseases, such as CF, and because of the major contribution of long-term immunosuppressive treatments following lung transplantation, bone loss and severe osteoporosis are enhanced in post-transplanted CF patients, even if no correlation between BMD and biochemical markers of bone turnover has been found (Tschopp *et al.*, 2002). It has been suggested that an early diagnosis and prevention of osteoporosis of CF patients in the lung pre-transplant period should have a high priority in reducing additional post-transplantation bone loss. Indeed, bisphosphonates treatments gave encouraging results in adolescent and adult CF patients (Brenckmann and Papaioannou, 2001).

10.4.9 Fertility and post-menopausal symptoms

In some animals, consumption of a phytoestrogen-rich diet can cause temporary infertility and reproductive system disorders (Irvine, 1999). In humans, lower testosterone levels and a decline in human semen quality over the past century have been linked to increased exposure to environmental endocrine disruptors (EDCs) (Sharpe and Skakkebaek, 1993). Furthermore, cases of sexual impotence have been reported in males exposed to synthetic estrogens in the pharmaceutical industry (Mattison *et al.*, 1990). If this might be the case, the fetal-prepubertal period and Sertoli cell development would be of critical importance (Sharpe and Skakkebaek, 1993). However, an adverse effect of phytoestrogens on male fertility has yet to be proven. Recent work (Mitchell *et al.*, 2001) addressing this point led to the conclusion that up to 40 mg/day of isoflavones over a two-month period had no effects on gonadotrophin and

sex hormone levels and on semen quality. The authors did not exclude possible adverse effects at higher concentrations of isoflavones for longer times of exposure or during sexual development.

In premenopausal women, a suppression of mid-cycle peaks of luteinising hormone (LH) and follicle-stimulating hormone (FSH), an increment of plasma estrogen levels, and a stimulation at different levels of breast tissue was observed (Cassidy *et al.*, 1994). All those effects were not observed in postmenopausal women (Baird *et al.*, 1995) suggesting that the background levels of endogenous estrogens play a determinant role in mediating the effects of phytoestrogens. The cancer preventive effect of soy consumption in premenopausal women has been related to increased menstrual lengths, increased serum level of sex hormone-binding globulin (SHBG), and decreased estrogen levels (Kurzer, 2002).

10.4.10 Cognitive functions and neurological disorders

Estrogens act on the development, genderization and function of the brain (Belcher and Zsarnovszky, 2001). Animal studies show a link between estrogens and cognitive function (Koenig, 2001). In female rats, memory skills are under the direct control of estrogen-dependent signalling. The mechanisms underlying the cognitive-enhancing effects of estrogens might be due to an increased activity of neurons or to a general protection of neurons from apoptosis. An estrogen-dependent increase in spine density is observed in female rats but not in male ones. Nonetheless, both sexes express estrogen receptors in the brain and the selective effect of estrogens in females is an intriguing challenge for future work. Along with increased connectivity, cellular plasticity of the neuroendocrine tissue during sexual development appears also to be under the direct control of estrogens. Different neurotransmitters, neuronal receptors and growth factors have been reported to be modulated by estrogens, dictating future correct sexual differentiation and proper onset of puberty (Koenig, 2001).

Isoflavones have been shown to act as anti-estrogenic compounds in the neuroendocrine tissue. In rats, phytoestrogens (genistein and coumestrol) antagonized the ER α -dependent binding of the oxytocin receptor and affected the ER β transcription levels in different areas of the hypothalamus (Whitten *et al.*, 2002), as well as the sexual behaviour of male offsprings and the proper ovulation of female offsprings (Whitten *et al.*, 2002). Moreover, isoflavones have been shown to produce anxiolytic effects and to invert the typical sexually dimorphic pattern of visual-spatial memory (Lephart *et al.*, 2002). All these reported alterations observed in animal models have not been investigated in humans (see also Section 10.4.1). Neurobehavioural effects of phytoestrogens (Belcher and Zsarnovszky, 2001) – even at small, but physiologically relevant, exposure levels – have to be carefully considered in the use of soy-based infant formula.

Epidemiological studies of postmenopausal women on estrogen replacement

therapy (ERT) showed a significantly lower risk of developing Alzheimer's disease (AD). In AD, as well as in other human dementias, an important hallmark is the hyperphosphorylation of the microtubule-associated protein tau. In a primate model of menopause, isoflavones – in a soy protein matrix – have been reported to decrease selected AD-relevant tau phosphorylation (Kim *et al.*, 2000). A second relevant hallmark of AD is the formation of senile plaques. *In vitro* experiments have shown that estradiol protects cells against the toxic effects of β -amyloid, the major component of plaques in the brains of AD patients. Estrogens and phytoestrogens have become potential candidates in the treatment of neurodegeneration. Indeed, in cell cultures both estradiol and kaempferol have been reported to possess a protective effect on β -amyloid peptide-induced toxicity (Roth *et al.*, 1999).

10.4.11 Age-related macular degeneration (AMD)

The ability of isoflavones to act as anti-angiogenic and anti-mitotic factors can be used to prevent the development and progression of several diseases (Fotsis *et al.*, 1998). In order to investigate the usefulness of dietary phytoestrogens in the treatment of ocular neovascularization, genistein and two structurally related flavonoids, fisetin and luteolin, were dissolved in microemulsions and applied topically at concentrations corresponding to the lower micromolar range (Joussen *et al.*, 2000). All substances significantly inhibited corneal neovascularization and no significant side-effects were reported. Fisetin showed the maximum effect, but all three substances were shown to act as potent inhibitors (between 1.85 and 3.7 μ M) of corneal angiogenesis *in vivo* (Joussen *et al.*, 2000). In a rabbit model of age-related macular degeneration (AMD), genistein was shown to block the regeneration of choriocapillaris without affecting the wound healing of retinal pigment epithelial (RPE) cells (Lutty *et al.*, 1999). Choriocapillaris degeneration is a typical pathological hallmark in vaso-occlusive disorders like diabetes mellitus and sickle cell disease. The ability of genistein to inhibit choroidal neovascularization (CNV) in rabbits suggests that overall tyrosine kinase inhibitors might be useful as a pharmacological treatment of CNV in a variety of diseases including histoplasmosis and pathological myopia leading to central loss of vision (Lutty *et al.*, 1999).

10.4.12 Thyroid function

Isoflavones have been implicated in goiter induction. Soybean extracts inhibit reactions catalyzed by thyroid peroxidase (TPO), essential to the synthesis of thyroid hormones (Divi *et al.*, 1997). Genistein and daidzein (at about 1–10 μ M of IC₅₀) may act as alternative substrates for tyrosine iodination (Divi *et al.*, 1997). Furthermore, genistein and daidzein have also been shown to cause the irreversible inactivation of TPO in the presence of hydrogen peroxide. Genistein also inhibits thyroxine synthesis in the presence of iodinated

casein or human thyroglobulin (Divi *et al.*, 1997). On the other hand, several animal studies found that soy consumption had no effects on triiodothyronine (T_3) and thyroid-stimulating hormone (TSH), whereas thyroxine (T_4) was increased (Potter *et al.*, 1996; Balmir *et al.*, 1996). Iodine status influences isoflavone action. Iodine deficiency greatly enhances the anti-thyroid effects of soy, while iodine supplementation prevents such effects (Sheehan, 1998; Doerge and Sheehan, 2002). In a six-month double-blind trial on postmenopausal women, a small effect of soy proteins on thyroid function measurements was demonstrated (Persky *et al.*, 2002). Such action on thyroid hormones may play a protective role.

An inverse correlation between thyroid cancer risk and phytoestrogens was recently proposed as a result of a multi-ethnic population-based case control study conducted in the San Francisco Bay Area (Horn-Ross *et al.*, 2002). In this study, dietary habits and phytoestrogen consumption were assessed by a food-frequency questionnaire and by a nutrient database. The outcome of the study was that soy-based foods and alfalfa sprouts were associated with a reduction of thyroid cancer risk, whereas a Western diet did not influence cancer risk. No difference was observed between American and Asian women or between pre- and postmenopausal women. Furthermore, among the few compounds examined, the isoflavones genistein and daidzein and the lignan secoisolariciresinol were the phytoestrogens most frequently associated with risk reduction (Horn-Ross *et al.*, 2002).

The link between soy consumption and goiter might be independent from the known estrogenic effects of phytoestrogens. The consistency of results of studies conducted in pre- (Duncan *et al.*, 1999a) and postmenopausal (Duncan *et al.*, 1999b; Persky *et al.*, 2002) women suggest that the goitrogenic effect of isoflavones is independent from estrogenic status (Doerge and Sheehan, 2002). However, an estrogenic effect on thyroid function cannot be totally excluded since estrogens can modulate the activity of the pituitary on the thyroid gland (Faglia *et al.*, 1973; Gershengorn *et al.*, 1979) and because, in selected target tissues, estrogens and thyroid hormones might interact in the regulation of gene expression (Zhu *et al.*, 1996; Persky *et al.*, 2002).

10.5 Establishing appropriate intake levels for isoflavones

Functional foods have no universally accepted definition. Only the Japanese Ministry of Health and Welfare has introduced a legislative definition of functional foods as 'processed foods containing ingredients that aid specific bodily function in addition to nutrition' (Osawa, 1998). In Europe, a definition of functional foods is still evolving, but functional ingredients are commonly defined as 'safe dietary substances that beneficially affect specific targets in the body beyond providing adequate nutrition' (Diplock *et al.*, 1998). In the context of the FUFOSE (Functional Food Science in Europe) Concerted Action, a working definition of functional foods was recently proposed as follows:

...foods can be regarded as functional if they can be satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way relevant to an improved state of health and well-being and/or reduction of risk of disease...functional foods must remain foods and they must achieve their effects in amounts normally consumed in a diet (Contor, 2001).

While soy-based products are widely available and consumed in South East Asia, North America and Australia, in Europe they are still considered to be niche products. At best, soy is used as an ingredient in baked products. Soy isoflavones can be added to food in the form of soy protein isolates or soy protein concentrates. Different categories of products can be considered for enrichment, such as baked products (breads, biscuits, cakes), breakfast cereals, beverages, milk and dairy products (milk, yoghurt, cheese) and soups. Foods with good organoleptic properties can be obtained, although isoflavone concentrates tend to give a metallic aftertaste that has to be masked with flavours (Drewnowski and Gomez-Carneros, 2000). Isoflavones are otherwise rather stable compounds that are resistant to most processes and do not shorten the shelf life of most commercial products.

10.5.1 Safe dose levels phytoestrogens

Data on safety have been obtained from *in vitro* as well as *in vivo* animal and human studies (see also Section 10.4). About 50 years ago, Australian farmers observed an infertility syndrome in sheep associated with the consumption of clover species (Bennets *et al.*, 1946). The clover compounds shown to cause the infertility (genistein, daidzein, equol, biochanin A, formononetin) were members of the isoflavone family (Bradbury and White, 1951; Shutt and Braden, 1968), raising the question of whether soy might cause infertility in humans (see also Section 10.4.9). A variety of reports further supported adverse effects of isoflavones on animal reproductive systems (Santell *et al.*, 1997; Flynn *et al.*, 2000a,b).

Genistein and daidzein have been demonstrated not to be mutagenic in the Salmonella/mammalian microsome reverse mutation assay (Ames test) (Bartholomew and Ryan, 1980). In controlled animal feeding studies, rat fed diets with 12.5 mg dietary genistein/kg body weight/day over a 13-month study (Rao *et al.*, 1997), or with 15–17 mg aglycone soy isoflavones/kg body weight/day over a 22-month study (Anastasia *et al.*, 1990), or with 39, 15, and 11 mg aglycone soy isoflavones/kg body weight/day over a 9.5-month study (Rackis *et al.*, 1979) have not shown any effect on body weight or organ weight and/or pathology and histopathology. Even if no toxic effects were reported at the maximum dose administered, it is important to stress that no studies determined the experimental 'no observed effect level' (NOEL). Instead, the 'lowest observed effect level' (LOEL) of 86 mg aglycone/kg body weight/day was reported in a reproductive study in female rats, and it was not associated with any adverse toxicological or reproductive effects

(Gallo *et al.*, 1999). Finally, pre-clinical studies to assess the sub-chronic toxicity of genistein were performed at the US National Cancer Institute over a three-month period in beagle dogs using two purified soy products with a genistein content of 43% and 90%. The two soy products were tested at doses ranging from 4.3 to 63 mg genistein/kg body weight/day and no clinical or histological signs of toxicity were reported (NCI, 1996).

Many clinical studies have been performed on human subjects to assess the effect of soy isoflavones on chronic disease risk factors with no ill-effects (see Section 10.4). The safety profile of isoflavones is, however, difficult to establish because of the limited sample sizes and short periods of investigation of such studies. At present the upper tested limits are:

- an ingestion of total isoflavones of up to 3 mg/kg body weight consumed by some human infants without significant clinical effects (Irvine *et al.*, 1998, but see also Section 10.4.1);
- an ingestion of up to 16 mg/kg body weight used in human adults with little significant toxicity observed in any subject (Busby *et al.*, 2002; NCI, 1998).

For long-term exposure, questions have been raised on proliferative effects of breast duct epithelial cells in premenopausal women (Messina and Loprinzi, 2001, and refs therein).

10.5.2 Phytoestrogen levels associated with health benefits

Useful guidelines on safety and health effects are given by studies on South-East Asian populations with the highest levels of intake. Epidemiological studies have suggested no ill-effects but rather correlated high dose consumptions of soy isoflavones with multiple 'beneficial and safe effects' on human health (Barnes, 2003). It has been estimated that approximately 10% of Asian populations consume routinely about 100 mg isoflavones/day (Seow *et al.*, 1998, Chen *et al.*, 1999; Wakai *et al.*, 1999; Ho *et al.*, 2000). Based on mean intakes observed in Japan and China, a suggested beneficial isoflavone intake would be 30–40 mg aglycones per day (Messina, 1995; Kimira *et al.*, 1998; Chen *et al.*, 1999; Wakai *et al.*, 1999; Nakamura *et al.*, 2000).

In order to evaluate the effect of phytoestrogen administration in the context of studies on functional foods, the blood level of isoflavones observed in South-East Asian populations can be considered. The blood concentration of isoflavones has been estimated to reach a mean value of 276 nM in Japanese men (Adlercreutz *et al.*, 1993), while a recent report on Japanese women using more sensitive techniques established higher values corresponding to 407 nM genistein and 118 nM daidzein (Uehara *et al.*, 2000). In clinical trials, it was shown that after a daily consumption of two soy protein servings, corresponding to 20 g of soy proteins and equivalent to 42 mg of aglycones, the plasma concentrations of isoflavones measured 6.5 hours after the first serving reached 800–1000 nM (Coward *et al.*, 1996; Urban *et al.*, 2001).

Such evidence indicates that it might be unnecessary to reach the same intake of soy products as Japanese or other Asian populations to reach the same plasma levels of isoflavones. The rationale for such a difference could be a different bioavailability of the ingested isoflavones. Indeed, in most Asians a deficiency of an intestinal lactase, responsible for β -glucosides hydrolysis, might explain the lower isoflavones concentrations in the blood of the Japanese (Day *et al.*, 2000).

An international consensus panel of experts has been established to set proper isoflavone food fortification levels. After reviewing human studies in the area of the menopause, osteoporosis, cardiovascular diseases and cancer, it reached the conclusion that the recommended isoflavone intake for health benefits should fall between 60 and 100 mg aglycones per person per day, with the lower dose considered as 'reasonable and responsible' (Anderson *et al.*, 2000). Intakes close to the lower limit may be appropriate for cardiovascular risk reduction or for the relief of menopause symptoms, while intakes close to the upper limit are needed to achieve an effect on bone health. In order to decrease serum LDL-cholesterol, a minimal intake of about 40–60 mg aglycones/day, depending on prior cholesterol status, is suggested, which represent about 25 g of soy proteins/day (Nestel *et al.*, 1997). For the relief of menopausal symptoms a consumption of 60 mg aglycones/day has been suggested since some reduction was already seen at about 50 mg aglycones/day (Duncan *et al.*, 1999a,b). Studies reporting a positive effect on bone health and calcium metabolism have used 90 mg IF/day (Potter *et al.*, 1998; Weaver *et al.*, 1999). Finally, for cancer prevention an intake between 50 and 110 mg aglycones/day is considered beneficial to reduce risks of breast, colon and prostate tumours in humans (Bennink and Om, 1998).

Soy diets or enrichment of food have, therefore, to achieve a daily intake of 60–100 mg total isoflavones per day. A short-term toxicity limit is probably ten times higher (16 mg/kg body weight; Busby *et al.*, 2002), although the information on such a limit has been obtained in the context of short-term studies with a limited numbers of participants. Given the limited information on long-term health benefits and possible risk in other groups of population, it is probably wiser for the time being to restrict enrichment to specifically targeted products that could be taken, for example, as snacks. Products that are widely consumed, such as bread or milk, are difficult to monitor and may be more inclined to induce to excessive or insufficient intakes. The simultaneous presence on the market of several different products, belonging to different food categories, may also be a problem, as different categories of consumers might be exposed to widely variable amounts of phytoestrogens.

10.6 Future trends

Beneficial or adverse effects of phytoestrogens have to be more rigorously evaluated and long-term studies are necessary for most, if not all, diseases in

which their action has been claimed. Moreover, although lignans have been identified as protecting against breast cancer in women and prostate cancer in men, a complete view of their beneficial or adverse effects on estrogen-dependent diseases is still lacking. So far, researchers have concentrated their interest on soybean and soy isoflavones. Given the widespread distribution of lignans in the plant kingdom and their resulting occurrence in cereal grains (as well as in fruit and vegetables), they form the basis of the diet of many individuals. Future studies may, therefore, concentrate on the importance of lignans for public health.

Genetic modification (GM) has already enhanced isoflavone content in soybean varieties such as the Roundup Ready variety (Taylor *et al.*, 1999). GM technology might also open the possibility of enriching the isoflavones content of cereal grains, which represent a major food staple for both humans and livestock. Production of isoflavones in non-legume plants has already been achieved via the introduction of a key enzyme of isoflavone synthesis – the cytochrome P450 monooxygenase, isoflavone synthase (IFS) – resulting in the production of isoflavones in plants that do not naturally synthesize them (Yu *et al.*, 2000 and refs therein). The synthesis of isoflavones in cereal transgenic crops, for instance, may potentially lead to an improved food nutritional quality.

Nevertheless, the use of genetically modified organisms (GMOs), and of their derived technology in health care, agro-food and the environment, opens new questions on the determination of long-term effects of foods derived by GM crops. Phytoestrogens are secondary metabolites, whose natural functions in plants are in plant defence responses, plant resistance to pathogens and plant-microbial interactions (Straney *et al.*, 2002). Since isoflavones levels in plants are genetically and environmentally influenced, the possible modulation of the phytoestrogens content in a GMO should always be assessed on a case-by-case basis to avoid any safety problems such as allergenicity. Finally, the interactions among environmental endocrine disrupters compounds (EDCs), GMOs-resistance genes, food chains and chronic diseases should never be forgotten, since there has not yet been a complete and independent evaluation of food chains as vehicles of EDCs introduced by pollution and pesticide-resistant genes in GM vegetables (Bontoux, 2001; Amaral Mendes, 2002).

10.7 Sources of further information and advice

Besides the literature mentioned in the references, we would like to direct readers to a series of websites, whose purpose is to diffuse information on the current research on food and nutrition, endocrine disrupters, hormonal-dependent diseases, and biotechnologies, to a wider community of scientists and physicians as well as to a non-scientific audience.

A general overview of endocrine disrupters and estrogens mechanism(s)

of action is available at the websites, <http://e.hormone.tulane.edu/>, of the Center for Bioenvironmental Research (CBR) of the Tulane and Xavier Universities; and <http://www.endodisru.iss.it>, of the Italian National Health Institute.

To obtain an updated set of data on the phytoestrogens content in foods and diets, we suggest checking the following websites: <http://www.venus-ca.org/database.htm> (phytoestrogen database of the EU-funded project VENUS); <http://www.nal.usda.gov/fnic/foodcomp/Data/isoflav/isoflav.html> (USDA-Iowa State University database on the isoflavones content of foods); and <http://www.ifr.bbsrc.ac.uk/phytochemicals/Links.htm> (Institute of Food Research Database on the levels of bioactive compounds in plant foods).

In order to get recent advances on the effects of phytoestrogens on hormonal-dependent diseases as well as on human supplementation trials, it might be useful to refer to: <http://www.venus-ca.org/> (EU-funded project on dietary exposure to phytoestrogens and related compounds and effects on skeletal tissues); <http://www.phytos.org> (EU-funded project on the prevention of osteoporosis by nutritional phytoestrogens); <http://www.phytoprevent.org> (EU-funded project on the role of phytoestrogens in the prevention of breast and prostate cancer); and <http://www.nutrition.tum.de/isoheart.htm> (EU-funded project on cardiovascular health of postmenopausal women).

On the GM debate and biosafety research, a review of results performed under the European Commission supervision ('EC-sponsored Research on Safety of Genetically Modified Organisms', edited by C. Kessler and I. Economidis) is available also online at the EU-website <http://europa.eu.int/comm/research/quality-of-life/gmo/>. An update on current research in food safety, nutrition and food-related disease might be found in the websites of the World Health Organization, <http://www.who.int/fsf/GMfood/index.htm>, and of the UK Food Standards Agency, <http://www.foodstandards.gov.uk/>.

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11

Testing the safety of phytochemicals

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11.1 Introduction: the health benefits of phytochemicals

Plant foods are important sources of nutrients and are the overwhelming source of food for the world's population. Apart from their function in ensuring healthy growth and development, there is growing evidence, from observational studies on humans consuming varying amounts of plant foods, including grains and pulses and fruit and vegetables, that they play an important role in reducing the risks of diseases of ageing, particularly cardiovascular disease and cancer (World Cancer Research Fund, 1997; van't Veer and Kok, 2000).

There have been countless studies designed to determine which specific compounds in plant foods might be responsible for their health protective effects. There is good evidence to support the hypothesis that plant foods contain phytochemicals that are either direct-acting antioxidants or which act indirectly as antioxidants by up-regulating the antioxidant phase II enzymes in cells (EUROFEDA, 2002). Since many of the diseases of ageing are associated with oxidative stress, arising as a result of a loss of cellular control over the generation of reactive oxidative species, it is hypothesised that these processes might be mediated through the phytochemicals present in plants.

It is estimated that there are in excess of 20 000 unique chemicals present as plant primary and secondary metabolites (Ohlrogge, 1994). The levels present in foods can vary quite considerably depending on the variety and on agronomic and environmental factors. To attribute benefits to any one chemical or group of chemicals seems a daunting task. Thus, in spite of intensive work in the last decade or so, there is still insufficient evidence with which to support the 'antioxidant hypothesis', or any other hypothesis, and to attribute

the health protective effects observed to any one class of phytochemicals. The most that can be said at the moment is that there may well be multiple mechanisms in operation, affected by a range of the phytochemicals present in foods, that could explain the observations.

Laboratory studies have shown a wide range of compounds to have some form of anti-carcinogenic activity acting at multiple stages in the complex process leading to cancers (Kohlmeier *et al.*, 1995). Whilst protective effects are seen for some chemicals, the results are not always consistent. Some anti-carcinogens also show potentially detrimental effects such as mutagenicity or tumour promotion. In large part, the lack of progress is attributable to the design of the experimental conditions that have been used to attribute benefits to phytochemicals. Many of the studies are *in vitro*. Those that have been undertaken *in vivo* have used doses that are pharmacological and very different from those that are likely to occur in the human physiological dose range through the consumption of plants as food. Nor has there been evidence provided to show that the animal models chosen show similar metabolic behaviour to humans. The human studies that have been undertaken have often failed to support the results obtained in animals or in cell systems, or at least have not shown strong consistency (EUROFEDA, 2002; Kall *et al.*, 1996).

In few, if any, cases has there been any dose-response data generated – this is because of the cost involved in undertaking lifetime studies with a range of doses. The limited studies that have been undertaken are mostly confined to substances that are used as natural additives in foods. In many cases, regulatory bodies have accepted that natural chemicals can generally be regarded as safe, since they have a history of consumption by humans, and no adverse effects have been reported. Consequently no toxicological studies have been undertaken. In addition there is growing evidence and support for the concept of a Threshold of Toxicological Concern (TTC) that assumes that there is a level of intake for any chemical substance below which there is no significant risk to health. Many natural chemicals in food are present in low amounts and would fall within the scope of this concept.

It remains the case that if the observational studies are valid, and not just a surrogate for other factors that contribute more significantly to a healthy life-style, the overall effect of consuming the highly complex and numerous phytochemicals found in our diet is to protect humans against the major age-related diseases. In addition, since none of these studies have specifically used organically-grown plant foods, it must be assumed that, if there are any adverse effects from consuming low residues of toxic natural compounds or infrequent residues of chemicals used in food production, these are outweighed by the beneficial properties of other constituents. This illustrates the problems associated with any attempt to ascribe a benefit, or a risk, to a single chemical when it is present in low amounts in a complex mixture of other chemicals.

In all cases the process of risk assessment of food chemicals has up to now ignored the effect of the other constituents present in that food. The scientific approach to risk (or benefit) assessment has so far demanded a

reductionist approach, rather than assessing the risk–benefit of consuming a substance when present in a food. The food matrix usually exerts a modulating effect, but an effective assessment of the safety of whole foods remains a major challenge.

Because of the requirement by public health authorities that any chemical used in the production of food should pose no risk to the consumer, the safety of food chemicals has mostly been approached from the perspective of whether or not the chemical poses a toxicological hazard. If it does so then it is argued that it is likely to present some degree of risk to the consumer even if humans are exposed to very low levels of that chemical in their diets. However, if exposure is minimal, depending on the nature of the toxicity, it is likely that the risk is ‘acceptable’ because it is so low. What is not considered, or even tested in the experimental systems designed to study the toxicological effects of chemicals, is whether there are levels of exposure where there might be potential health benefits. The assumption is made that any non-natural, adventitious substance that can be shown to be toxic is unlikely to have health benefits.

11.2 Evaluating the safety of phytochemicals in food

The safety of a diet could be defined as the overall risk–benefit of consuming that diet over a lifetime. This concept is not one that is applied in determining the safety of a chemical in food. Safety is almost invariably considered as the absence, or minimisation, of risk and not as the maximisation of benefit. Consequently, the scientific basis for regulating food chemicals is based on principles that were developed for assessing the risks posed by pharmaceuticals and industrial chemicals, and minimising these, rather than for maximising the benefits.

Since fruit and vegetable consumption is recommended as part of a healthy lifestyle, it could be argued that there is no need to obtain detailed safety data on individual phytochemicals that are present in these items of the diet. This ignores the fact that:

- the intake of specific phytochemicals might not be optimal in the protection against age-related disease;
- there could be important additional improvements in health if more was known about the benefits and risks of consuming increased amounts of specific compounds;
- there could be important applications in the treatment of disease;
- plant breeders are likely to give more attention to breeding naturally-resistant varieties of plants that will contain higher levels of bioactive phytochemicals, but they have no means of knowing what the ‘target’ levels of these phytochemicals should be, or how to improve the composition of the plant to improve the health benefits for consumers;

- the information will be increasingly sought by regulatory authorities as more and more food products use natural ingredients in food manufacture or market specific products on the basis of implied health claims;
- nutritional supplements containing phytochemicals are in widespread use and may present risks to sections of the population, or at least provide no benefit; and
- there is an increasing requirement to inform consumers about food issues.

11.3 Risk evaluation of food chemicals

At present the approach to assessing the potential risks of exposure to a chemical in the diet involves the application of a standardised risk assessment approach consisting of three main elements – hazard identification and characterisation and exposure assessment.

11.3.1 Hazard identification

Assessment of whether a chemical has the potential to cause adverse effects in humans arises usually from direct observation of an effect in animals or humans, such as the acute poisoning episodes that have occurred when potatoes contain high levels of glycoalkaloids. Epidemiological studies have also been used to infer a possible relationship between intake of a particular type of food, or constituent of that food, and the potential to cause an adverse effect. Such observations led to the characterisation of the aflatoxins as human carcinogens. However, natural toxic substances that occur in plant foods have often been identified through observations in animals, particularly farm animals. It was observations of adverse effects in farm animals that led to the further characterisation of the phytoestrogens and the mycotoxins. In other instances, the concern arises from the chemical similarity to other known toxins.

For chemicals in general the identification of a potential hazard normally arises from the application of *in vitro* tests or from short-term toxicity studies undertaken in laboratory animals (up to a period of 90 days in the case of the rat where the test material normally should not exceed 1% of the total diet). This usually enables a critical effect to be assessed.

11.3.2 Hazard characterisation

The characterisation of the toxicity associated with a specific chemical is invariably dependent on feeding the chemical to laboratory animals in statistically-based lifetime bioassay, guidelines for which have been agreed at the international level by the OECD (OECD, 1981) and the IPCS (IPCS, 1999), and which are undertaken strictly in compliance with the principles of good laboratory practice (GLP). The animals used in long-term bioassays are

in-bred and genetically homogeneous. Variations in results occur depending on the species and strain chosen. These bioassays involve the administration of high doses of the compound to animals in a design that enables any differences from control animals to be statistically evaluated. The dosing regime involves the use of from three up to five dose levels selected to produce a range of responses. The maximum dose of the chemical which is selected is usually that dose which does not result in >10% loss in body weight gain (the maximum tolerated dose, MTD). Other dose levels are set as fractions of the MTD to enable a dose–response relationship to be established. The lowest dose is ideally chosen to correspond with a no observable adverse effect level (NOAEL). In many instances the doses utilised are orders of magnitude in excess of those to which an individual might be exposed.

A typical dose–response is shown in Fig. 11.1. This assumes that a dose exists which has no effects due to the capacity of the body to reverse minor changes and maintain cellular homeostasis. The threshold dose is normally taken to be the observed experimental NOAEL, but the NOAEL could be lower than the threshold dose. The NOAEL chosen is the one that represents the most sensitive species studied since all international protocols require that the chemical is tested on at least two species (frequently the rat and the mouse).

The determination of an acceptable dose for humans involves the application of uncertainty factors to reflect the fact that, unlike the experimental animal, there is wide variability and susceptibility of response in the genetically diverse human population. Variations in gender, age, hormonal and disease status can affect the response to a chemical. In order to minimise any potential risks, uncertainty factors are applied to the NOAEL to arrive at a reduced exposure that is considered tolerable – namely the acceptable daily intake or ADI. These are usually tenfold for variations in susceptibility amongst the human population (the intra-species factor) and tenfold for the potential

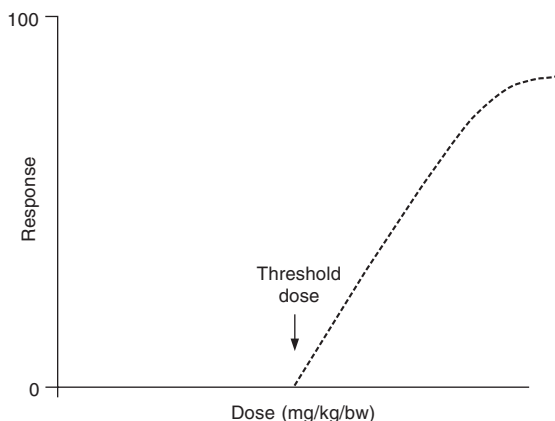


Fig. 11.1 A hypothetical dose–response curve for a toxic chemical.

difference in susceptibility between the experimental animal and humans (the inter-species factor). These factors can be higher or lower depending on the strength of the information available. In the case of macro- or micronutrients they are usually much lower since, if the safety of some nutrients were evaluated according to the protocol described, the ADI would be much lower than the daily allowance recommended to ensure healthy growth.

In the case of food chemicals, where there is likely to be significant exposure, information is invariably required about the mechanism of action that results in the toxicity. This involves a large amount of extra effort to determine if the effects observed are due to the utilisation of high doses only, or whether or not the mechanisms are likely to occur in humans.

11.3.3 Exposure assessment

This requires detailed information on the amount of the substance that is consumed, or to which a person might be exposed from other environmental sources. Often the data available on patterns of food consumption is of variable quality and accuracy. All of the methods that have been utilised, such as food-frequency questionnaires, duplicate diet studies, total diet studies and food balance sheets, have significant errors associated with them, although some methods are better than others (Bingham, 1987). There is a growing trend towards the use of statistical techniques which express exposure data in terms of probability distributions, thus allowing the variability and uncertainty inherent in the data to be quantified and providing for a more realistic assessment that can be applied to a range of situations (Lambe, 2002). But in general, the data used to estimate the potential risks are based on the level of consumption likely to be found in the 95th percentile of the population. Comparison can then be made between the amount of chemical ingested (as amount/kg body weight per day) and the ADI found in animal studies.

11.4 Potential food carcinogens

It has been known since the 1940s that the lifespan of laboratory animals could be increased if they were restricted in their food intake and were not allowed to feed *ad lib* (Tannenbaum, 1942). Since then many other studies have confirmed this observation (Roe *et al.*, 1995). The principal observation was that there was a reduced incidence of spontaneous tumours. These observations are of critical importance in understanding the benefits or risks of consuming food. Although the mechanism behind the observations still remains to be proved, it is not explainable on the basis of a reduced intake of exogenous mutagenic substances. It suggests that spontaneous tumours arise from endogenous processes, such as oxidative damage which increases with age and which can be influenced by energy intake. This contrasts with the

overwhelming scientific effort which has focussed on characterising and studying the exogenous mutagens present in the diet.

Many chemicals, when tested in long-term animal bioassays, can be shown to increase the incidence of spontaneous tumours that occur in that species, often in a dose-related manner, and this has led to the classification of many chemicals as carcinogens. In an analysis of data from the US National Toxicology Program it was shown that, in some of the studies, there was both an increase and a decrease in tumour incidence when all of the tumours that resulted were considered. But, as the concern in risk assessment only resides with an increase in tumour incidence, these facts have not been taken into account in the risk assessment (Davies and Monroe, 1994). In another analysis of these data, it has been shown that over 50% of the chemicals tested were found to result in an increased cancer incidence in test animals (Ames and Gold, 1990). Some phytochemicals act as natural pesticides, and many of these compounds have enabled the plants to survive in the environment. The development of modern plant varieties has probably reduced their concentrations in plants, and synthetic pesticides have to be used to maintain their viability. Even though only a small proportion of natural pesticides have been tested, over 50% were found to be rodent carcinogens and are ubiquitous in fruits, vegetables, herbs and spices (Ames *et al.*, 1990). Thus there appears to be a lack of consistency between animal cancer bioassays on individual substances and the final overall outcome of consuming plant products.

Cancer bioassays have caused an inordinate amount of concern. In many cases, however, it is not possible to demonstrate that these chemicals are mutagenic when tested in *in vitro* and *in vivo* mutagenicity tests. Many tests now exist to determine, with a good degree of confidence, whether a chemical is a genotoxic carcinogen, i.e. is capable of causing a permanent change in the genetic material leading to a change in phenotype in the organism. It is prudent to assume that substances that show mutagenicity in a variety of tests, particularly *in vivo* tests, are likely to be potential human carcinogens.

However, the observation that there are many compounds that increase tumours in animals, but are non-genotoxic, is likely to occur through a number of mechanisms. The most common is that of sustained cytotoxicity and cell proliferation, arising as a consequence of the high doses that are often administered to the animals that lead to cytotoxicity. An increase in tumour incidence results (Ames and Gold, 1990). But the tissue sites where the tumours are induced are frequently those where there is a significant incidence of spontaneous tumours. Effects on hormonally-induced tumours and other receptor-related mechanisms that can lead to enzyme induction can also result in increased cancer incidence. These so-called non-genotoxic carcinogens induce tumours as a secondary event following an effect that has a threshold, and as such it is usually possible to determine a no-effect level and arrive at an ADI.

In contrast, the calculation of human risk for genotoxic carcinogens from

animal bioassay data is more problematic. It is a cornerstone of regulatory toxicology that, for genotoxic carcinogens, there is no threshold dose below which there would be no carcinogenic effects, although there is evidence that this is an overly conservative assumption (Henderson *et al.*, 2000). Risk management in such cases often attempts to reduce human exposure to the minimum feasible. In the case of a substance added to food, this would mean in practice that the chemical would not be permitted for use. However, in the case of naturally occurring compounds, this presents a real difficulty that is rarely satisfactorily addressed. Whilst it is understandable that a highly conservative approach is adopted, the problem can lead to regulation aimed at controlling minute amounts of such substances in the food chain. The effect of this regulation on improving public health is likely to be minute and is certainly not quantifiable.

The fact that there may be other substances in a food that can block the adverse effects of such carcinogens through a variety of mechanisms is ignored, as indeed is the fact that there are a variety of repair mechanisms in operation that control the potential of genotoxins to cause permanent mutation. But, given the difficulty in managing the risks of chemicals naturally present in food, there is a greater need to focus on determining benefits, since these could have major public health outcomes that would be measurable.

11.5 Problems in assessing safety: the example of β -carotene

One of the few phytochemicals that has been subjected to the rigorous testing procedures required by food safety authorities is β -carotene, a naturally-occurring carotenoid that is also a pre-cursor of vitamin A in humans. It is increasingly used as a food colour since the food product can be claimed to contain all natural ingredients. For this reason, detailed toxicological studies were undertaken that enabled the Joint FAO/WHO Expert Committee for Food Additives (JECFA) to set an ADI of 0–5 mg/kg/bw/day based on a NOAEL of 50 mg/day and the application of an uncertainty factor of 10 (JECFA, 1974). This low factor was used because it was argued that the compound occurred naturally in food, that its use as a food additive would not lead to a substantial increase in the total amount normally consumed, and that there had been no reports of adverse effects in humans. The ADI would correspond to an acceptable intake in humans of up to 350 mg/day.

More recently, large human intervention trials were undertaken with β -carotene alone, or in combination with non-dietary amounts of vitamin E. These trials were undertaken because of promising animal studies that suggested that these antioxidants could offer chemo-preventive action against oxidative stress. The results, which are summarised in Table 11.1, were disappointing. Although the study population in two of the studies (ATBC and CARET)

Table 11.1 Summary of human intervention trials with β -carotene

Study	No of participants	Dose	Increase in plasma levels (fold)	Effect on incidence of CVD	Effect on incidence of cancer
ATBC (Albanes <i>et al.</i> , 1996)	29133 male smokers	20 mg β -carotene + vitamin A	17.5	Increase	Increase
CARET Omenn <i>et al.</i> , 1996)	18314 smokers	30 mg β -carotene + vitamin A	12		Increase
PHYS (Hennekens <i>et al.</i> , 1996)	22071 males	50 mg β -carotene every other day	4	No effect	No effect

ATBC = Alpha Tocopherol Beta Carotene Prevention Study;

CARET = The Beta Carotene and Retinol Efficacy Trial;

PHYS = Physicians' Health Study.

were smokers, an increased risk of developing lung cancer was found. In the other study (PHYS), when a healthy population was studied, some of whom were smokers, no reduction in the incidence of cancer or cardiovascular disease was observed with doses of β -carotene of 50 mg/day every other day, but the rise in plasma β -carotene was significantly less than in the other studies. The increased risk for smokers on intakes some 10 times less than the ADI set by JECFA suggested that the animal models chosen for the toxicological work were inappropriate.

Subsequent studies have confirmed that the reason for this discrepancy is that the rat is able to rapidly metabolise β -carotene to retinol in the intestine, through the action of intestinal dioxygenase. In contrast humans absorb β -carotene systemically such that plasma levels of β -carotene increase to levels not found in the rodent. A more appropriate animal model is the ferret, which shows a similar metabolism to humans. High levels of plasma β -carotene in the ferret induce the cellular transcription factors c-fos and c-jun, and squamous metaplasia is seen in the lung with or without exposure to cigarette smoke (SCF, 2000). Even after the investment of all these resources it has not been possible for the EU Scientific Committee on Food to set an ADI.

The above example illustrates the inherent problems that can arise in the use of standardised protocols for assessing chemicals naturally occurring in the food chain. Had work on comparative metabolism and pharmacokinetics been undertaken before any animal bioassay work, it could have given more useful information. The extrapolation of effects obtained in high-dose animal studies to a large number of people exposed to a low dose is not the most effective use of resources. Nor are such experiments consistent with biological reality. There are few chemicals that would not cause illness or death if the daily intake was increased some 100–1000 fold as is the situation in many

animal bioassays. In addition, the costs associated with such tests severely limit their use to only those compounds where there is commercial pressure to undertake the work.

Ideally any process of risk assessment should be based on a prioritisation process which is able to analyse the chemicals to which we are exposed daily in our diets and which are ranked according to their likely intake and their potential to cause toxic or beneficial effects. However, this is not what is done since nearly all the emphasis is on undertaking detailed risk assessments of chemicals that are added to food and not those that are naturally present in it. The process results in an estimation of risk for a single chemical, but does not relate this in any way to the risks that may be derived from the chemicals that comprise the diet and to which consumers are likely to be exposed to a significantly greater extent. More serious still is the failure to relate the process to any measurable health effect for which there is epidemiological evidence in humans that the diet is a significant risk factor. The process is highlighting risks from those chemicals that are used in the production of food and sensitising the general public to what are generally perceived to be very low risks in relation to the known health risks from consuming poor diets. It is also helping to encourage the belief that all chemicals added to food have some degree of risk and no health benefits, whilst natural constituents of foods are mostly of benefit. This has partly fuelled the growth in the sale of organic foods. In practice, the situation is that there are risks and benefits associated with all chemical constituents of the food chain and the approach should be to determine this balance.

11.6 Improving risk assessment of phytochemicals

An inadequate intake in the diet of those food chemicals that are essential nutrients results in health risks. Indeed these risks are by far the most important in terms of the world's population where malnutrition is a major public health problem. But, unlike the 'toxic' chemicals, they would show a very different dose-response if they were subject to similar animal bioassays. At very low doses there would be a high risk of disease that would decrease as the dose was increased, the curve would then plateau until exposure was at such a level that toxicity could occur. Figure 11.2 shows this relationship which is U- or J-shaped rather than the essentially linear dose-response that is assumed for chemicals that are only toxic. The plateau region reflects what is commonly regarded as the homeostatic region where the cell is able to maintain its function and any excess nutrient is excreted, or mechanisms are induced that are completely reversible.

There is good evidence to suggest that certain chemicals, other than nutrients, show a J- or U-shaped dose-response relationship at levels of exposure which are lower than those where there is some impairment of the inherent

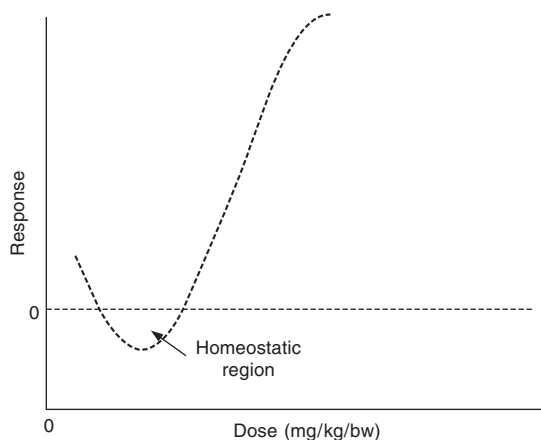


Fig. 11.2 A hypothetical dose – response curve for an essential nutrient.

protective responses (Calabrese *et al.*, 1999). These low levels of exposure correspond more closely to those which are consumed in practice.

For some toxins it is possible to demonstrate an apparent improvement in functional response at levels of exposure which are below a threshold. This effect, which has been termed ‘hormesis’, is most effectively demonstrated in the consistently improved longevity of animals whose caloric intake is restricted rather than allowing them to feed *ad lib* (Tannenbaum, 1942). Clearly in this instance, the observed effects are the result of exposure to a complex mixture of chemicals whose metabolism determines the total amount of energy available to the organism. But it is also possible to show similar effects when single chemicals such as alcohol (Maclure, 1993), or caffeic acid (Lutz *et al.*, 1997) are administered, as well as for more toxic chemicals such as arsenic (Pisciotta and Graziano, 1980) or even tetrachloro-p-dibenzodioxin (TCDD) (Huff *et al.*, 1994) when administered at very low doses. It is possible that there are ‘toxins’ that effect a modest, reversible disruption in homeostasis which results in an over-compensation, and that this is the mechanism of the beneficial effect observed. These effects would not be observed in the animal bioassays since to show them it would be necessary to have at least three dose groups below the NOAEL. In addition, the strain of animal used would have to have a very low incidence of disease to show any effect.

It is, therefore, perfectly feasible to consider that phytochemicals, at the levels present in foods, are capable of showing a similar dose–response. But, in assessing the benefit–risk associated with intake of a specific level in a food, it is important to establish the responses at doses that are below, or slightly above, the plateau region of the dose–response curve.

Given the problems associated with using standard animal bioassays and doses above the level where hormetic effects might be observed (which

might be termed the 'top-down' approach to hazard evaluation), an alternative approach might be to undertake studies at doses that better reflect likely human exposures. The stages in any such evaluation would need to be:

- 1 an assessment of human exposure;
- 2 information about the absorption, distribution, target organ and cellular concentrations of the chemical at similar dose levels;
- 3 information about the nature of the metabolites at the target sites;
- 4 *in vitro* and *in vivo* model systems that are sensitive to particular effects; and
- 5 detailed mapping of the genes and proteins induced as a consequence.

However, before any such studies could be usefully applied, it is necessary to know how beneficial effects are to be defined. In the case of risk evaluation, a large body of data has been assembled on the pathology of the laboratory rodent that enables the toxic effect to be fully characterised – in terms of functional effects that lead to toxicity and the end points of these effects – and thus makes it possible to determine whether or not there has been a statistically significant change in any of these parameters. No such body of evidence of changes in physiology that could be considered to lead to a beneficial effect has been assembled, other than a final outcome such as a reduction in the number of spontaneous tumours, or an increase in the average lifespan. Other beneficial effects might be an improvement in behavioural performance, a reduced incidence of birth defects, a reduction in somatic and reproductive mutation load, or a decreased incidence of disease. But in terms of characterising what biochemical changes might result in an eventual beneficial health outcome, there is almost no data and experimenters would have to undertake lifetime studies in every case.

11.7 Future trends

One of the most promising developments in recent years has been the potential that the application of genomics (DNA microarrays), proteomics and associated information technology can bring to assist in a systematic understanding of the functional consequences of exposure to a specific chemical. Microarrays are constructed by copying individual cDNAs of around 600–2400 base pairs, each representing all or part of the mRNA of an expressed gene, onto glass slides or nylon membranes at high density using high-speed robotics. Current technology allows for the simultaneous expression monitoring of around 10 000 genes.

Proteomics, the measurement of the global changes in proteins produced as a result of gene expression, bridges the gap between genome sequence and cellular behaviour and takes into account the post-translational modifications that often result in the functional effect. It has the potential to determine the role of protein–protein complexes in the complex signalling cascades that

exist within cells and which genomics cannot directly determine. However, proteomics is still in its infancy, and further technical developments will be necessary before the full potential of the technology is achieved.

Metabolomics studies the entire metabolism of an organism. It is possible to consider characterising the complex pattern of cellular proteins and metabolites that are excreted in urine. Pattern recognition techniques of nuclear magnetic resonance spectra have been applied to determine the dose–response using certain classical liver and kidney toxicants (Robertson *et al.*, 2000). This could well provide a signature of the functional state of the kidney, and perturbations in the pattern as a result of exposure to a chemical could be observed. But first it would be necessary to understand how compounds with known effects on the kidney affect these processes.

The ability to obtain a quantitative transcription profile of a cell should, ultimately, enable the mapping of changes with various low-dose levels of a chemical and the determination of a dose–response relationship without any assumptions being made about its potential to produce either benefits or harm. Not only should information about likely mechanisms behind an observed effect be obtained, but direct comparison should also be enabled between species, individuals, disease states and *in vitro* and *in vivo* methods. The development of early biomarkers of risk or benefit should be possible. The techniques allow for a more holistic approach, and should give better information on effects on individuals, enabling segmentation of consumers and better targeting of their dietary needs. However, major challenges remain to be resolved before they can be adopted for determining food chemical benefit–risk relationships. These include the need for sophisticated computational tools to handle the large amounts of data that are generated and the need for suitable models that take into account the complex interactions that occur in biological systems

The combination of these technologies is likely to provide useful biomarkers that will be better than those presently available. They will also provide the potential to address the major difficulty faced in classical toxicology, namely the ability to study the effect of either whole foods or mixtures of chemicals present in those foods. But they will still be limited by the difficulty of knowing how relevant the *in vitro* or *in vivo* systems chosen are in reflecting human responses and the effects of polymorphism. Gene expression profiles could be obtained on individuals exposed to specific chemicals, but it would be necessary to compare expression profiles in the same individual since there is likely to be wide intra-individual variations that could mask the exposure to a specific chemical, making it difficult to distinguish effects from the background noise. Studies in humans could be compared with similar studies in animals in which the correlations with specific end points had been made. But it is evident that huge amounts of data will need to be collected and it is to be hoped that there will be a major international effort to do this.

The exciting possibilities opened up with these technologies are well

Table 11.2 Some general classes of genes affected in terms of health risks or benefits

Ageing processes		Health-protective mechanisms (CR)	
Stress response genes		Stress response genes	
• Heat shock protein	↑	• Heat shock protein	↓
• Oxidative stress related		• DNA repair	
• DNA repair		• Detoxification enzymes	
• Lysosomal protease		• NF-kB signalling	
		Protein synthesis & metabolism genes	
		• Protein synthesis	↓
		* tRNA synthesis	
		* Elongation factors	
		• Protein turnover	↑
		• Proteosome pathway	
Energy metabolism genes	↓	Energy metabolism genes	↑
Inflammatory response genes		Immune modulation genes	
• Complement cascade	↑	• Interferon induction	↑
• MHC molecules		• Suppression of inflammatory peptides	
• Inflammatory peptides			
• Microglial activation factors			

CR = caloric restriction.

illustrated by recent work on the ageing animal that has been calorically-restricted (Lee *et al.*, 1999, Lee *et al.*, 2000, Cao *et al.*, 2001) and subject to radiation stress (Fornace *et al.*, 1999). In almost all of the living species examined caloric restriction is known to lead to health benefits in terms of increased longevity and the decrease in age-related diseases. These studies have shown the importance of up- or down-regulation of certain classes of genes in both the ageing and the oxidatively stressed animal. Some of the main classes of genes affected are shown in Table 11.2, and a comparison is drawn with animals that are calorically restricted.

To define the beneficial (or adverse) effects of dietary phytochemicals research is required which can study effects at doses that reasonably reflect likely human exposures and which will:

- determine the likely ranges of human exposures to specific phytochemicals;
- investigate suitable animal models to determine the relationships between blood and tissue levels of major classes of bioactive phytochemicals in humans;
- study how food processing or preparation influences the release and uptake of phytochemicals;
- study the metabolism and degradation in humans and appropriate animal models;
- identify tissue-specific patterns of gene expression;
- use knowledge from quantitative expression analysis and proteomics to

- identify sensitive biomarkers of responsiveness to phytochemicals using various doses and establish dose–response relationships;
- relate these data to biochemical end point and disease outcome.

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12

Investigating the health benefits of phytochemicals: the use of clinical trials

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12.1 Introduction

For any intervention intended to impact favorably upon human health, it is important to evaluate its safety and efficacy in order to demonstrate that it does not cause harm and it does provide the expected benefit. The ‘gold standard’ method for evaluating any intervention, whether it be a botanical product, dietary supplement, drug, medical device or medical procedure, is the randomized, clinical trial (RCT). A ‘clinical trial’ is a type of experiment conducted in human subjects where the effects of at least two interventions are compared. Often, the clinical trial takes the form of an active treatment compared to an inactive control or placebo.

‘Randomization’ refers to the process of assigning subjects by chance to treatments. This eliminates known and unknown sources of bias that could interfere with accurate interpretation of the study results. The main problem that randomization is intended to prevent is bias in subject selection. Without randomization, investigators might consciously or subconsciously select subjects to receive the active treatment, which, they believe, are most likely to respond. History shows that uncontrolled studies are much more likely to provide exaggerated support in favor of the effectiveness of a treatment than properly controlled trials (Pocock, 1983). Therefore, whenever possible, randomization should be used in order to help insure a fair and unbiased evaluation of the intervention under study.

In addition to randomization, blinding and placebo controls are safeguards often used to insure that the results obtained are not subject to bias or confounding. ‘Blinding’ refers to methods used to keep subjects and/or investigators unaware of which treatment a subject is receiving in a clinical

trial. A double-blind study would be conducted in such a way that both the subjects and investigators were unaware of treatment assignment throughout the study. Single-blind usually means that only the subjects are unaware of treatment assignment. Placebo controls are often the method through which blinding is accomplished. A placebo usually takes the form of a pill, capsule or tablet, that is indistinguishable from the active treatment and which allows the treatments to be administered in such a way that subjects and investigators are unaware of treatment assignment. This chapter is intended to provide both an overview of fundamental concepts in the design and conduct of RCTs and practical recommendations for application of these principles to the study of phytochemical products.

12.2 Types of clinical trials

Phytochemical products are occasionally marketed as drugs, but more often fall into the category of botanicals or dietary supplements. Regulation of these products varies from country to country, but they generally require fewer clinical trials and less extensive evidence than drug products before marketing is allowed. In the United States, the Federal Trade Commission requires that results from two well-controlled clinical trials be available to support health-related statements that are made in advertising (FTC, 1998). These trials are generally ‘proof-of-concept’ studies that are similar to Phase II trials in the drug development process which is described below.

In pharmaceutical and medical device development, clinical trials are classified into four main phases designated with Roman numerals: I, II, III and IV. The various phases of development trials differ in purpose, length and number of subjects involved. Phase I trials are conducted to determine safe dose levels of a medication, treatment or product (National Institutes of Health, 2002). The main purpose is often to determine an acceptable single dosage – how much can be given without causing serious side-effects. Phase I trials will also involve studies of metabolism and bioavailability (Pocock, 1983). The sample size of a Phase I clinical trial is usually small, ranging from 10–80 subjects (National Institutes of Health, 2002; Pocock, 1983).

Phase II trials are initial clinical studies to assess treatment efficacy. These may require sample sizes of 25–100 per group (National Institutes of Health, 2002; Pocock, 1983). Often, Phase II trials incorporate a range of doses in order to define the dose–response characteristics of the compound under study. Phase III trials are full-scale evaluations of a treatment to assess longer-term benefits (usually 6–12 months). A Phase III trial is the most rigorous and extensive type of scientific investigation of a new agent (Pocock, 1983). As a result, Phase III trials involve a large number of subjects, ranging from several hundred to several thousand, and may include a comparison with an already approved treatment, such as another drug in the same class. Phase IV trials are conducted after a product has been cleared for marketing

by regulatory authorities. These are typically done to evaluate hypotheses that were not tested during the Phase I–III development program, such as to evaluate response in subgroups or to demonstrate superiority against another product.

12.3 Hypothesis testing, endpoints and trial design

All clinical trials should have a pre-specified research question, which may be stated in the form of a primary hypothesis (or possibly a few primary hypotheses). An objective outcome measure or measures should also be clearly identified, such as the results of a biochemical test or the score on a validated scale. This allows statistical tests to be applied in order to assess the likelihood that any differences in response between treatment groups resulted from the active treatment and were not due to chance.

With investigations of phytochemicals and functional foods, the outcome measure is generally going to be a biomarker of disease, such as serum cholesterol level as a marker of heart disease risk, or indicators of bone turnover as markers of osteoporosis risk. Alternatively, markers of exposure may also indicate the benefit from a functional food by demonstrating bioavailability, such as increased serum levels of vitamins or carotenoids. Some components will be measurable in both ways. For instance, effects of a folic acid-fortified food could be measured via decrease in plasma homocysteine levels, or increase in red blood cell folate.

There are two main types of clinical trial design, parallel and cross-over. In a parallel study, subjects are assigned to one of two or more treatments, e.g. active and placebo, and proceed through the trial concurrently. In a cross-over design, subjects act as their own controls, undergoing two or more treatments in sequence (see Fig. 12.1).

Whenever practical, a parallel study design is preferred when evaluating efficacy. The reason for this is that the parallel design is less subject to sources of confounding that may complicate the interpretation of results from cross-over studies (described below). In fact, the US FDA will not accept data from cross-over studies as pivotal efficacy trials. The major disadvantage of the parallel design is that, compared with cross-over studies, more subjects are required in order to detect a given difference between treatments. This is the case because between-subject variability is almost always greater than the variability within subjects. However, since active and control treatments are administered concurrently to different subjects in a parallel study, the total time required to complete the trial is often less than is the case for a cross-over study.

Because they require fewer subjects and are usually less costly to complete, cross-over trials are commonly used to investigate the effects of phytochemical products. Cross-over designs may be used effectively for studying conditions that are relatively stable so that a similar baseline status can be established at

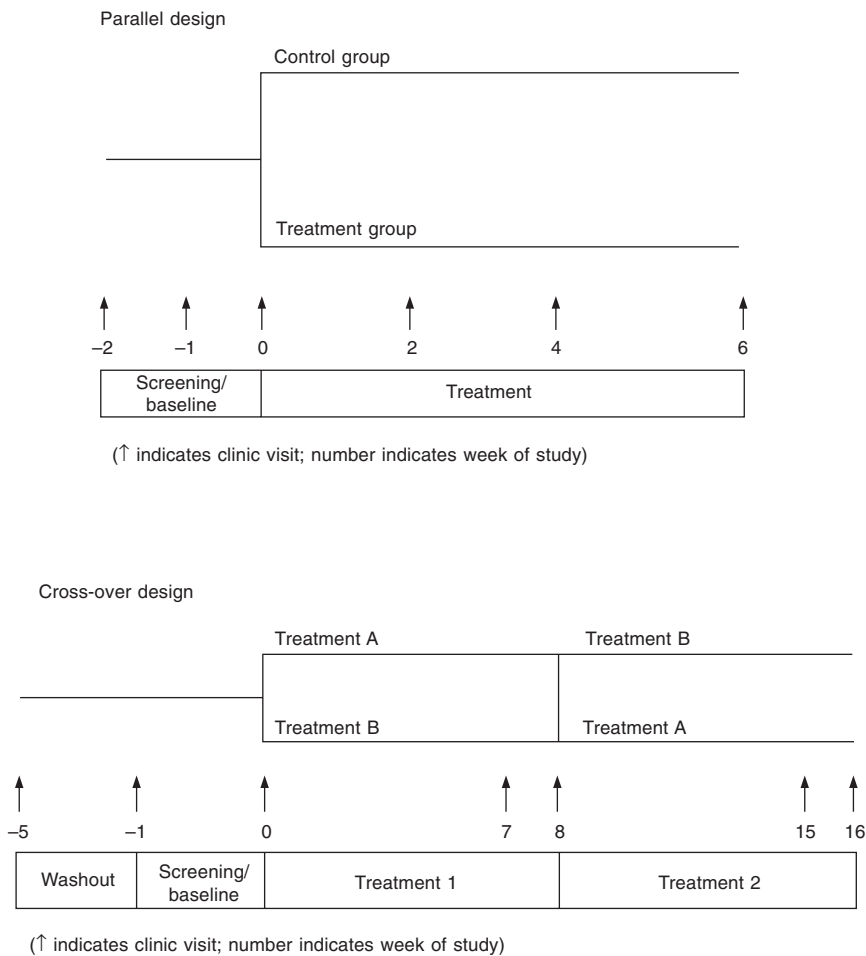


Fig. 12.1 Flow diagram examples.

the start of each treatment period (Spilker, 1991). Of significance in the cross-over design is the assumption that there will not be carryover (residual) effects after either treatment.

For example, if two treatments (a phytochemical product vs a placebo) are being evaluated to assess the influence of the active product on blood pressure, half of the subjects might be in the active-placebo sequence and the other half in the placebo-active sequence. If the active product lowered blood pressure and the effect continued after the treatment was stopped, then blood pressure might be lower during the placebo period for subjects in the active-placebo sequence than would be the case for those in the placebo-active sequence. This might be detected as a lower level of blood pressure before the start of the second treatment among subjects in the active-placebo treatment.

However, it is difficult to differentiate between true carryover effects and differences that occur due to chance.

The carryover effect is only one type of issue that may complicate the interpretation of results from a cross-over study. A more complete description of the potential pitfalls in the analysis of cross-over studies is beyond the scope of this chapter. The reader should simply be aware that, although cross-over studies may be less expensive to conduct than parallel trials initially, they also carry a higher risk of producing results that are difficult or impossible to interpret, thus requiring that the study be repeated to obtain clear results.

12.4 Assessing sample size

The determination of the proper sample size for a clinical trial is often as much art as science. Of course, selecting the number of participants has many practical and economic implications. Having too many subjects can increase study costs and the length of the trial unnecessarily, while having too few subjects can result in a non-significant result, even though the treatment may be effective. Additionally, ethical considerations dictate that only as few subjects as are necessary be exposed to a test product to determine efficacy (Edwards *et al.*, 1990). In general, larger samples are required to detect small differences between treatments and fewer subjects are required to detect large differences.

The appropriate number of patients to be recruited for a trial is dependent on several factors:

- number of treatment groups;
- expected comparison group response;
- anticipated benefit;
- significance level;
- statistical power, which is the risk the researcher is willing to accept that a difference that is truly present will be missed, producing a non-significant result (Campbell and Machin, 1999).

12.4.1 Number of treatment groups

Often there is a desire to compare responses to multiple treatments rather than simply evaluate active against a placebo control. For instance, it may be useful to evaluate several doses or to assess a product against another marketed product. Increasing the number of treatments will increase the sample size required overall, but will also increase the number of subjects required per treatment arm because the number of statistical comparisons is larger. If all between-group comparisons are to be made, the number of statistical tests increases dramatically as the number of treatment arms increases. With two groups, only one comparison is possible. With three groups, the number

increases to three, and with four groups, the number increases to six. Because the probability of showing statistical significance by chance increases as the number of comparisons goes up, it is necessary to increase the sample size per group in order to maintain the same statistical power (statistical power is discussed below).

12.4.2 Comparison group response

For some conditions, a large placebo effect can be anticipated. For example, studies of hormone replacement therapies for hot flashes in postmenopausal women consistently show a 50% decline from baseline in the number of daily hot flashes in the placebo group. Therefore, in order to show significance, an active treatment must produce an effect that is substantially larger than 50%. A marked placebo response is commonly observed with any condition that has a subjective component, such as chronic pain (e.g. arthritis), episodic pain (e.g. headaches), psychological states (e.g. anxiety), and certain physiologic measurements (e.g. blood pressure).

12.4.3 Anticipated benefit

The larger the benefit, the smaller the number of subjects required to show statistical significance. For example, if a herbal supplement is expected to produce a 5% lowering of the blood cholesterol level vs placebo, this will require many more subjects than a study evaluating the effects of a drug that is expected to produce a 50% reduction vs placebo.

12.4.4 Significance level

The significance level relates to the risk of designating a chance occurrence as statistically significant. Usually a 5% level is utilized for testing treatment effects. If a p-value of 0.04 is reported for a treatment effect, this means that there is only a 4% chance that the difference in response between the active and control treatments occurred due to chance. Keep in mind, however, that if many tests are run in a trial, it is entirely possible that one or two might be significant due to chance. As an extreme example, consider a study in which 100 statistical tests are run. We would expect five of those tests to show significance with a p-value of 0.05 or less due to chance. Therefore, it is essential to specify the main tests to be run in the protocol. Any tests that are conducted after the trial has been completed should be clearly labeled as *post hoc* exploratory analyses.

12.4.5 Statistical power

Statistical power is an expression of the risk that the researcher is willing to accept that the study will fail to find significance when an effect is truly present. Usually the number of subjects utilized will provide 80% or 90%

power. This means that if an effect of the specified size is truly present, the study will have an 80% or 90% probability of showing statistical significance. For a given significance level, statistical power can be increased by increasing the sample size (Chow and Liu, 1998).

One of the main determinants of the number of subjects required to reach the desired statistical power is the precision of the measurement tool utilized. More precise measurements will reduce the number of subjects required. As an example, if a study is being conducted to assess the influence of a dietary supplement on body fat, several measurement tools could be used to assess this outcome. These tools range from low levels of cost and precision (e.g. skinfold measurements) to moderate levels (e.g. bioelectrical impedance) to high levels of cost and precision (dual x-ray absorptiometry – DXA). A study that uses skinfold measurements to measure the outcome will require many more subjects than one which employs DXA. Therefore, it is often less expensive in total to utilize a more expensive measurement tool, because the more precise tool will allow the study to have sufficient power with a smaller number of subjects.

Statistical methods are often employed to determine the study sample size and optimize power. Outlining the methods for calculating sample size and power for clinical trials is beyond the scope of this chapter. Interested readers are referred to texts by Chow and Liu (1998), Hulley and Cummings (1988), and Shuster (1990) for specific information on sample size and power estimation methods.

12.5 Other issues in making trials effective

12.5.1 The clinical trial protocol

The clinical trial protocol is a detailed written plan that outlines how the study procedures are to be carried out and how the data are to be collected and analyzed. It insures the quality and integrity of the trial, particularly when multiple research sites are participating in data collection (Chow and Liu, 1998). The purposes of the protocol are to outline:

- 1 study question(s);
- 2 rationale for conducting the trial;
- 3 details of how the trial will be conducted;
- 4 subject description and eligibility criteria;
- 5 data to be collected;
- 6 a data analysis plan.

12.5.2 Inclusion and exclusion criteria

Inclusion and exclusion criteria are used to define eligibility parameters for study participation. These criteria are established to insure that the study

sample is reflective of the target population for the intervention, as well as to prevent participation by those who may have characteristics that could confound the interpretation of the study's results. For example, a trial examining the effect of a phytochemical on cancer chemoprotection might exclude subjects with a current or previous malignancy. Similarly, a trial testing the effect of soy isoflavones on menopausal symptoms would exclude women taking drugs or dietary supplements intended to reduce menopausal symptoms.

Examples of parameters used to define inclusion and exclusion criteria often include:

- demographic variables, such as age and sex;
- health-related variables, such as current medical conditions and body weight;
- illness-related variables, such as use of certain medications, laboratory results;
- social variables, such as smoking status and alcohol use.

While clearly outlined criteria are essential, a lengthy list of exclusions can also impede subject recruitment. Therefore, a balance must be struck between what is scientifically desirable and that which is still practical with regard to the number of study sites and length of time required to complete the trial.

12.5.3 Setting for conduct of the trial

Clinical trials can be conducted at a growing number of settings including a clinic, hospital, university, ambulatory care center, an independent research center, or contract research organization. The choice of setting often depends on the purpose and timeframe as well as the clinical trial methodology. Clinical trials that are assessing the effects of an intervention that has the potential for producing dangerous side-effects, such as changes in cardiac rhythm, may need to be conducted in a hospital setting. More commonly, the study will be conducted at an ambulatory center or outpatient clinic. The most appropriate setting for a specific clinical trial is determined by several factors, including the expertise of investigators, research experience of the site, study timeline and costs. Contract research organizations or independent research centers are often able to coordinate and manage studies more quickly than is the case for trials conducted in an academic setting, but usually have a higher cost.

The number of study sites to be used for a clinical trial depends on the characteristics and number of subjects that need to be recruited. Often, a sufficient number of participants cannot be enrolled from a single site, especially if the study inclusion criteria are restrictive and the timeframe for recruitment is limited. In order to complete the study within a reasonable period of time, an inclusion of multiple research centers is often necessary (Chow and Liu, 1998). The selection of study sites depends on several factors including:

- access to subjects who meet the eligibility criteria;

- experience and training of personnel at the site;
- availability of required equipment (e.g. radiographic or nuclear scanning capabilities); and
- location.

The use of multiple study sites can facilitate enrollment and ensure diversity in the study sample. However, as the number of study sites increases, so too does the cost and complexity of managing the trial. Therefore, the proper balance must be struck between issues of cost, logistics, scientific considerations and practicality when deciding on the appropriate number of research sites.

12.5.4 Study product packaging and blinding

Whenever possible, trials should be conducted in a double-blind fashion. The packaging and labeling of study products should be performed in a manner that protects the blinding. Ideally, the active and control products should be indistinguishable with regard to appearance, taste, smell and feel. Study product blinding of phytochemical products can be complicated, as these are often delivered in forms other than tablets and capsules, e.g. in a food or beverage. Other times, the product may have a distinctive feature, which makes blinding difficult. For one trial we conducted with a preparation containing garlic, a garlic odor was added to the desiccant in the bottles so that the placebo and active tablets would have a similar odor. In another case, we studied a botanical product in a blinded trial with a matching placebo. Much to our surprise, we found that the active product altered the color of the participants' urine, and subjects were sitting in the lobby of the clinic discussing urine color. Unfortunately, this led to some participants guessing the group to which they were assigned.

12.5.5 How much will it cost?

The cost of completing a clinical trial to answer a particular research question will depend on a number of factors including:

- the setting in which the research is conducted;
- the number of treatment conditions;
- the number of subjects needed;
- the availability of qualified participants; and
- the number and complexity of study visits and tests.

In general, it is less expensive to conduct a clinical trial in a university setting than with a professional research company. In part, this is because graduate students will often perform some portion of the work. However, the time required to complete the study will typically be longer. Whereas it may take just 2–3 weeks to get a trial started at a for-profit research center, investigations conducted in an academic setting will more often take 2–3

months to get underway. If multiple sites are required, the cost will be more than is the case for a single site trial with the same number of subjects because each site will have fixed start-up costs such as submission to an institutional (ethics) review board.

Obviously, the greater the number of subjects studied, the larger the cost. Nevertheless, having too few subjects may lead to inconclusive results, requiring that the study be repeated. Another important consideration is the availability of qualified participants. If the inclusion and exclusion criteria are very restrictive, the cost of recruiting subjects may exceed that of the actual testing. In pharmaceutical development trials, it is not unusual to see recruitment budgets of US \$500–\$1000 per randomized subject. Thus, for a Phase III development study with several hundred participants, often more than US \$500 000 in cost is allotted to efforts to identify qualified subjects who are interested in participation.

Some clinical trials can be completed with only a few visits. Others require more frequent contact with the study staff. As an example of the former, our clinic has conducted many studies intended to assess blood insulin and glucose responses to test products such as snack bars and beverages. These are usually conducted using a cross-over design and may require only three visits: one for screening, one for consumption of the control product, and one for consumption of the active product. In contrast, we have also completed several trials to assess dietary and pharmaceutical interventions intended to promote weight loss. These usually require frequent clinic visits over a period of at least 12 weeks and sometimes as long as two years.

The number and complexity of tests completed and factors controlled will also have a major impact on the cost of a clinical trial. Often, a trade-off is required between the degree of control and the number of subjects. For instance, when our clinic was studying the effects of a fat substitute on vitamin status, a high degree of control was needed to insure compliance. Accordingly, all food consumed by the subjects during the study period was provided and consumed under supervision. This was a very costly and logistically challenging investigation, which it would not have been practical to perform on a large scale for an extended period. Thus, although it might be scientifically ideal to provide all of the food consumed by subjects in a study of a botanical product intended to induce weight loss, doing so would be so expensive that it might severely limit the number of subjects that could be enrolled. A more practical approach would be to randomize a much larger number of subjects to compensate for the fact that some will be likely not to comply with the prescribed diet.

Similarly, the number and types of tests completed will influence the cost in ways that can be very complex. Recently, the author was involved in designing a proof-of-principle study intended to assess the ability of a dietary supplement to enhance weight loss among subjects instructed to follow a reduced energy diet. Sample size calculations were run for two scenarios, the first using change in body weight as the primary outcome variable, the

second using change in fat mass measured by DXA. Based on results from a previous trial, the response was expected, as well as the variability in the measurements. Body weight can be measured very inexpensively, whereas DXA scans are relatively expensive. However, because of the greater precision of the DXA measurements, using that approach required only half the number of subjects as would have been the case with body weight. As a result, the overall cost was much lower with the more expensive measurement tool.

12.5.6 Subject burden

Many scientists were trained in academic settings where graduate students were the primary source of study participants. Graduate students will put up with more inconvenience than most people recruited from the general community. While students may be willing to spend several hours filling out extensive questionnaires or undergoing complex metabolic tests on several occasions, most working people are not. Therefore, it is imperative to take into account the degree of subject burden to insure that it is appropriate for the population under study. Also, if a stipend is provided to participants, it should be adequate to compensate for the amount of inconvenience that will be necessary to complete all of the study procedures.

12.5.7 The need for expert guidance

Clinical trials are costly to conduct, and results are often critical to the commercial viability of a phytochemical product. Seemingly minor decisions, such as which measurement tool to use or a single entry criterion, can produce thousands of dollars in additional costs. Likewise, a great deal of time, effort and money can be saved by having experts review the study protocol to provide feedback regarding ways to improve efficiency, reduce subject burden and insure that the objectives are being met in the most scientifically sound and cost-effective manner possible. In particular, I recommend that an expert statistician is consulted regarding sample size and power and that the assumptions used in these calculations are reviewed carefully with one or more clinicians. It is not uncommon to see two studies with very similar objectives, which vary by two-fold in the number of subjects under study. Often this can be explained by differences in the assumptions employed in the sample size calculations.

12.6 Ethical issues

When conducting a clinical trial, the well-being of the study subjects is primary. Subjects must be treated fairly and with respect. The two primary methods of ensuring fair treatment of study subjects are review of the study protocol by an Institutional Review Board (IRB) or Ethics Committee and

obtaining informed consent. An IRB or Ethics Committee is an independent body that assesses the potential risks vs the potential benefits of a research study. IRB approval must be obtained prior to the start of a trial. Subjects also must be advised individually of the potential risks vs the potential benefits of a research study, as well as alternative treatments that are available (if any), the confidentiality of the information obtained from them during the study, and their right to withdraw from the study at any time. These issues are generally provided to subjects in the form of a written document, as well as explained to them verbally by study personnel. Once the subjects feel they understand the study issues and have had an opportunity to have any questions answered, they sign the informed consent document as an agreement to participate in the study. Ethical guidelines for the US are outlined by the Department of Health and Human Services.

12.7 Sources of further information and advice

Further information and advice related to the use of the clinical trial design can be found in a variety of sources including textbooks, manuscripts, organizations and Internet sites. In addition to the chapter reference list which cites helpful sources of information related to clinical trial protocol development, design and analysis, the following sources are also recommended.

Clinical trials listings

National Institutes of Health Clinical Trials Listing

<http://www.clinicaltrials.gov/>

National Cancer Institute Clinical Trials Listing

http://www.cancer.gov/clinical_trials

Center Watch Clinical Trials Listing Service

<http://www.centerwatch.com>

OncoLink – clinical trial listing of oncology trials, sponsored by the University of Pennsylvania

<http://www.oncolink.com/templates/treatment/matching.cfm>

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SPIPKER B, CRAMER J A (1992) *Patient recruitment in clinical trials*, New York, Raven Press.

Websites

Research Randomizer: instant random sampling and random assignment:

<http://www.randomizer.org>

Power Calculator: provides sample size programs for various models, including normal, exponential, binomial, and correlation models:

<http://home.stat.ucla.edu/calculators/powercalc/>

Power Analysis for ANOVA Designs: can be used to calculate sample size for one and two-way factorial designs with fixed effects:

<http://eval1.crc.uiuc.edu/fpower.html/>

Effect Size Calculator – online source for calculating effect size:

<http://web.uccs.edu/lbecker/Psy590/escalc3.htm>

Regulatory guidelines

U S Department of Health and Human Services. Food and Drug Administration. Center for Drug Evaluation and Research. Center for Biologics Evaluation and Research. Guidelines for industry: Good clinical practice: consolidated guidance. 1996. Rockville Maryland.

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<http://www.fda.gov/cder/guidance/4155fnl.pdf>

US Department of Health and Human Services. Food and Drug Administration. Code of Federal Regulations. Title 21 – Food and Drugs. Part 314.126 Adequate and well-controlled studies – Applications for FDA Approval to Market a New Drug or an Antibiotic Drug. 1998. Rockville Maryland.

http://www.access.gpo.gov/nara/cfr/waisidx_98/21cfr314_98.html

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13

The genetic enhancement of phytochemicals: the case of carotenoids

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13.1 Introduction

The carotenoids are the most widespread group of pigments in nature, with an estimated yield of 100 million tonnes per annum. They are present in all photosynthetic organisms and responsible for most of the yellow to red colours of fruits and flowers. The characteristic colours of many birds, insects and marine invertebrates are also due to the presence of carotenoids, which have originated in the diet. Animals are unable to synthesise carotenoids *de novo*, and so rely upon the diet as the source of these compounds. Carotenoids found in the human diet are primarily derived from crop plants, where the carotenoids are located in roots, leaves, shoots, seeds, fruit and flowers. To a lesser extent, carotenoids are also ingested from eggs, poultry and fish. Commercially, carotenoids are used as food colourants and in nutritional supplements (Table 13.1). Over recent years there has been considerable

Table 13.1 Examples of plant extracts used commercially

Plant	Carotenoids	Use
<i>Bixa orellana</i> seeds	Bixin, norbixin (annatto)	Food colouring
Carrot root	Carotenes, mainly β -carotene	Dietary supplement
<i>Capsicum annuum</i> fruit	Capsanthin, capsorubin (paprika)	Food colouring
<i>Crocus sativus</i> petals	Crocin, crocetin (saffron)	Food colouring
Marigold petals	Lutein, zeaxanthin	Dietary supplement
Tomato fruit	Lycopene	Dietary supplement
Palm oil	Carotenes	Dietary supplement, colouring

interest in dietary carotenoids with respect to their potential as antioxidants that alleviate chronic diseases. The large amount of research in this topic has been paralleled by the significant advances in cloning most of the genes involved in carotenogenesis and our increased understanding of factors that regulate carotenoid formation and deposition in plants. Taken together, these advances in knowledge have opened the way to enhancing carotenoid levels in crop plants. The aim of this chapter is to summarise our current understanding of carotenoid formation in plants, to explain the perceived benefits of carotenoids in the diet and to review the efforts that have been reported to increase carotenoids in crops.

13.2 Carotenoids in plants: structure

Carotenoids are members of the isoprenoid group of compounds and generally consist of eight isoprene units joined together so that the linking of units is reversed at the centre of the molecule to give methyl groups 20 and 20' with a 1,6 positional relationship, whereas the remaining methyl groups are 1,5 (Fig. 13.1). The most obvious feature of the carotenoid molecule is the polyene chain, which may extend from 3 to 15 conjugated double bonds. The length of the chromophore determines the absorption spectrum of the molecule and hence its colour to the human eye.

Although there are well over 500 different carotenoids in nature (Straub, 1987), all are based on seven different end groups of which only four (β , ϵ , κ , ψ) are found in higher plant carotenoids (Fig. 13.1). Cyclisation of the carbon skeleton occurs at one or both ends of the molecule, whilst xanthophylls

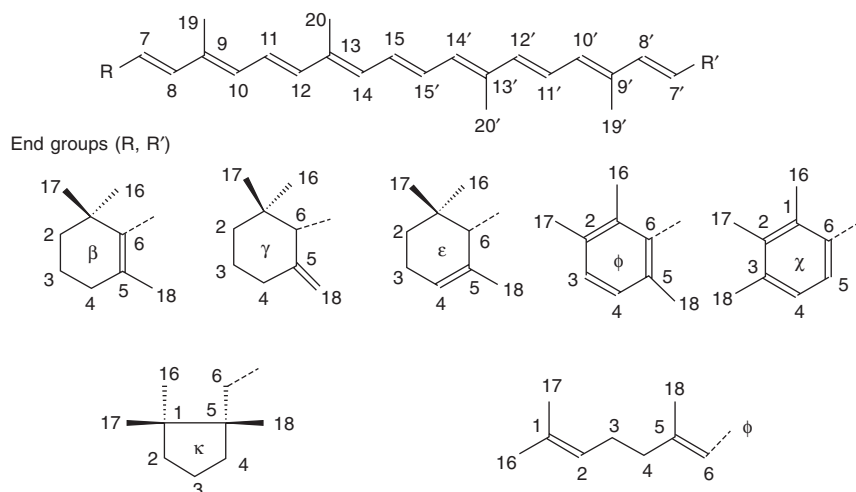


Fig. 13.1 General structures of carotenoids with numbering and end groups.

are formed from the hydrocarbon carotenes by the introduction of oxygen functions. In addition, modifications involving chain elongation or degradation can occur. Many of the commonly used trivial names of carotenoids are related to the original sources from which they were isolated, e.g. β -carotene from carrots, but there is a standardised nomenclature (Weedon and Moss, 1995). In this review, the trivial names will be used.

13.3 Carotenoids in plants: distribution

13.3.1 Photosynthetic tissues

Carotenoids are found in the chloroplasts of all green leaves, and all species so far examined contain β -carotene, lutein, violaxanthin and neoxanthin (Fig. 13.2). Minor carotenoids include α -carotene, β -cryptoxanthin and zeaxanthin. It is thought that this constancy reflects the common ancestry of higher plants. Although quantitative differences are found, in general the carotenes account for 25% and lutein 45% of the total carotenoids. All leaves of green vegetables contain qualitatively similar carotenoids, as part of the photosynthetic apparatus (Young, 1993), although there are significant quantitative differences between species (Table 13.2). Some unusual carotenoids are found in certain vegetables, e.g. lactucaxanthin in lettuce (Siefermann-Harms *et al.*, 1986). Those with the highest concentrations of carotenoids, such as parsley, spinach and watercress, tend to be rather minor components of the human diet. Lists of unusual carotenoids found in higher plants can be found in Goodwin and Britton (1988) and Britton (1991).

The carotenoids are located in photosynthetic pigment-protein complexes (PPCs) in the thylakoid membranes (Young, 1993), with minor amounts in the chloroplast envelope (Joyard *et al.*, 1991) and the envelope of amyloplasts (Fishwick and Wright, 1980). In all plastid envelope membranes, violaxanthin is the major carotenoid. Carotenes are also found in plastoglobuli (Lichtenthaler and Peveling, 1966).

13.3.2 Fruits

The distribution of carotenoids in fruits is extremely complex and subject to considerable variation. Patterns of them change during fruit ripening (e.g. the tomato). The number can vary from relatively few to over 50 as found in citrus fruits. Unripe fruits contain the same pigments as other photosynthetic tissues, but upon ripening the chloroplasts differentiate into chromoplasts and there is often, but not always, *de novo* synthesis of new carotenoids. Comprehensive tables of carotenoids in fruits can be found in Goodwin and Goad (1971), Goodwin (1980) and Gross (1991) and typical examples are shown in Table 13.3. Two plants of dietary importance that are particularly well characterised with respect to the impact of genotypes are the carrot and the tomato.

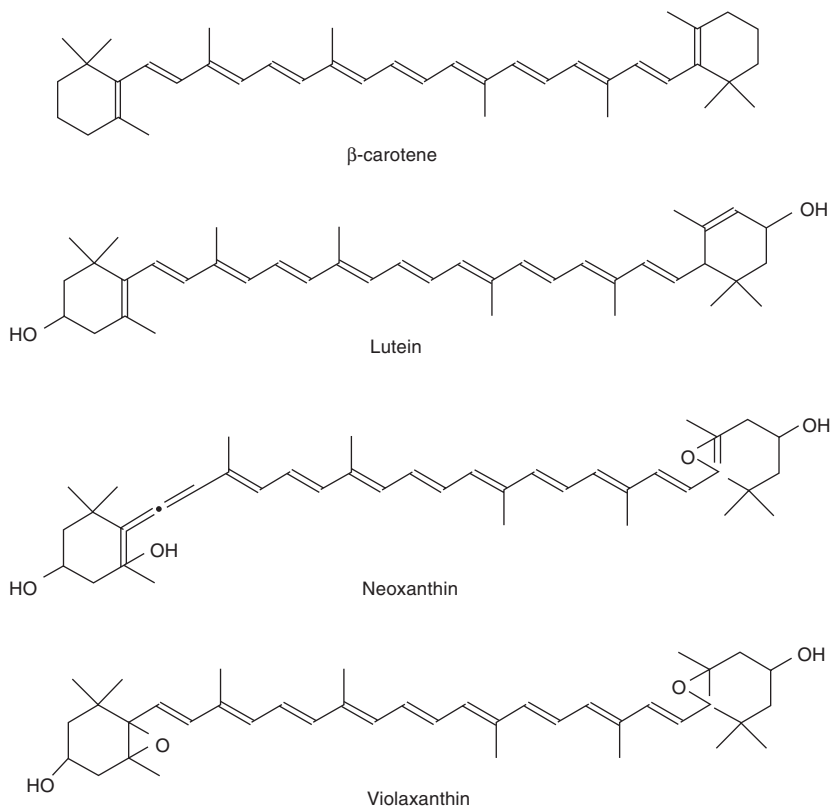


Fig. 13.2 Structures of β -carotene, lutein, violaxanthin and neoxanthin.

Table 13.2 Carotenoid content of raw leafy green vegetables

Species	Carotenoid ($\mu\text{g/g}$ fresh weight)			
	Total	Lutein	α -Carotene	β -Carotene
Brussels sprout	1163	610	—	553
Green bean	940	494	70	376
Broad bean	767	506	—	261
Broccoli	2533	1614	—	919
Green cabbage	139	80	—	59
Lettuce	201	110	—	91
Parsley	10335	5812	—	4523
Pea	2091	1633	—	458
Spinach	9890	5869	—	4021
Watercress	16632	10713	—	5919

From Scott and Hart (1994). Values include *cis* and *trans* isomers.

Table 13.3 Carotenoid content of fresh fruits, roots and seeds

Species	Carotenoid ($\mu\text{g/g}$ fresh weight)						
	Total	Lutein	Zea	β -cryp	Lyc	α -Car	β -Car
Apricot	2166	101	31	231	—	37	1766
Banana	126	33	4	Nd	—	50	39
Carrot (May)	11427	170	—	—	—	2660	8597
Carrot (Sept)	14693	283	—	—	—	3610	10800
Orange	211	64	50	83	—	Nd	14
Pepper	2784	503	1608	90	—	167	416
Peach	309	78	42	86	—	Tr	103
Sweet corn	1078	522	437	—	—	60	59
Tomato	3454	78	—	—	2937	—	439

From Scott and Hart (1994).

Nd = not detected; Tr = trace; β -cryp = β -cryptoxanthin; lyc = lycopene; α -car = α -carotene; β -car = β -carotene; zeax = zeaxanthin.

Carrot

The carrot derives its name from the large amount of β -carotene in its roots. Analyses of the carotenoids of carrot have been conducted since the 1930s. Generally, β -carotene accounts for at least 50% of the total carotenoids, with α -carotene as the second major compound. The ratio of β : α is variable, and depends upon the variety. Selective plant breeding over 60 years has favoured the dark orange carrot varieties, although some red, Japanese varieties contain lycopene. Carrot was the first crop to be genetically modified for increased carotenoid levels, resulting in an increase in β -carotene (see Section 13.7).

Tomato

The tomato has probably been the most thoroughly investigated fruit with respect to its carotenoid content, varietal differences and genetic mapping of the mutant genes, with the first studies reported in 1950 (Porter and Lincoln, 1950). The levels of lycopene, the principal carotenoid of ripe tomato fruit, vary considerably between varieties and ripening conditions, with a typical value of about 2.9 mg/g fresh weight (Table 13.3). Minor amounts of other carotenoids are also present, especially β -carotene. Besides the common red tomato, there are a number of strains and mutants of various colours. These range from those having no carotenoids (e.g. the *r*, *r* mutant, with a yellow skin due to flavonoids) to those that are deep orange, due to high levels of β -carotene, e.g. the high β mutant (Ronen *et al.*, 2000; Table 13.4). These mutants have been of considerable importance in the cloning of carotenoid genes (see Section 13.5).

13.4 The functional benefits of carotenoids

The prime reason for elevating or altering carotenoids in crop plants is linked

Table 13.4 Tomato mutants with altered carotenoid levels

Mutant	Phenotype	Mutation	Chromosome
r (yellow fruit)	No carotenoids	Phytoene synthase-1	3–29
hp (high pigment)	Increased carotenoids	?	12
t (tangerine)	Increased poly <i>cis</i> -lycopene	?	10–24
og ^c (old gold crimson)	Increased lycopene, lower β -carotene	B cyclase	6–106
B (high beta)	Increased β -carotene, very low lycopene	B cyclase	?
del (delta)	Increased δ -carotene, reduced lycopene	?	?
at (apricot)	Apricot fruit colour	?	5
sh (sherry)	Yellow flesh fruit	?	10L

to their potential benefit to human health. The best documented function of carotenoids in humans is their provitamin A activity, which is restricted to β -carotene and other carotenoids with β -end groups, e.g. β -cryptoxanthin and zeaxanthin. Evidence has been documented to suggest that humans with low carotenoid levels are more susceptible to degenerative diseases (Mayne, 1996). Much of this evidence for the alleviation of chronic diseases such as coronary heart disease and certain cancers has come from epidemiological studies (e.g. Giovannucci *et al.*, 1995; Gann *et al.*, 1999; Ascherio *et al.*, 1999). Lycopene has been particularly implicated in the reduction of prostate cancer, although the evidence indicates that a high tomato diet, rather than lycopene alone, is beneficial (Giovannucci, 1999; Giovannucci *et al.*, 2002; Bramley 2000). The benefits of carotenoids in the diet are thought to be primarily due to their powerful antioxidant capacity, which has been demonstrated in a wealth of *in vitro* studies (Miller *et al.*, 1996; Woodall *et al.*, 1997; Farombi and Britton, 1999; Rehman *et al.*, 1999, Mortensen *et al.*, 2001). However, whether this is their sole role *in vivo* remains uncertain (Rice-Evans *et al.*, 1997; Collins, 2001). They have also been reported to affect gap junction communication by up-regulation of connexin 43 (Zhang *et al.*, 1991) and to induce cell cycle arrest and apoptosis in human adenocarcinoma cell lines (Palozza *et al.*, 2002). Epidemiological studies have also shown an inverse association between antioxidant/carotenoid intake or blood level and the risk of cataract and age-related macular degeneration (AMD). Cataract risk was reported to decrease with higher intakes of lutein and zeaxanthin, but not other carotenoids (Brown *et al.*, 1999). Lutein and zeaxanthin are the principal components of the macula pigments, and their contents can be modified by dietary changes, thus reducing the amount of blue light reaching those eye tissues vulnerable to AMD by 30–40% (Landrum and Bone, 2001).

Based upon such reports, there is an increasing view that functional foods (nutraceuticals), in which carotenoid levels are high or modified qualitatively, would be beneficial to human health, especially with respect to degenerative

diseases in an ageing population or as dietary sources of provitamin A in populations with vitamin A deficiency. One strategy for increasing the levels in plants is by genetic engineering of the pathway in crops (Hirschberg, 1999; Grusak *et al.*, 1999).

13.5 Carotenoid biosynthesis and encoding genes

Carotenoid biosynthetic pathways in bacteria, fungi, algae and plants have been mainly elucidated from biochemical analyses using labelled precursors, specific inhibitors and characterisation of mutants, especially the tomato. The use of recombinant DNA technology has facilitated studies of the pathways through characterisation of genes that encode the biosynthetic enzymes. This approach has facilitated an insight into the structure and function of carotenogenic enzymes and elucidated some of the mechanisms that regulate carotenoid biosynthesis and deposition.

The vast majority of genes encoding enzymes in the biosynthesis of carotenoids from photosynthetic bacteria, fungi, cyanobacteria, green algae and higher plants have been cloned (reviewed by Armstrong, 1994; Bartley and Scolnik, 1995; Armstrong and Hearst, 1996; Harker and Hirschberg, 1998; Cunningham and Gantt, 1998; Hirschberg, 1998, 2001). Unfortunately, the nomenclature of the genes or cDNAs that have been cloned has yet to be standardised. The first genes, from the bacterium *Rhodobacter capsulatus*, were designated by the prefix *crt*, with the corresponding protein CRT (Marrs, 1981). However, subsequent workers have also used other prefixes that are acronyms of the enzymic step (e.g. *Psy* for phytoene synthase, *Pds* for phytoene desaturase). Both types of nomenclature are currently in use and this can lead to some confusion. Wherever possible, both types of nomenclature have been included in this article.

Although carotenogenesis in plants takes place in plastids, all of the carotenoid biosynthesis genes are nuclear encoded and their polypeptide products are imported into the plastids. Therefore, they contain a N-terminal transit peptide sequence. For example, the size of the transit peptide of PSY from ripe tomato fruit is approximately 9 kDa, corresponding to about 80 amino acid residues (Misawa *et al.*, 1994).

13.5.1 Biosynthesis of phytoene

Since carotenoids are isoprenoids, they share a common early pathway with other biologically important isoprenoids such as sterols, gibberellins, phytol and the terpenoid quinones (Fig. 13.3). In all cases, these compounds are derived from the C₅ isoprenoid, isopentenyl diphosphate (IPP). Until a few years ago it was believed that a single pathway from the C₆ precursor mevalonic acid (MVA) formed IPP, which itself was synthesised from hydroxymethylglutaryl coenzyme A (HMG CoA) by the action of HMG

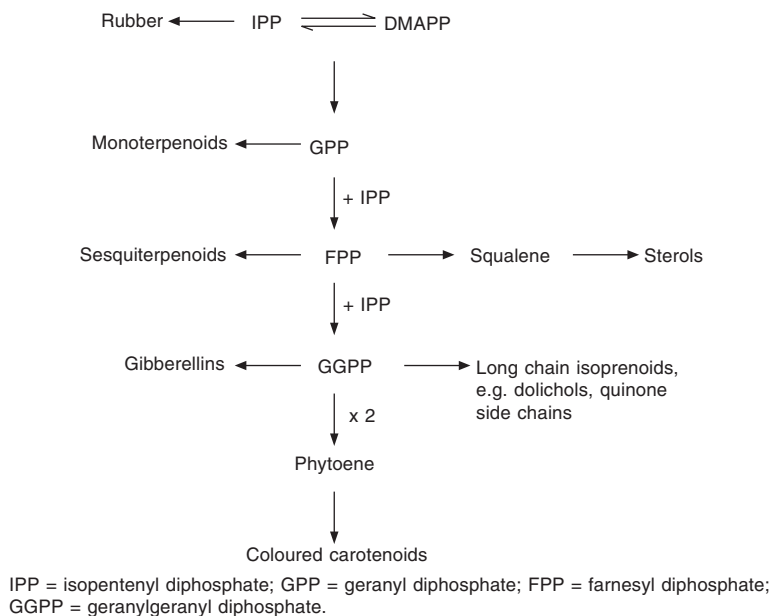
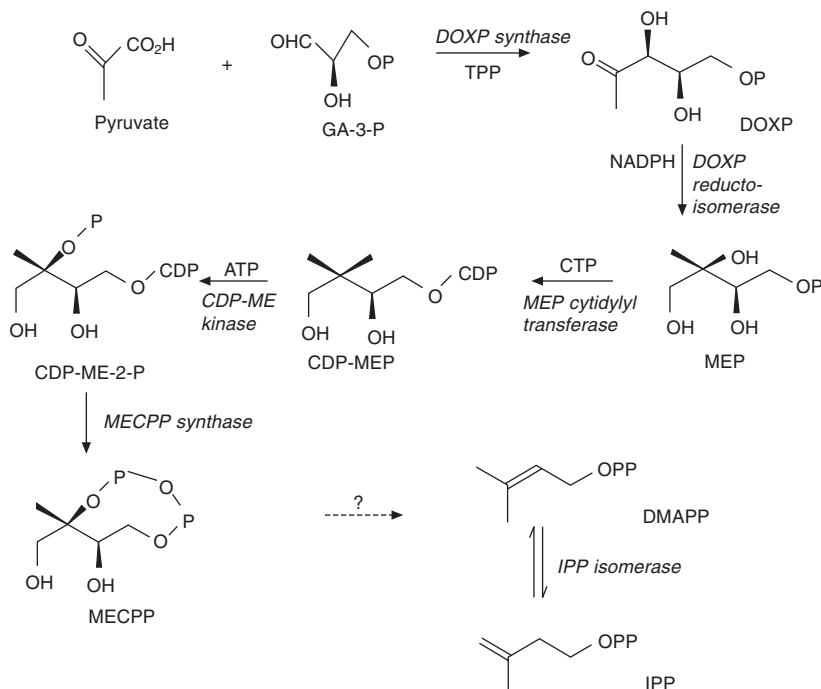


Fig. 13.3 Biosynthetic relationships between the isoprenoids.

CoA reductase. However, it has now been shown that an alternative pathway exists from glyceraldehyde 3-phosphate and pyruvate. This is known as the non-MVA or deoxyxylulose phosphate (DOXP) pathway and is found in higher plants and algae. The complete set of reactions still remains to be elucidated (Fig. 13.4). It has now been established that the DOXP pathway is responsible for producing IPP that is then converted into carotenoids within the plastid, whereas the MVA pathway to IPP results in the synthesis of other isoprenoids (reviewed by Lichtenthaler, 1999). The discovery is significant for the genetic manipulation of the early reactions of carotenogenesis. It has been shown, for example, that expression of *dxps* genes in lycopene-producing *E. coli* significantly increases carotenoid and ubiquinone formation (Harker and Bramley, 1999).

Once IPP is formed, it is isomerised to dimethylallyl diphosphate (DMAPP). This soluble enzyme is rate-limiting in yeast, and may be so in higher plants (Kajiwarra *et al.*, 1997). Sequential addition of three IPP molecules yields the C₂₀ isoprenoid geranylgeranyl diphosphate (GGPP), the reactions being catalysed by GGPP synthase (Fig. 13.3). At least five isoforms of GGPP synthase are present in *Arabidopsis*, but their individual roles are unknown. It is possible that individual isoenzymes catalyse the metabolism of GGPP to different products, e.g. carotenoids, phytol, terpenoid quinones. GGPP synthases from higher plants exhibit a significant sequence similarity to one another (Kuntz *et al.*, 1992; Aitken *et al.*, 1995), but individual GGPP synthases exhibit different affinities with respect to the chain length of the allylic substrate (Math *et al.*, 1992).



GA-3-P = glyceraldehyde 3-phosphate; TPP = thiamine pyrophosphate; DOXP = 1-deoxy-D-xylulose; CTP = cytosine triphosphate; ATP = adenosine triphosphate; CDP-ME-2-P = 4-diphosphocytidyl-2-methyl-D-erythritol 2-phosphate; CDP-MEP = 4-diphosphocytidyl 2-C-methyl-D-erythritol 4-phosphate; MEP = 2-C-methyl-D-erythritol 4-phosphate; MECPP = 2-C-methyl-D-erythritol 2,4-cyclodiphosphate; DMAPP = dimethylallyl diphosphate.

Fig. 13.4 The 1-deoxyxylulose 5-phosphate (DOXP) pathway for IPP formation.

The first committed step in the isoprenoid pathway to the carotenoids is the head-to-head condensation of two GGPP molecules to produce phytoene (Fig. 13.5) and catalysed by phytoene synthase (designated as PSY or CRTB). At least two isoforms exist in tomato (PSY-1 and 2), of which *Psy-1* is upregulated in ripening fruit, whilst *Psy-2* appears to predominate in green tissues (Fraser *et al.*, 1999). The enzyme is loosely associated with plastid membranes and forms a functional, soluble complex with GGPP synthase in tomato (Fraser *et al.*, 2000). Comparison of the amino acid sequence of phytoene synthases from various organisms indicates that these enzymes are both structurally and functionally conserved in eukaryotes and prokaryotes (reviewed by Armstrong, 1994).

13.5.2 Desaturation and isomerisations reactions

The desaturation of 15-*cis* phytoene into lycopene occurs in four stepwise dehydrogenations, yielding phytofluene, ζ -carotene, neurosporene and lycopene

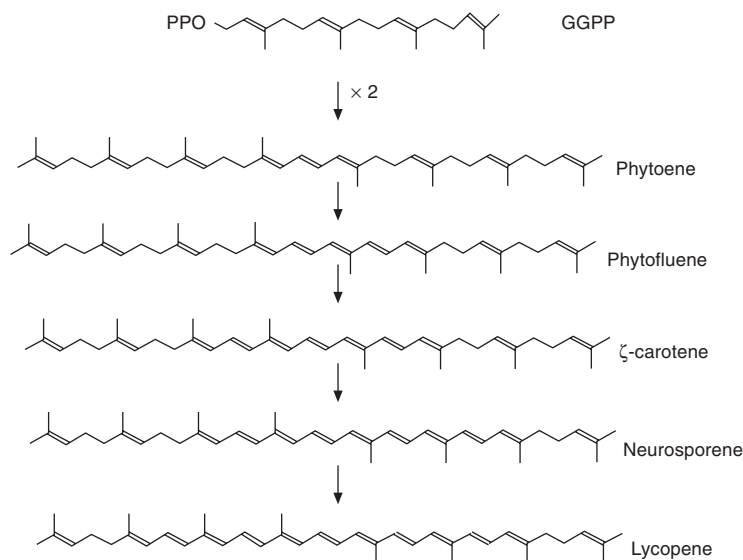


Fig. 13.5 Formation of phytoene and desaturation reactions.

(Fig. 13.5). The introduction of double bonds extends the chromophore to 11 conjugated double bonds in lycopene, giving the molecule its characteristic red colour. The four dehydrogenations are catalysed by a single enzyme in bacteria (named CRTI or AL-1), but two are required in higher plants; phytoene desaturase (PDS, CRTP), forming ζ-carotene from phytoene (Pecker *et al.*, 1992), and ζ-carotene desaturase (ZDS, CRTQ), which catalyses the remaining two reactions to lycopene (Linden *et al.*, 1994; Albrecht *et al.*, 1995). They are membrane bound, possibly in both thylakoids and envelope of plastids (reviewed by Bramley, 1993). A dinucleotide-binding motif has been found in all phytoene and ζ-carotene desaturases. Comparison of PDS/CRTP-type to CRTI-type enzymes reveals less than 22% sequence conservation, most of which is in the FAD/NAD(P) binding motif found in the amino termini of the two types of enzymes. Both desaturases require quinones as redox cofactors (Mayer *et al.*, 1992; Schulz *et al.*, 1993) and a NAD(P)H-dependent respiratory redox pathway operates in chromoplasts for both carotene desaturation and to provide chemiosmotic energy (Morstadt *et al.*, 2002).

Isomerisation of 15-*cis*-phytoene to the all-*trans* configuration must occur during the desaturation steps, since most desaturated carotenoids are in the all-*trans* form. The CRTI type desaturases appear to be able to carry out this isomerisation themselves (Fraser *et al.*, 1992; Bartley *et al.*, 1999), but mutants of PDS/ZDS-type organisms accumulate *cis* isomers of unsaturated carotenoids, suggesting the presence of a separate isomerase (Clough and Pattenden, 1983; Ernst and Sandmann, 1988). Three recent publications have reported the cloning of a carotene isomerase (*CrtISO*) from tomato (Isaacson *et al.*, 2002), *Arabidopsis* (Park *et al.*, 2002) and *Synechocystis* 6803 (Breitenbach

et al., 2001). In each case, the isomerase, which is a redox enzyme structurally related to CRTI, facilitates the formation of all-*trans* lycopene and its further cyclisation to β -carotene.

13.5.3 Cyclisation

The cyclisation reactions of carotenoids represent an important branching point in the biosynthetic pathway, with one route leading to β -carotene, zeaxanthin and violaxanthin, while the other route leads to α -carotene (ϵ , β -carotene) and lutein (Fig. 13.6). Cyclisation of lycopene is initiated by proton attack at C-2 and C-2'. The gene *crtY*, which encodes lycopene β -cyclase, has been cloned from *Erwinia* (Misawa *et al.*, 1990), whilst the cDNA of the higher plant equivalent, *crtL-b*, has been cloned from several plants including tomato (Pecker *et al.*, 1996) and *Arabidopsis* (Scolnik and Bartley, 1995). In both prokaryotes and eukaryotes this enzyme catalyses two cyclisation reactions that convert lycopene into β -carotene. The gene for lycopene ϵ -cyclase (*crtL-e*) has been cloned from *Arabidopsis* (Scolnik and Bartley, 1995) and tomato.

13.5.4 Xanthophyll formation

The oxidation of carotenes results in the formation of a diverse array of xanthophylls (Fig. 13.7). Zeaxanthin is synthesised from β -carotene by the hydroxylation of C-3 and C-3' of the β -rings *via* the mono-hydroxylated intermediate β -cryptoxanthin, a process requiring molecular oxygen in a mixed-function oxidase reaction. The gene encoding β -carotene hydroxylase (*crtZ*) has been cloned from a number of non-photosynthetic prokaryotes (reviewed by Armstrong, 1994) and from *Arabidopsis* (Sun *et al.*, 1996). Zeaxanthin is converted to violaxanthin by zeaxanthin epoxidase which epoxidises both β -rings of zeaxanthin at the 5,6 positions (Fig. 13.7). The

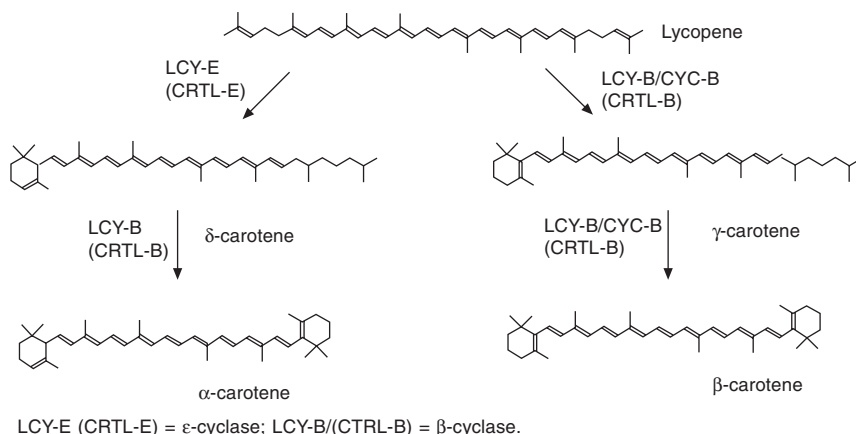
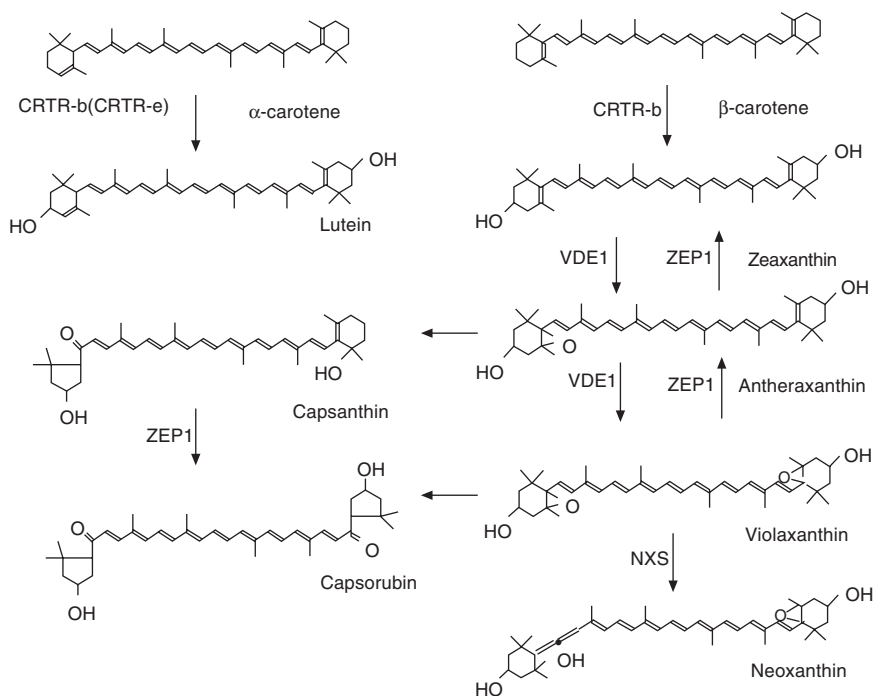


Fig. 13.6 Cyclisation reactions.



CRTR-e = ϵ -ring hydroxylase; CRTR-b = β -ring hydroxylase; ZEP-1 = zeaxanthin epoxidase; NXS = neoxanthin synthase; VDE 1 = violaxanthin de-epoxidase.

Fig. 13.7 Formation of xanthophylls.

zeaxanthin epoxidase gene of *Nicotiana plumbaginifolia* has been cloned (Marin *et al.*, 1996), as has the homologous gene from pepper (Bouvier *et al.*, 1996). The violaxanthin de-epoxidase (*Vde*) cDNA has been cloned from lettuce (Bugos and Yamamoto, 1996) and the properties of this enzyme reviewed by Rockholm and Yamamoto (1996). Neoxanthin synthase has been cloned from tomato and found to be closely similar to lycopene cyclase and capsanthin-capsorubin synthase (Bouvier *et al.*, 2000). The products of violaxanthin/neoxanthin cleavage are the substrates for abscisic acid synthesis, and the gene for the cleaving enzyme has been cloned (Schwartz *et al.*, 1997), as has a novel carotenoid cleavage dioxygenase (Schwartz *et al.*, 2001).

Ketocarotenoids, e.g. echinenone, canthaxanthin, adonirubin and astaxanthin, are oxygenated derivatives of β -carotene (Fig. 13.7). The cDNAs encoding β -carotene-4-oxygenase have been cloned from *Agrobacterium auranticum* and *Alcaligenes PC-1* (Misawa *et al.*, 1995) and the green alga *Haematococcus pluvialis* (*CrtO*, Bkt; Lotan and Hirschberg, 1995; Kajiwarra *et al.*, 1995), which accumulates astaxanthin under stress conditions. The enzyme catalyses the introduction of keto groups to C4 and C4' of β -carotene to form canthaxanthin via the mono keto intermediate echinenone (Fig. 13.7).

13.5.5 Regulation of carotenoid biosynthesis

Since carotenoids are derived for the central isoprenoid pathway (Fig. 13.3), the regulation of their formation must involve a co-ordinated flux of isoprenoid units into this branch of the pathway as well as into others such as the biosynthesis of sterols, gibberellins, phytol and terpenoid quinones. An understanding of the complexities of regulation of the pathway is necessary in order to target the regulatory steps for genetic manipulation.

The discovery of gene families for several isoprenoid and carotenoid steps implies that there may be unique roles for each member of a family. This has been well documented for the multiple forms of HMG CoA reductase (reviewed by Chappell, 1995), but our understanding of the roles of isoenzymes for later steps, e.g. GGPP synthase, is poor. Although carotenoids are produced exclusively in plastids, it is likely that exchanges of cytoplasmic and plastidic metabolites, especially prenyl diphosphates, occur and that these exchanges vary depending upon the type and developmental stage of the tissue (reviewed by McCaskill and Croteau, 1998). The concept of metabolic channelling, with isoenzymes dedicated to the formation of specific classes of isoprenoids that may be regulated independently from one another, has been suggested by Chappell (1995). Furthermore, it has been shown, from work with transgenic tomatoes that perturbations of the carotenoid pathway using either up- or down-regulation of *Psy-1* lead to changes in the levels of gibberellins. Increases in phytoene formation cause a reduction of gibberellin levels and dwarfing of the progeny (Fray *et al.*, 1995). Such cross-talk between classes of isoprenoids suggests interdependence between isoprenoid metabolic channels.

Carotenoid formation itself in plants is a highly regulated process. For example, the composition of leaf xanthophylls is affected by light intensity (Demmig-Adams *et al.*, 1996), and the accumulation of specific carotenoids in chromoplasts of fruits and flowers is developmentally regulated (reviewed by Bramley, 1993). The signalling pathways mediating these transformations are unknown, but reactive oxygen species act as second messengers mediating carotenoid formation during chromoplast differentiation (Bouvier *et al.*, 1998). Although carotenoid biosynthesis is necessary for the development of chloroplasts, expression of carotenoid genes is neither light-dependent nor controlled by cryptochrome 1 genes (Ninu *et al.*, 1999). During de-etiolation of tomato seedlings carotenoid levels increase several-fold, but the transcript levels of *Pds* and *Psy-1* remain relatively unchanged (Giuliano *et al.*, 1993). This indicates that transcriptional control of these two genes does not play a major role in the regulation of carotenoid biosynthesis during greening and chloroplast development. All of these factors suggest that manipulation of the pathway to change the levels of carotenoids will be difficult.

Carotenoid accumulation during fruit ripening in tomato has been studied extensively and is a good model system to elucidate the regulation of the process. During ripening the concentration of carotenoids increases between 10 and 15-fold due mainly to a 500-fold increase in the concentration of lycopene (Fraser *et al.*, 1994; Table 13.5). Accumulation of lycopene begins

at the breaker stage of fruit ripening after the fruit has reached the mature green stage. The mRNA levels of *Psy* and *Pds* increase significantly at this stage (Pecker *et al.*, 1992; Fraser *et al.*, 1994; Giuliano *et al.*, 1993) due to transcriptional control that is developmentally regulated (Corona *et al.*, 1996). In contrast, the mRNA of lycopene β - and ϵ -cyclase genes decreases, probably accounting for the reduction in cyclic carotenoids in ripe fruit (Pecker *et al.*, 1996; Ronen *et al.*, 1999). Evidence for transcriptional regulation of carotenoid genes has also been found in flower development where the steady-state levels of mRNA for *Psy*, *Pds* and *crtL-b* increase dramatically in the petals of tomato flowers as they mature (Giuliano *et al.*, 1993). Similarly, differences in mRNA transcription or stability underlie the differences in carotenoid formation in marigold flowers (Moebs *et al.*, 2001).

Clearly, the control of gene expression at the transcriptional level is a key regulatory mechanism controlling carotenogenesis *in vivo*. However, post-transcriptional regulation of carotenoid biosynthesis enzymes has been found in chromoplasts of the daffodil. The enzymes phytoene synthase (PSY) and phytoene desaturase (PDS) are inactive in the soluble fraction of the plastid, but are active when membrane-bound (Al-Babili *et al.*, 1996; Schledz *et al.*, 1996). The presence of inactive proteins indicates that a post-translational regulation mechanism is present and is linked to the redox state of the membrane-bound electron acceptors. In addition, substrate specificity of the β - and ϵ -lycopene cyclases may control the proportions of the β , β and β , ϵ carotenoids in plants (Cunningham *et al.*, 1996).

The carotenoid pathway may also be regulated by feedback inhibition from the end products. Inhibition of lycopene cyclisation in leaves of tomato causes increase in the expression of *Pds* and *Psy-1* (Giuliano *et al.*, 1993; Corona *et al.*, 1996). This hypothesis is supported by other studies using carotenoid biosynthesis inhibitors where treated photosynthetic tissues accumulated higher concentrations of carotenoids than untreated tissues (reviewed by Bramley, 1993). The mechanism of this regulation is unknown. A contrary view, however, comes from studies on the phytoene-accumulating *immutans* mutant of *Arabidopsis*, where there is no feedback inhibition of phytoene desaturase gene expression (Wetzel and Rodermel, 1998).

13.6 Strategies and methods for transformation to enhance carotenoids

Broadly speaking, there are two strategies for enhancing carotenoids in plants: conventional plant breeding and genetic engineering (often called metabolic engineering or genetic manipulation). Although both have resulted in increased levels of carotenoids in crop plants, the procedures are quite different from each other.

Table 13.5 Carotene changes during Ailsa Craig tomato fruit ripening

Fruit	Carotenes				Xanthophylls			Total ^a
	P	PF	ζ-C	L	γ-C	β-C	Lut	
Mature green	0	0	0	0.1	0	2.1	4.7	10.1
Breaker	1.9	0	0	3.7	0.4	5.6	1.5	15.9
24 d.p.b.	40.6	22.2	7.5	70.5	11.3	36.8	6.4	207

From Fraser *et al.*, 1994.
P = phytoene; PF = phytofluene; ζ-C = ζ-carotene; L = lycopene; γ-C = γ-carotene; β-C = β-carotene; Lut = lutein; Viola = violaxanthin; Neo = neoxanthin; d.p.b. = days post breaker.
All values are µg/g fresh weight. ^a = includes all minor carotenoids.

13.6.1 Conventional plant breeding

Modern agriculture has used conventional plant breeding to increase successfully productivity and yields of crop plants (the so-called 'Green Revolution'). However, this broad objective has tended to obscure the importance of the micronutrients in crops, including the carotenoids. In cases where carotenoid levels have been analysed, significant genotypic variation has been observed, e.g. the tomato (Table 13.5). Such variation can be used to develop cultivars with enhanced carotenoid levels and to assist in identifying the genetic and biochemical basis for such variations.

13.6.2 Genetic engineering

The ready availability of most genes encoding carotenoid biosynthetic enzymes (Section 13.5), the elucidation of the pathway in plants and the ability to transfer genes to plants by *Agrobacterium*-based protocols provides the basis for the genetic modification of crops with respect to carotenoid content. Such modifications are now well established in microorganisms (reviewed by Cunningham and Gantt, 1998; Misawa and Shimada, 1998; Schmidt-Dannert, 2000). However, the complexity and plasticity of higher plant metabolism, combined with the branched nature of the biosynthetic pathway, has meant that genetic modifications have proved to be more difficult than in microorganisms. The current range of crops transformed with carotenoid genes is shown in Table 13.6.

One key difference between conventional breeding and genetic engineering involves the ability to transform additional gene(s) into the plant from unrelated species, including microorganisms. Similarly, the gene promoter can also be from an unrelated organism. In order to achieve changes in the carotenoid pathway there are four prerequisites:

- 1 a detailed knowledge of the pathway (see Section 13.5);
- 2 the availability of cloned genes encoding the enzymes of the pathway (see Section 13.5 and below);
- 3 the availability of suitable promoters for expression of the transgene(s) *in planta* (see Section below);
- 4 ideally, an understanding of the regulation of the pathway and the cellular compartmentation of the metabolites (see Section 13.5.5).

Choice of genes and promoters

Most of the genes encoding the biosynthetic enzymes for carotenoid formation have now been cloned from a range of organisms, including higher plants (Section 13.5). The few remaining structural genes (e.g. α -carotene hydroxylase) are likely to be isolated within the next year. At the present time, however, no regulatory genes have been isolated.

The selection of which gene(s) (or cDNA) to use depends upon the end product required, the carotenoid content of the host tissue and the need to

Table 13.6 Higher plants transformed with carotenoid genes

Plant	Inserted gene/cDNA	Promoter	Phenotype	Reference
Rice	<i>Psy</i> cDNA from daffodil	CaMV 35S	Phytoene accumulation in endosperm	Burkhardt <i>et al.</i> , 1997
	<i>Psy</i> , <i>crtI</i> , <i>crtL-B</i>	CaMV 35S, glutelin	β -carotene accumulation in endosperm	Ye <i>et al.</i> , 2000
Tomato	Antisense <i>Psy-1</i> from tomato	CaMV 35S	Reduced carotenoids, increased gibberellins	Bird <i>et al.</i> , 1991
Tomato	<i>crtI</i> from <i>E. uredovora</i>	CaMV 35S	Increased β -carotene, decreased lycopene	Römer <i>et al.</i> , 2000
Tomato	<i>crtB</i> from <i>E. uredovora</i>	Tomato polygalacturonase (PG)	Increased total carotenoids	Fraser <i>et al.</i> , 2002
Tomato	Yeast <i>ySAMdc</i>	E8 (ripening inducible)	3-fold increase in lycopene	Mehta <i>et al.</i> , 2002
Tomato	<i>Psy-1</i> cDNA from tomato	CaMV 35S	Sense suppression, pre-mature lycopene accumulation, dwarf plants	Fray <i>et al.</i> , 1995
Tomato	<i>CrtL-b</i> , sense and antisense	<i>Pds</i>	Increased (up to 7-fold) β -carotene in sense plants	Rosati <i>et al.</i> , 2000
Tomato	Lycopene β -cyclase (<i>CrtL-b</i>) and β -carotene hydroxylase (<i>b-Chy</i>)	Tomato <i>Pds</i> (fruit specific)	Increased β -carotene, β -cryptoxanthin and zeaxanthin	Dharmapuri <i>et al.</i> , 2002
Oil seed-rape	<i>crtB</i> from <i>E. uredovora</i>	napin	50-fold increase in seed carotenoids	Shewmaker <i>et al.</i> , 1999
Carrot	<i>crt</i> genes from <i>E. herbicola</i>	CaMV 35S	2–5 fold increase in root carotenoids	Ausich <i>et al.</i> , 1991; Hauptmann <i>et al.</i> , 1997

avoid co-suppression. For tissues that do not normally contain carotenoids (but will form sterols) it is necessary to transfer genes that encode enzymes that convert prenyl diphosphates into phytoene as well as any subsequent steps needed (Figs 13.3, 13.5, 13.6, and 13.7). In some cases, a single gene transformation does not elevate carotenoid levels, but does alter their

composition. The range of crop plants that have been transformed with carotenoid genes is described in Section 13.7 and shown in Table 13.6.

Transformation of a plant with an extra copy of the endogenous gene usually leads to the phenomenon of co-suppression (sense suppression, gene silencing). Under these circumstances, expression of the transgene causes down-regulation of the endogenous gene and hence a phenotype similar to that shown in antisense transgenic plants. For example, transformation of tomato with its own *Psy-1* gene caused a reduction in ripe fruit carotenoids (Truesdale, 1994). In order to overcome this problem, genes from different species, and especially bacteria such as *Erwinia* spp., are used as they have very low homologies to the plant carotenoid genes and hence do not cause co-suppression. However, since the bacterial genes have no leader sequence for targeting the protein to the plastid, a chimeric gene construct is used, typically with the small sub-unit of Rubisco as the leader. Alternatively, a synthetic cDNA can be made, with every third base of the triplet code changed to reduce homology by 33%. This approach has been successful with a synthetic *Psy* transformed into tomato (Bramley, 1997). Clearly the bacterial enzymes are active in the plant plastid and must associate into the endogenous carotenogenic enzyme complex.

A further consideration in the choice of gene relates to those that are members of a gene family. Only one member of such a family may be involved in carotenogenesis in a particular tissue. A good example of this is *Psy-1* and -2 of tomato. *PSY-1* is responsible for phytoene synthesis in ripening fruit (Fraser *et al.*, 1999) whereas *PSY-2* is not functional in chromoplasts, even if the protein is produced.

A number of promoters have been used successfully with carotenoid genes or cDNAs (Table 13.6). These include constitutive promoters such as CaMV 35S, or tissue-specific promoters such as napin, polygalacturonase (PG) and glutelin. The *Pds* promoter, which is chromoplast-specific, has also been used (Corona *et al.*, 1996). The choice of promoter is a very important part of the strategy, since it controls the dynamics, level and tissue specificity of expression of the gene of interest. The advantage of tissue-specific promoters is that transgene expression can be focused on a particular tissue or at the time of development, thus minimising unwanted effects on isoprenoid metabolism elsewhere in the plant or at the wrong time of development. The use of PG rather than CaMV 35S with phytoene synthase in tomato has changed a dwarf phenotype with 35S (Truesdale, 1994) to one with no dwarfism and elevated carotenoids (Fraser *et al.*, 2002). The strength of the promoter in triggering transcription may also influence the mRNA levels and phenotype, sometimes detrimentally.

13.7 Examples of genetically modified crops with altered carotenoid levels

Several crop plants have been transformed with the aim of increasing or

altering the carotenoids in the edible fruit, seed or root (Table 13.6). At the present time none of these transgenic lines is used commercially, despite the significant changes to the carotenoid profiles in each case. In the main, this is due to the resistance of consumers towards GM foods, and the consequent reluctance of plant biotechnology companies to apply for licenses to market such products.

13.7.1 Tomato

There are several publications detailing the successful alteration of carotenoids in tomato fruit (Table 13.6). In most cases, these studies have aimed to elevate the levels of lycopene or β -carotene. Typically, lycopene levels have been increased some 2–3 fold. The amount of β -carotene has also been elevated using either the *crtI* gene from *Erwinia* (Römer *et al.*, 2000) or the *Lcy-b* cDNA (Rosati *et al.*, 2000). The former example is intriguing, as *crtI* encodes phytoene desaturase, and so the expected phenotype was an increase in lycopene. However, these authors have shown that introduction of *crtI*, under a constitutive promoter, causes up-regulation of the endogenous *Lcy-b*, thus channelling more lycopene in β -carotene. Therefore, cross-talk occurs between the expression of genes of the same pathway. More recently, Dharmapuri *et al.* (2002) have shown that it is possible to metabolise β -carotene in the fruit to hydroxy carotenoids (zeaxanthin and β -cryptoxanthin). The study of Mehta *et al.* (2002) indicates that altering other traits in the tomato fruit may have pleiotrophic effects that include elevated levels of lycopene. In this study, the tomato was transformed in order to increase levels of polyamines for enhanced vine life and fruit juice quality. However, these transgenic fruit also contained increased lycopene levels, presumably because of the longer ripening times for the fruit.

13.7.2 Rice

The formation of β -carotene in rice endosperm, where carotenoids are normally absent, was achieved by the simultaneous transfer of three genes: *Psy* from daffodil, *crtI* from *Erwinia herbicola* and *crtL-B* from daffodil (Ye *et al.*, 2000). The progeny, called ‘Golden Rice’, has received massive publicity for its potential to alleviate vitamin A deficiency in developing countries (Potrykus, 2001).

13.7.3 Carrot

The first reports of genetic manipulation of a crop plant were using the carrot. The introduction of *crt* genes from *Erwinia herbicola* by a group from Amoco (Ausich *et al.*, 1991; Hauptmann *et al.*, 1997) resulted in elevated levels of β -carotene.

13.7.4 Canola

A single gene transformation (*crtB* from *Erwinia*) was sufficient to increase carotenoid levels some 50-fold in seeds of canola (*Brassica napus*), which already contains low levels of carotenoids (Shewmaker *et al.*, 1999). This is the most spectacular increase in carotenoid levels of any plant to date.

13.8 Future trends

Plant biotechnology is at an exciting stage with respect to the alteration of carotenoid levels by genetic engineering. As described in Section 13.7, the ready availability of genes from plants and microorganisms, together with an array of suitable promoters, has facilitated the successful transformation of several key crops in recent years (Table 13.6). These transgenic crops can provide the basis for producing functional foods across the world with elevated or altered carotenoid levels that have the potential to alleviate vitamin A deficiency (rice, oil seed rape, tomato), increase lycopene intake (tomato) or provide zeaxanthin to alleviate AMD (tomato). In the near future, it is anticipated that a further generation of transgenic crops will be produced, perhaps with increased levels of lutein (for prevention of AMD) or greater increases than those presently reported. The latter will require us to understand the regulation of carotenogenesis more thoroughly than we do at present and also perhaps require the isolation and use of transcription factors (Chen *et al.*, 2002) and regulatory genes that control the expression of carotenoid genes. It is also likely that multiple gene transformations, perhaps using alternative promoters, will be needed in order to overcome bottlenecks in the pathway, and the use of genes from the DOXP pathway is very likely in the near future. Somewhat further into the future will be the use of directed evolution (DNA shuffling) that increases genetic diversity and may allow the construction of chimeric genes that encode enzymes with greater catalytic activities (Cramieri *et al.*, 1998; Zhao *et al.*, 1998; Gibbs *et al.*, 2001; Lassner and Bedbrook, 2001).

At present, we do not understand why changes in carotenoid content in transgenic plants vary so dramatically between species, some of which may have been transformed with the same gene. These differences may be linked to the type of tissue in which expression is targeted (e.g. embryo, endosperm, vegetative) or the photosynthetic capacity of the tissue (chloroplast or chromoplast). In addition, the availability of precursors of the carotenoids may differ in these tissues. Further studies on the metabolic flux through the pathway, similar to those reported by Fraser *et al.*, (2002), are essential in order to effectively target the rate-limiting steps in the pathway (Wiechert *et al.*, 2001; Visser and Heijnen, 2002). The development of proteomic studies to elucidate the association of enzymes with partner proteins will also enable more refined transformation events in the next generation of transgenic crops.

13.9 Sources of further information

There is a wealth of information about carotenoids, their biosynthesis and regulation, as well as health-related aspects. Key reviews on the formation of carotenoids and regulation of the pathway include: Spurgeon and Porter, 1983; Bartley and Scolnik, 1995; Cunningham and Gantt, 1998; Harker and Hirschberg, 1998; Sandmann, 2001; Hirschberg, 2001; Bramley, 2002. Additional information on the health benefits of carotenoids, can be found in Cooper *et al.*, 1999; Bramley, 2000; Mortensen *et al.*, 2001; Landrum and Bone, 2001; as well as web-based comment that can be located by using the appropriate keywords in any search engine.

Sequences of the genes/cDNAs can be retrieved from databases on the Internet at various web sites. For example, GeneBank (at the National Center for Biotechnology Information, NCBI) is at <http://www.ncbi.nlm.nih.gov/Web/Search/index.html>. The EMBL Nucleotide Sequence database (through the European Bioinformatic Institute, EBI) can be found at <http://www.ebi.ac.uk/queries/queries.html>, whilst that of the DNA Data Bank of Japan is at <http://www.ddbj.nig.ac.jp/>.

General reviews on nutritional genomics include one by DellaPenna (1999), whilst further details of plant metabolic engineering, including its application to carotenoids have been written by Tengerdy and Szakacs, 1998; Hirschberg, 1999; Sommerville and Dangel, 2000; Giuliano *et al.*, 2000; DellaPenna, 2001; Fraser *et al.*, 2001; Lessard *et al.*, 2002; Huang *et al.*, 2002 and Hanson and Shanks, 2002.

13.10 Acknowledgements

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Developing phytochemical products: a case study

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14.1 Introduction

Phloem powder is produced from the inner bark layer of the pine tree. It was used to compensate for a shortage of flour for bread-making during times of famine in Finland and in other Nordic countries until the early twentieth century. In the late 1970s the Finnish army also studied the potential of phloem as a source of nutrition in times of crisis. Recently, a lot of attention has been given to phloem as a possible raw material for functional foods. Results from nutritional analyses indicate that phloem powder has a low energy content and that it is a rich source of fiber. Furthermore, it contains high amounts of different polyphenols such as catechins and lignans. Reduced risk of cardiovascular disease (CVD) and of different cancers has been partly explained with fiber intake (Liu *et al.*, 2002, Wolk *et al.*, 1999), lignans (Vanharanta *et al.*, 1999) or flavonoids (Hertog *et al.*, 1993, Knekt *et al.*, 1996). Despite the fact that phloem is rich in fiber and polyphenols, and may thus be beneficial to health, these benefits have to be proven in human studies. Until now, no studies have been made to address the health effects of phloem.

The aim of our project was to study phloem as a source of fiber and polyphenols, and to develop a method to improve its taste without losing the potentially bioactive polyphenols. In addition, we wanted to investigate the bioavailability, cholesterolemic and antioxidative effects and safety of phloem and its phytonutrients in humans in a randomised double-blind trial.

Phloem is a common term used to describe the inner layer of the pine tree. As already mentioned phloem powder was used to compensate for the shortage of flour in bread-making during times of famine in Finland and other Nordic

countries. Bark bread formed a part of peoples diet and it was used even during the twentieth century (World Wars I and II) when food was rationed.

The inner bark of the pine tree is harvested in the spring when it is easier to be rossed from the tree trunk and when the vitamin content is at its highest. The phloem is derived preferably from straight, boughless and at least 50-year old pine trees by rossing the bark layer. A flap of the outer bark as well as the green layer is cut away with a knife and folded back, exposing the light beige inner bark, which is about 2 mm thick. After the inner bark is removed, it is dried outside in the open air until it hardens and starts to resemble cardboard. Then the phloem plates are heated briefly to prevent microorganism growth. After the heating the inner bark is ground up to produce phloem powder.

When compared to whole meal rye flour (280 kcal/1160 kJ) and to wheat flour (320 kcal/1320 kJ), phloem powder (140 kcal/580 kJ) contains approximately 50% less energy. As is typical for all flours, phloem powder also contains a low amount of fat (total amount 2.3 g/100 g). The protein content of phloem is only 2.5 g (per 100 g), whereas the respective amount in whole meal rye flour is 8.8 g and in wheat flour 12.1 g. The content of carbohydrates in phloem (~30 g/100 g) is about 50% less than in rye (55 g) and wheat flours (59 g). The relatively low energy, protein and carbohydrate content of phloem when compared with commonly used flours, is related to its high content of different fiber. Detailed nutritional data for phloem and phloem breads used in our trial are presented in Table 14.1.

Phloem powder is rich in different types of fibers, lignans and polyphenols (Table 14.1). Phloem contains ~58 g of fiber per 100 g, of which ~51 g is insoluble and a lesser amount (~7 g) is water-soluble. The total amount of different lignans in phloem is 79.3 mg/100 g, consisting mainly (98%) of secoisolariciresinol. The main flavonoids in phloem are catechins, and the

Table 14.1 The nutrient content of phloem powder and study breads per 100 g¹

Nutrient	Phloem powder	Placebo bread	LP bread	HP bread
Energy (kcal)	140.0	243.3	233.5	224.9
Protein (g)	2.5	9.0	8.5	8.0
Carbohydrates (g)	26.9	46.3	44.4	42.8
Fat (g)	2.3	2.1	2.1	2.0
Fiber, total (g)	57.5	8.4	12.0	14.7
Total amount of catechins (mg)	336.4	0.6	25.3	51.2
Catechin (mg)	296.9	0.2	24.2	48.9
Epicatechins ² (mg)	9.1	0.4	1.1	2.3
Total amount of lignans (mg)	79.3	0.5	6.7	10.4

¹LP = Low phloem bread, HP = High phloem bread.

² Sum of epicatechin and epigallocatechin.

major compound is (+)-catechin with a lesser amount of (–)-epicatechin and (–)-epigallocatechin. In total phloem powder contains 336 mg of catechins per 100 g. In addition to flavonoids, phloem contains some simple phenols such as cinnamic acid and benzoic acid derivatives. The total amount of simple phenolic substances is 1.78 mg/g. The most abundant phenolic acids in phloem are ferulic acid and p-coumaric acid, which occur in equal amounts. Phloem may contain also other phenolic acids, but only these two have been reliably identified. Analysed simple phenols were caffeic, chlorogenic, sinapinic, ferulic, p-coumaric, gallic and protocatechuic acid. Phloem also contains unwanted and strong-flavoured substances such as terpenes, which cause the characteristic woody taste of phloem. One of the aims in our phloem project was to remove unwanted compounds while keeping those with potential health benefits.

14.2 Chemical enhancement of phytochemicals: the case of phloem

Phloem contains high amounts of different terpenes (Sjöström, 1992). These compounds have a characteristic odour and are mainly responsible for the bitter taste of phloem (Hiltunen and Holm, 2000). So far, only whole grain rye bread has been found to have a sufficiently strong flavour to mask the taste of phloem added to it. To maximize the usability of phloem in food industry it was necessary to modify the taste to make it milder. When compounds with strong and bitter taste are removed, the loss of phenolic compounds with expected bioactivity has to be minimized. Phloem is already heated during the manufacturing process, but only for 20 seconds at 200°C. This may evaporate some of the terpenes, but still a great deal of the bitter tasting compounds remain. Simple heating and some chemical methods were tested in order to modify the taste of phloem. The aim of these tests was to develop a treatment which improves the taste with minimum loss of lignans and catechins.

These two groups of polyphenols were the only ones monitored during this taste modification, because they were the ones of direct interest to our clinical trial (Vanharanta *et al.*, 2002a). The phenolic compounds in phloem were described earlier in this chapter. If the loss of lignans and catechins can be minimised, no significant loss of other phenolic compounds is likely to occur. Lignans are thermally labile (Ayres and Loike, 1990) and catechins are very polar, as polar as, for example, phenolic acids which may also be an important group of phenolic compounds in phloem. Even although they are as polar as catechins, phenolic acids are far more tightly bound into the woody matrix and therefore their loss would also be lower than that of catechins.

14.3 Heating and extraction of phenolic compounds

14.3.1 Heating

As described earlier, phloem is heated during the manufacturing process at 200°C for 20 seconds. The boiling or sublimation points of monoterpenes ranged approximately from 150–200°C, when the values were available. To modify the taste of phloem, the material was heated at 100, 150, 200 and 250°C for 30 minutes in each case. Heating was carried out in ventilated conditions to remove all the evaporating compounds. The taste of the phloem was evaluated after the samples were cooled. Three persons tasted the samples and freely described the flavour. The taste of the heated phloem samples was compared to that of the original sample. We found that heating for 30 minutes at 200°C removed the bitter taste of phloem. Lower temperatures had no effect on the taste and the higher temperature caused a strong flavour of tar and coal. The colour of the phloem heated at 200°C for 30 minutes was darker than that of the original sample. After a week the colour of the heated sample was the same, but the taste was different. There was no bitter taste, but the slight, pleasant taste of smoke, observed immediately after heating, was now strong and disturbing. Long heat treatment at a high temperature might have changed the original woody material and may have induced the formation of carcinogenic compounds. Detailed data of lignans and catechins after heat treatment are presented in Table 14.2.

14.3.2 Solvent extractions

Various extraction methods for phenolic compounds in plant material have been published (Ayres and Loike, 1990; Arts and Hollman, 1998; Andreassen *et al.*, 2000; Fernandez *et al.*, 2000). In this case phenolic compounds were an important part of the plant material and all the published methods were optimised to remove those analytes from the matrix. Our interest was to find the solvents to modify the taste, but not to extract the phenolic compounds of interest. In each test the technical treatment of the sample was similar. Extraction was carried out at room temperature (approximately 23°C) for 30 minutes in a horizontal shaker with 200 rpm. Samples were weighed into extraction vials and solvent was added. The vials were closed with caps to minimise the evaporation of the extraction solvent. After 30 minutes the samples were filtered to separate the solvent from the solid. Filter papers were placed on aluminium foil and, after the solvent evaporation, were removed. Extracted samples were dried at 100°C for 30 minutes to evaporate all the solvent traces. The solvents tested were chloroform, ethanol, diethylether, butanol, ethylacetate, heptane, n-hexane and cyclohexane and they were tested with different solvent/solid ratios. Methanol (MeOH) and acetonitrile (ACN) were not considered because of the high solubility of catechins and lignans to MeOH and ACN. The extracted phloem samples were tasted in the same way as the heated ones. Detailed results from each extraction experiment are presented in Table 14.2.

Table 14.2 Lignans and catechins in heat- and solvent-treated phloem

Sample	Lignans ¹	Catechins ²	Taste
Original phloem	1.000	1.000	Bitter, long, disturbing
<i>Heating</i>			
100 °C, 30 min	1.020	1.084	Same as original
150 °C, 30 min	1.009	0.712	Same as original
200 °C, 30 min	0.602	0.074	Mild, slight smoke, later tar and coal
250 °C, 30 min	0.198	0.010	Tar, coal
<i>CHCl₃</i>			
50 l/kg	not analysed	1.040	Slightly bitter
20 l/kg	not analysed	1.071	Less bitter than original
10 l/kg	not analysed	1.004	Same as original
5 l/kg	not analysed	0.973	Same as original
<i>EtOH</i>			
10 l/kg	0.650	0.734	Short bitter, then neutral
5 l/kg	not analysed	not analysed	Long bitter
<i>Other solvents</i>			
Diethylether 10 l/kg	0.868	1.026	Solvent like
Diethylether 5 l/kg	not analysed	not analysed	Not tested
Butanol 20 l/kg	0.736	0.916	Not tested
Ethylacetate 20 l/kg	1.023	1.107	Not tested
n-heptane 20 l/kg	1.014	1.097	Not tested
n-hexane 20 l/kg	0.951	0.961	Not tested
Cyclohexane 20 l/kg	0.759	0.900	Not tested

¹The amount of lignans (i.e. secoisolariciresinol) is marked with number 1 in the original phloem sample.

²The amount of catechins (i.e. (+)-catechin, (-)-epicatechin, (-)-epigallocatechin) is marked with number 1 in the original phloem sample.

Chloroform (CHCl₃)

Chloroform is too non-polar to dissolve the phenolic compounds under study, but it dissolves many of the monoterpenes, at least to some extent. Because the solubility of some monoterpenes into chloroform was low, different solvent/solid ratios were tested. These were 50, 20, 10 and 5 l/kg of dry phloem. The extracts were bright yellow and the strongest colour was with the smallest solvent/solid ratio (5 l/kg). The colour of the solvent indicated that the solubility of the extractable compounds was not restricting the reaction even with the smallest solvent volume. The taste of the dry samples was evaluated by comparing them to the original phloem sample. The results showed that the mildest taste was in the phloem extracted with a solvent/solid ratio of 50 l/kg and 20 l/kg also had some effect on the taste. The taste of the chloroform-extracted phloem was stabile and it was the same after a week.

Ethanol (EtOH)

Many of the monoterpenes are soluble into alcohols. Methanol and alcohols with three or more carbons are poisonous, but ethanol (EtOH) in moderate amounts is not. EtOH is also an economical choice when compared to other possible solvents. The only disadvantage is that lignans and catechins, the analytes of interest, are also soluble into EtOH. Solubility is dependent on extraction efficiency, i.e. shaking power, temperature and time. The solvent/solid ratios tested for EtOH and diethylether were 10 l/kg and 5 l/kg. The EtOH extracts had a strong yellow or slightly brown colour. The taste of the dry samples extracted with EtOH was evaluated by comparing them to the original phloem sample. Phloem extracted with EtOH using a solvent/solid ratio of 10 l/kg had only a short-lived bitter taste which disappeared quickly. After that the taste of EtOH extracted phloem was neutral. The taste depended on the amount of solvent used for extraction. Only 10 l/kg solvent/solid ratio improved the taste of phloem. The taste was more pleasant than it was after chloroform extraction and the bitterness was significantly reduced. The characteristic odour and smell of resin and the unpleasant sour feeling in the mouth were not observed.

Other solvents

Diethylether was tested with solvent/solid ratios of 10 and 5 l/kg. Other solvents (i.e. butanol, ethylacetate, heptane, n-hexane and cyclohexane) were tested using a solvent/solid ratio of 20 l/kg. The higher solvent/solid ratio compared to EtOH was chosen because of the very limited solubility of polar compounds to moderately polar or non-polar solvents. The taste of the dry samples extracted with diethylether was evaluated by comparing them to the original phloem sample. Other solvent extracted phloem samples were subjected only to the catechin and lignan analyses described in Section 14.4. Diethylether evaporates easily already at room temperature, but the ether extracted phloem had a strong, pungent solvent odour and the taste was solvent-like. The original bitterness of phloem was not observed, but the taste of the solvent probably covered it.

14.3.3 The best extraction technique

EtOH extraction was the most efficient way to improve the flavour of the phloem. A solvent/solid ratio of at least 10 l/kg was needed to achieve a significant change in the taste. The loss of catechins was approximately 27% and that of lignans was 35%. All the catechins and lignans were found from the EtOH extract. Losses of lignans and catechins were smaller with other solvents, but either the taste was not modified or the cost of solvent treatment would be too high. Phenolic compounds like lignans and catechins also have a bitter taste and some improvement in flavour may have occurred because of the lower concentration of these. The disappearance of the characteristic

woody taste of phloem must have been due to a reduced amount of terpenes and resins.

14.4 Measuring phenolic compounds

Only secoisolariciresinol, the main lignan found in phloem, was analysed. Other lignans occur in phloem, but in low amounts compared to secoisolariciresinol and, furthermore, they are quite labile at higher temperatures. Lignan analyses were carried out using high performance liquid chromatography (HPLC) with coulometric electrode array detection (CEAD). The chromatographic conditions were presented earlier by Nurmi and Adlercreutz (1999), and pretreatment was a simplified version of the protocol used in the gas chromatographic–mass spectrometric (GC–MS) method (Mazur *et al.*, 1996). The results of the lignan analyses are presented in Table 14.2.

Catechins were analysed using the same HPLC with CEAD as for lignans. The analytes were separated by gradient elution. The mobile phase consisted of two eluents: a) 50 mM $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ buffer pH 2.3:MeOH (90:10, v/v); and b) 50 mM $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ buffer pH 2.3:MeOH:ACN (40:40:20, v/v/v). The total flow rate was 0.3 ml/min and the composition of the gradient was the following: 0 min/20%B, 5 min/20%B, 7 min/30%B, 12 min/30%B, 18 min/50%B, 23 min/50%B, 25 min/20%B, 40 min/20%B. The column was Inertsil C 18 with dimensions 150×3 mm (GL Sciences, Japan). The packing material was deactivated with C 2 end capping and the particle size was 3 μm . The pre-column was packed with Inertsil C 18, particle size 5 μm , and the dimensions of the column were 10×3 mm. Both columns were placed into the thermostated chamber at 37°C. The samples were pretreated using the recently developed protocol for the chocolate samples. A quantity of 20–30 mg of dry sample was weighed into the extraction vial and 2.5 ml of 0.1 M HCl in 50% MeOH was added. The sample was incubated at 50°C for two hours. The supernatant was taken into the volumetric flask and filled with 50% MeOH. All the analyses were done in duplicate. The results of the catechin analysis are presented in Table 14.2.

Absolute values of secoisolariciresinol were not determined, but the treated phloem samples were compared to the original sample. Value 1 corresponds to the lignan content of the original phloem sample. Values slightly above 1 may occur due to a variation between the assays. The results for catechins are also presented in relation to the untreated, original phloem sample. Six different catechin forms were analysed and three of them were present in phloem. (+)-catechin is the major catechin in phloem and two minor catechins are (–)-epicatechin and (–)-epigallocatechin. Value 1 corresponds to a sum of three catechins detected in original phloem. Absolute values are not given, because the sample pretreatment was not optimised for phloem. When the results for treated phloem were compared to the results for original phloem, sufficient information about the effect of treatment on lignans and catechins was obtained.

14.5 The functional benefits of phloem

Phloem powder contains very high quantities of fiber (almost 60 g/ 100 g) most of which is insoluble (Table 14.1). According to current nutritional recommendations, the amount of dietary fiber should be increased to 30–35 g/day. This recommendation is based on the evidence from several epidemiological studies suggesting that a high intake of dietary fiber decreases the risk of CHD by 20–40% (Liu *et al.*, 2002; Wolk *et al.*, 1999). The mechanisms behind the health effects of dietary fiber have not been fully explored, but several explanations have been proposed. Experimental studies show that an increased intake of soluble fiber reduces plasma cholesterol concentrations (Brown *et al.*, 1999; Leinonen *et al.*, 2000). It has also been suggested that insoluble fiber (the main fiber in phloem), even without affecting cholesterol concentrations, may reduce the risk of CHD by slowing the absorption of food or by reducing the clotting factor (Marckmann *et al.*, 1990). However, adequate intake of dietary fiber is a problem in many populations and adding phloem to the diet could easily increase it.

Besides fiber, phloem is also rich in polyphenols such as lignans and flavonoids, which are often postulated to explain some of the association between diets high in whole grain, fruit and vegetables and reduced risk of CVD (Anderson and Hanna, 1999; Ness and Powles, 1997). One of the leading hypotheses is that polyphenols can protect against CVD by decreasing the lipid peroxidation in the human body (Bravo, 1998). It has been suggested that oxidative modification of low-density lipoprotein (LDL) plays an important role in atherogenesis (Steinberg *et al.*, 1989; Salonen *et al.*, 1992; 1997), and it is possible that agents preventing oxidation of LDL in the arterial wall also attenuate the development of atherosclerosis (Fuhrman and Aviram, 2001).

Previous studies have shown that by increasing the daily consumption of fruit and vegetables lipid peroxidation can be partly inhibited (Miller *et al.*, 1998). Among the most potent diet-derived antioxidants are flavonoids. Flavonoids are hydrophilic polyphenols widely found in vegetables, fruits and chocolate and beverages such as tea and wine, and thus consumed daily by most people (Bravo, 1998; Scalbert and Williamson, 2000). Out of more than 5000 described flavonoids, the sub-group of catechins has particularly attracted scientific attention. Evidence from epidemiological studies suggests that a high intake of flavonoids and tea catechins may decrease the risk of CHD (Hertog *et al.*, 1995; Geleijnse *et al.*, 1999). Catechins have been demonstrated to possess a relatively high antioxidant capacity *in vitro* (Serafini *et al.*, 1996; Lotito and Fraga, 1998; Hodgson *et al.*, 1999). Additionally, evidence from animal studies suggests that ingestion of catechins or proanthocyanidins retards the progression of atherosclerosis (Hayek *et al.*, 1997; Yamakoshi *et al.*, 1999). In humans, controlled long-term catechin supplementation studies have resulted in inconsistent findings concerning the effect of tea, red wine or red wine extract on the resistance of LDL to oxidation (de Rijke *et al.*, 1996; Carbonneau *et al.*, 1997; Nigdikar *et al.*, 1998; Princen *et al.*, 1998).

Lignans are another group of polyphenols in phloem which are thought to contribute to the pool of dietary antioxidants. Although hardly anything is known of their antioxidant capacity, the process of lignan degradation and conversion in the colon by the intestinal bacteria has been described in detail (Setchell *et al.*, 1980). Plant lignans serve as pre-cursors to mammalian lignans such as enterolactone, in a process which is dependent on the existence of certain types of bacteria. Enterolactone concentration can be measured in human serum as well as in urine and other biological fluids (Adlercreutz and Mazur, 1997). The health effects of high serum enterolactone are not fully understood, but we have observed some interesting associations in Finnish men. In a nested case-control study of a male cohort (Kuopio Ischaemic Risk Factor Study), we have shown that serum enterolactone is protective against acute coronary events (Vanharanta *et al.*, 1999).

In cross-sectional setting in the ASAP (The Antioxidant Supplementation in Atherosclerosis Prevention) study, we have also found that high serum enterolactone concentration was associated with a lower level of lipid peroxidation, assessed by F₂-isoprostanes (Vanharanta *et al.*, 2002b). Because enterolactone is relatively stable compared to other lignans, it might serve as a useful marker of lignan intake. For the same reason, however, it might not be an active antioxidant. Nevertheless, other lignan pre-cursors have greater potential as antioxidants, which could also explain the association observed in the ASAP study (Vanharanta *et al.*, 2002b).

14.6 Testing functional benefits

14.6.1 A randomised double-blind phloem study

To study the bioavailability of polyphenols from the highly insoluble wood matrix and to address the question of the hypocholesterolemic and antioxidant effect of phloem we conducted a long-term placebo-controlled supplementation study. For this study we recruited 75 hypercholesterolemic, but otherwise healthy, non-smoking men (aged 31–70 years) from the Kuopio area in Eastern Finland. Potential participants were screened for the following inclusion criteria: 1) no severe obesity (BMI < 32 kg/m²); 2) increased serum cholesterol concentration (total cholesterol 6–9 mmol/L); 3) no regular use of any drug or supplement with antioxidative (β -carotene, vitamins C or E) or lipid-lowering properties; 4) no chronic diseases like diabetes, CHD or other major illness.

Subjects were randomly assigned to consume daily 70 g of normal dried rye bread (placebo group, $n = 30$), rye bread in which 8% of the rye flour was substituted with phloem powder (low phloem, LP, group, $n = 30$) or bread in which 14% of the rye flour was substituted with phloem powder (high phloem, HP, group, $n = 15$). Study breads used in our study were different in fiber, lignan and catechin content. The nutrient content of the phloem powder and

the study breads is presented in Table 14.1. The placebo group received 5.9 g, the LP group 8.4 g and the HP group 11.8 g of dietary fiber daily from the study breads. Daily amount of catechins were 1 mg in the placebo group, 18 mg in the LP group and 36 mg in the HP group. Daily amount of lignans were 0.4, 4.7 and 7.3 mg respectively. The subjects were advised to discontinue consumption of tea, red wine, cocoa and chocolate one week prior to the study, but otherwise to maintain their normal eating and exercise habits.

In this study we evaluated the bioavailability of polyphenols by measuring the enterolactone concentration in serum. Enterolactone analyses were carried out applying time-resolved fluoroimmunoassay (TR-FIA) (Adlercreutz *et al.*, 1998; Stumpf *et al.*, 2000). Lipid peroxidation was assessed by measuring the resistance of serum lipids and isolated plasma VLDL (very low-density lipoprotein) and LDL to oxidation after induction of copper as described previously (Nyyssönen *et al.*, 1997; Porkkala-Sarataho *et al.*, 1998). Briefly, serum was diluted to a concentration of 0.67% in 0.02 mol/L phosphate buffered saline (PBS), pH 7.4. Oxidation was initiated by addition of 100 µl of 1 mmol/L CuCl₂ into 2 mL of diluted, prewarmed (30°C) serum. The formation of conjugated dienes was followed by monitoring the change in 234 nm absorbance at 30°C on a spectrophotometer (Beckman DU-6401, Fullerton, California) equipped with a six-position automatic sample changer. The change in absorbance was recorded every 5 min for 4 h. The time required from the start to the maximal rate of the reaction (lag time) was determined. VLDL and LDL were isolated in a combined fraction from fresh EDTA plasma by ultra-centrifugation. EDTA and gradient salts were removed by gel permeation columns, VLDL + LDL was exposed to copper-induced oxidation and the lag time was determined. We also measured serum lipids (total cholesterol, LDL and high-density lipoprotein (HDL) cholesterol and triglycerides) and all measurements were done at baseline and after the four-week supplementation period. Subjects also kept a four-day food recording before the supplementation period and during the last week of the study.

14.6.2 Results and discussion

All the 75 recruited men completed the study, but two participants were excluded, one in the LP group due to a pathological concentration of serum triglycerides (8.8 mmol/L) and one in the placebo group due to insufficient dietary compliance. The physical characteristics or the dietary intake of various nutrients did not differ significantly between the study groups at entry (Table 14.3). According to the four-day food recordings and the questionnaire, the compliance of the 73 remaining volunteers with the given dietary and lifestyle instructions was good during the experiment and no adverse effects during the experimental period were reported.

In our study we used serum enterolactone as a marker of bioavailability of phloem polyphenols from the wood matrix. As a result of the four-week

Table 14.3 Baseline characteristics and estimated nutrient intakes at baseline based on four-day food recording¹

	Placebo group (n = 29)	LP (n = 29)	HP (n = 15)
Age (y)	51.9 ± 12.4	47.5 ± 9.4	54.5 ± 9.2
BMI (kg/m ²)	25.8 ± 2.7	25.3 ± 2.3	26.6 ± 3.4
Energy intake (MJ/d)	9.2 ± 2.3	9.4 ± 2.0	9.2 ± 1.9
Total fat (E %) ²	31.9 ± 5.1	32.8 ± 4.4	32.2 ± 4.4
SAFAs (E %)	12.8 ± 3.2	13.6 ± 3.2	12.8 ± 2.4
MUFAs (E %)	10.7 ± 2.0	10.6 ± 1.6	10.8 ± 2.2
PUFAs (E %)	5.1 ± 1.4	5.3 ± 1.0	5.2 ± 1.7
Fiber g/d	25.5 ± 8.6	27.3 ± 10.7	26.8 ± 9.2
Vitamin E intake (mg/d)	9.2 ± 2.9	10.2 ± 3.5	9.8 ± 3.2
Vitamin C intake (mg/d)	76.6 ± 51.2	103.1 ± 70.0	92.4 ± 56.5
β-carotene intake (mg/d)	2.1 ± 2.0	2.8 ± 1.7	2.6 ± 2.0
Folate intake (μg/d)	251 ± 66	321 ± 78	268 ± 69

¹= ± SD. LP = Low phloem group, HP = High phloem group.

²Percentage of total daily energy intake.

intervention the serum enterolactone concentration, which is a metabolic product of dietary lignans, increased in all groups (by 1.9 nmol/l in placebo, 25.3 nmol/l in LP and 27.1 nmol/l in HP), but particularly in the LP and HP groups (Vanharanta *et al.*, 2002a). The difference between the placebo and the LP group ($P = 0.009$) as well as between the placebo and the HP group ($P = 0.003$) was also statistically significant. These results show that polyphenols are bioavailable from highly insoluble fiber. Phloem is also rich in catechins and we will analyse plasma catechins in the near future to evaluate their bioavailability too.

To our knowledge, our finding showing that high serum enterolactone protects against CHD has not yet been confirmed in other populations. It can be estimated that among the Finnish whole grain rye bread accounts for a major part of lignan intake. In this population, already consuming high amounts of whole grain rye bread, it was interestingly shown that an additional amount of rye bread, which is rich in lignans, does not increase serum enterolactone level (Juntunen *et al.*, 2000). There are no previous phloem supplementation studies concerning the concentrations of serum enterolactone. Phloem is an exceptionally dense source of plant lignans and, by substituting 8% or 14% of the rye flour in the rye bread dough with phloem powder, the total amount of enterolactone pre-cursors increases 15–24-fold in the bread. This significantly higher content of plant lignans in our phloem breads explains the impact on serum enterolactone level as compared to the above-mentioned study (Juntunen *et al.*, 2000). Whole grain rye bread is widely consumed among Finnish people and it is a major source of lignans among the population. Therefore dense lignan sources like phloem may be the only way to further increase the concentrations of enterolactone, if such an increase is needed to achieve health benefits.

Substantial individual differences were observed in the response to study breads and the ranges of enterolactone concentration changes in the groups were as follows: -54.5 – 60.0 nmol/l (placebo), -26.2 – 101.3 nmol/l (LP), -19.6 – 81.8 nmol/l (HP). This was something that could have been expected as in several studies dietary factors have explained only $\sim 10\%$ of the variation in serum enterolactone (Vanharanta *et al.*, 2002b; Kilkkinen *et al.*, 2001). This gives further support to the major role of intestinal bacteria in the synthesis of enterolactone. Decreased concentrations of enterolactone may occur due to an increased fiber intake, which may shorten the retention time in the colon and lead to incomplete metabolism of plant lignans. Constipation was earlier shown to be associated with an increased level of serum enterolactone (Kilkkinen *et al.*, 2001).

In our study, consumption of rye bread or rye bread with phloem did not have an effect on serum lipids (total, LDL or HDL cholesterol or triglycerides) (Table 14.4). This is contrary to a recent finding suggesting that soluble fiber from rye bread decreased the concentrations of cholesterol (Leinonen *et al.*, 2000). In that study ingestion of rye bread (220 g/d) with naturally high amounts of insoluble (18 g/d) and soluble fiber (4 g/d) decreased the LDL concentrations by $\sim 8\%$ in hypercholesterolemic men. The researchers speculated that soluble fiber, maybe β -glucan, was responsible for the hypocholesterolemic effect. The amount of rye bread (70 g/d vs 220 g/d), the amount of total (5.9–11.8 g/d vs 22.1 g/d) and soluble fiber (0.6–1.3 g/d vs 4 g/d) ingested in this study was considerably less, and could explain the lack of effects on blood lipids in our study.

One of the main objectives of this study, besides testing the bioavailability of phloem polyphenols, was to study the effects of long-term (four-week) consumption of phloem on lipid oxidation. The results showed that the resistance of serum lipids to oxidation was enhanced in the group receiving the highest amount of phloem (HP group). No effect was seen in the low phloem group (LP group) or in the placebo group. In the HP group the oxidation resistance of serum lipids measured as lag time to maximal oxidation rate increased by $11 \pm 14\%$ and the increase in oxidation resistance was significantly greater compared with the LP group ($P = 0.005$) and with the placebo group ($P = 0.004$).

Ingestion of phloem resulted in increase in the oxidation resistance of serum lipids. This effect was probably not due to dietary lignans, because increase in serum enterolactone did not correlate with the increase in lag time (0.098, $P = 0.421$). The change in the resistance was probably due to catechins or catechins and other phenolic compounds present in phloem. Phloem contains considerably high amounts of catechins 668 mg/100 g, and being highly antioxidative compounds these could be responsible for the decreased lipid oxidation. Although we observed an effect on the susceptibility of serum lipids, neither the rye breads enriched with phloem powder nor the normal rye bread had any detectable effect on the susceptibility of isolated VLDL + LDL oxidation *ex vivo*. This might indicate that catechins as

Table 14.4 Serum ASAT, ALAT, creatinine, serum lipoproteins and oxidation kinetics before and after four week consumption of study breads¹

	Placebo group (n = 29)			LP (n = 29)			HP (n = 15)		
	Baseline	Change	P for change	Baseline	Change	P for change	Baseline	Change	P for change
ASAT (U/L)	44 ± 6 ²	0 ± 5	0.943	27 ± 7	-0 ± 6	0.731	31 ± 9	-3 ± 12	0.290
ALAT (U/L)	31 ± 11	30 ± 11	0.132	34 ± 18	-1 ± 15	0.800	44 ± 20	-5 ± 11	0.106
LDL cholesterol (mmol/L)	4.82 ± 1.15	-0.20 ± 0.59	0.082	4.96 ± 0.92	-0.11 ± 0.78	0.434	4.77 ± 0.90	-0.12 ± 0.58	0.423
HDL cholesterol (mmol/L)	1.38 ± 0.34	-0.02 ± 0.15	0.465	1.26 ± 0.24	0.00 ± 0.15	0.980	1.19 ± 0.23	0.02 ± 0.24	0.793
Triglycerides (mmol/L)	1.72 ± 0.85	-0.18 ± 0.75	0.217	1.74 ± 0.92	0.24 ± 0.68	0.069	2.23 ± 1.37	-0.01 ± 1.09	0.963
Serum lipid oxidation resistance (lagtime, min)	165 ± 25	-3 ± 17 (28)	0.394	180 ± 22	-2 ± 24	0.614	175 ± 27 (12)	20 ± 23	0.014
VLDL + LDL oxidation resistance (lagtime, min)	64 ± 5	3 ± 7	0.039	66 ± 6	2 ± 9	0.186	66 ± 6	-1 ± 3 (14)	0.522

¹n = 29 in placebo and LP group, 15 in HP group except where otherwise indicated in brackets. LP = Low catechin group, HP = High catechin group.
² ± SD.

hydrophilic compounds may not accumulate in LDL sufficiently to inhibit oxidation (van het Hof *et al.*, 1999).

In previous catechin supplementation studies in humans the oxidisability of lipoproteins *ex vivo* has usually been determined after isolation of LDL. These studies resulted in inconsistent findings on LDL oxidation (de Rijke *et al.*, 1996; Carbonneau *et al.*, 1997; Nigdikar *et al.*, 1998; Princen *et al.*, 1998). Some researchers (Hodgson *et al.*, 1999) suggested that the findings could be partly related to the method used to assess LDL oxidisability, speculating that isolation of LDL might not be appropriate when evaluating the effects of catechin ingestion on lipid oxidation *ex vivo*. For example, van het Hof *et al.*, (1999) found in their study that, after consuming eight cups of green tea for three days, most of the catechins were found to be associated with the hydrophilic fraction of plasma. Actually, less than 10% of the total amount of catechins found in plasma were distributed in LDL and more than 50% were recovered in the plasma protein fraction. Furthermore, Lotito and Fraga (1998) have shown that catechins have a stronger antioxidant capacity in the aqueous than in the lipid phase. These observations support the assumption that flavonoids may act in the aqueous phase, perhaps on the surface of the lipoprotein particles (Yamakoshi *et al.*, 1999; Carbonneau *et al.*, 1997).

Despite the fact that phloem is rich in catechins the amount of these ingested daily in our phloem study was rather small when compared to previously conducted studies. In similar studies the amount of catechins ingested in the form of tea or red wine per day has varied from 81 mg to as high as 2490 mg (de Rijke *et al.*, 1996; Carbonneau *et al.*, 1997; Nigdikar *et al.*, 1998; Princen *et al.*, 1998). It would be interesting to test in the future whether increasing the ingested amount of phloem would further reduce the inhibition of lipid peroxidation.

A major drawback of this study was that we measured lipid peroxidation *ex vivo*, but not *in vivo* using the latest and most promising methods such as F₂ isoprostanes (Roberts and Morrow, 2000). However, we are planning to do that soon, so hopefully future studies will bring us more detailed information about the effects of phloem on lipid peroxidation. In conclusion, our study showed that lignans are bioavailable from the wood matrix, that long-term consumption of phloem is safe and that ingestion of phloem can inhibit lipid peroxidation in humans.

14.7 Future trends

Nowadays food manufacturers, scientists and also consumers are interested in the health effects of foods. Therefore a lot of resources are being devoted to developing new food items with particular health effects for the market. However, in many cases scientific evidence authorising the health claims of such products is lacking. Phloem is one candidate as a functional food because

it is rich in fiber and phytochemicals such as lignans and catechins. Our aim was to study the usability of phloem as a food item, the bioavailability of the phytochemicals, and the safety and health effects of long-term consumption of phloem by humans. Our results show that these phytochemicals are bioavailable and that long-term consumption of phloem is safe and may decrease lipid peroxidation. Phloem powder can thus be added to different food items to improve their quality and nutritional value. However, our findings need to be confirmed in future studies.

14.8 Sources of further information and advice

The phloem project, which included the chemical analyses and modification of pine phloem powder and the randomised double-blind trial, was funded by and carried out at the Research Institute of Public Health, University of Kuopio, Finland. The phloem powder used in the phloem study was delivered by Finnbark Co-operative Society and the study breads were baked by Linkosuo Bakery Oy. Our research group will be pleased to give further information about the phloem project. Further information about the randomised double-blind trial can be also obtained from the recently published work of Vanharanta *et al.*, (2002a). Other published material consists of some leaflets and small books about the history of phloem and is available only in Finnish. Those books can be found using keyword 'pettu' in Finnish library databases, e.g. LINDA, which is a collective database for university and other professional libraries. Further information about dietary polyphenols and their role in health and disease can be obtained from the book edited by Rice-Evans and Packer (1998). This extensive book describes in great detail the chemical and biochemical properties of flavonoids as well as their occurrence and analysis and health effects in humans. Another excellent book containing further information about the metabolism of dietary polyphenols is written by Scheline (1991). Information on plant lignans can be obtained from the book by Ayres and Loike (1990), which clearly explains the structural diversities of different lignan compounds. Additionally, isolation, biosynthesis and synthesis are discussed illustratively in separate chapters.

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15

The impact of food processing in phytochemicals: the case of antioxidants

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15.1 Introduction: natural antioxidants present in foods

Most foods, especially those of plant origin contain antioxidants. They are almost exclusively phenolic substances, possessing two (more rarely three) hydroxyl groups in the 1,2-position of the benzene ring (pyrocatechol derivatives), less frequently in the 1,4-position (hydroquinone derivatives). Such systems are very reactive, especially in the presence of oxygen. Their content depends on the degree of plant ripeness, on the climate and on the cultivar. If the antioxidant content is too low, other natural antioxidants could be added, either in the form of natural food constituents rich in antioxidants or as antioxidant preparations. In this chapter we shall discuss only changes in antioxidants and their functionality during food processing.

In addition to phenolic substances, there are other components present in foods which have no antioxidant activity of their own, but which increase that of phenolic antioxidants. They are called synergists, and they should be accounted for in any discussion of antioxidant activity. Polyvalent organic acids, amino acids, phospholipids (lecithin) and various chelating agents belong to this group. Proteins may modify the efficiency of antioxidants as they react with the reaction products of both antioxidants and synergists.

15.2 Changes in antioxidants: mechanism of action

During food processing, interactions of antioxidants with proteins and other food constituents take place, and the activity of some antioxidants may change as a result of hydrolytical processes because glycosides and esters are converted

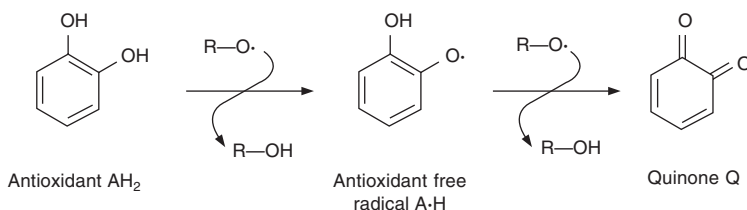


Fig. 15.1 Reactions of an antioxidant AH_2 with free radicals.

into free phenolic derivatives. Formation of complexes by reaction of phenolic substances with metals is also important. The most important reactions of phenolic antioxidants are, however, different oxidation reactions as they affect their functionality to a pronounced degree. Antioxidants (AH_2) react with lipidic free oxy ($R-O^*$) or peroxy ($R-OO^*$) radicals following reactions [15.1] and [15.2], respectively:



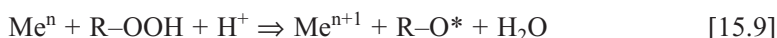
where Q is the respective quinone (Fig. 15.1).

Antioxidant free radicals (A^*H) produced in this way are relatively stable, but they may react with another lipid free radical to form a quinone [15.3] and [15.4]. Quinones can react with amine or thiol groups of proteins, forming polymerisable yellow or red coloured compounds (Pokorny, 1987). Antioxidant free radicals may also react with either another antioxidant free radical or a lipid free radical, forming dimers [15.5] or copolymers [15.6] and [15.7], respectively. Oligomeric or condensated products of antioxidant free radicals usually possess moderate antioxidant activities too (Pokorný *et al.*, 1974):



15.2.1 Inhibition reactions due to metal chelation

Metals of transient valency, particularly copper and iron, catalyse the lipid oxidation because they decompose lipid hydroperoxides with formation of free radicals [15.8] and [15.9]:



The valency of the metal ion changes in every step so that a single atom of heavy metal (Me) may produce many free radicals. Metal chelating compounds, such as citric, tartaric or phosphoric acids, ascorbic acid, phytin or phosphatidic acids, combine with metals to form non-reactive compounds so that the oxidation reactions are inhibited and natural food antioxidants are saved.

15.2.2 Effect of water phase on the functionality of antioxidants

In most foods, both aqueous and lipid phases are present. Polar antioxidants, such as ascorbic acid, are dissolved in the aqueous phase, and react with hydrophilic free radicals, such as HO* or HOO*. In contrast, lipophilic antioxidants, such as tocopherols, are dissolved in the lipidic phase, reacting with liposoluble free radicals produced during lipid oxidation or decomposition of lipid hydroperoxides. At the water-oil interface, antioxidants can accumulate forming an oriented mono-molecular layer, according to their polarity. This layer protects the lipidic phase against oxidation by oxygen dissolved in the aqueous phase (Frankel *et al.*, 1993). Therefore, the activity of antioxidants is very different in bulk fats and oils and in lipid emulsions. This behaviour should be taken into account when considering changes of antioxidant functionality.

15.3 Changes during heating: water as the heat transfer

Changes of antioxidant functionality during food processing, storage, and meal preparation depend on the processing conditions (Table 15.1). Energy is applied to the food material in many processes, while others do not require energy input. Changes in the antioxidant functionality depend not only on the energy requirement, but also on other factors such as air access, temperature, food composition, time and light access.

Table 15.1 Processes of food preparation and storage

Type of action	Processes
<i>Energy transfer medium</i>	
Water	Boiling, pasteurisation, sterilisation, blanching, evaporation, extrusion cooking
Air	Roasting, baking, drying
Oil	Shallow and deep fat frying
Waves	Microwave oven heating, irradiation
<i>No energy application</i>	
Chemicals	Smoking, curing
Enzymes	Fermentation
Storage	In air, in vacuum, in inert gas, refrigerated, frozen storage, transport

During boiling, oxidoreductases, especially lipoxygenases and polyphenol oxidases, increase their activity at the beginning, but they are soon inactivated because of denaturation as the temperature rises. Oxygen dissolved in water is rapidly consumed, and the diffusion of air into boiling water is negligible. Therefore, the oxidation does not appreciably proceed during prolonged boiling.

Some phenolic antioxidants, especially flavonoids, are present as esters or glycosides. They are partially hydrolysed during boiling, and these hydrolytical changes influence both their distribution between the lipidic and aqueous phases and their reaction with lipidic free radicals. The nutritional value is partially lost at the same time. Only approximately one-fifth of flavonol glycosides was retained after boiling the broccoli (Price *et al.*, 1998). Boiling reduced the anti-radical activity of mushroom juice, did not affect the activity of onions and yellow bell peppers, and partially increased the activity of white cabbage juice (Racchi *et al.*, 2002).

During boiling, relatively polar phenolic antioxidants are completely or partially dissolved in the aqueous phase, affecting the stability of the lipidic phase against oxidation. Antioxidants dissolved in water may still inactivate water-soluble hydrophilic free radicals, but they are lost for the stabilisation of the lipids phase as they are no longer in contact with lipophilic free radicals. A very important operation in the processing of fruits and vegetable is blanching. The heat treatment is less intensive than for example in heat pasteurisation or sterilisation, and the resulting changes in food quality are less dramatic. It involves rapid passing of saturated steam or hot water through solid foods so that only the water, whatever its physical state, is the carrier of heat. The heating should be very rapid, and need not be long. The destruction of phenolic antioxidants by polyphenol oxidases or reactions with lipid hydroperoxides is thus avoided. Two flavonoids with an antioxidative effect, quercetin and kaempferol, in onions, green beans and peas, were analysed with HPLC before and after blanching, cooking in water, cooking in a microwave oven, and frying (Ewald *et al.*, 1999). Onions had greater levels of the two flavonoids than green beans and peas; no kaempferol was detected in peas. Peeling and blanching of onions reduced the flavonoid content to a half. Further processing had only a small effect on flavonoid content. Blanching of fresh pepper cultivars and processed jalapenos decreased provitamin A activity by 25% and total ascorbic acid by 75% (Howard *et al.*, 1994).

Another important food processing technology is pasteurisation. It consists of rapid heating to temperatures between 60 and 65°C in order to destroy microorganisms. Oxidoreductases are inactivated at the same time. As the heating is short, the destruction of antioxidants is only moderate. Losses of ascorbic acid are a good indicator of the destructive changes. Losses of ascorbic acid and carotenes are minimised by deaeration.

Commercial sterilisation is a more severe process than pasteurisation as the temperature is higher (70–90°C). The process is used to protect food during long-term storage. The oxygen residue should be minimised before heating to reduce deterioration of antioxidants or, in some cases, antioxidants

may be added after the treatment. The stability of oxidoreductases during the process depends on the food material, especially on the pH-value. Added antioxidants can diminish the losses of viscosity and gel strength of polysaccharide systems occurring on autoclaving. Antioxidant treatments tended also to delay age-thickening and gelation processes in sterilised evaporated milk (Harwalker *et al.*, 1983). Rosemary extract could be an effective solution in inhibiting lipid oxidation and the formation of an undesirable sulphur off-flavour in heat-sterilised meat, when during filling in tray air is removed by nitrogen flushing.

Evaporation is the oldest process for the concentration of liquid foods. Temperatures are higher compared to those of the more modern membrane filtration or freeze concentration processes. Tocopherols, carotenes, ascorbic acid, flavonoids and other phenolic antioxidants are partially destroyed by heating. Therefore, it is necessary to minimise the time needed for evaporation, and heating to the evaporation temperature should be carried out very rapidly. The temperature may be decreased if the pressure is reduced. The process is then more expensive, but losses of antioxidants become substantially lower.

High pressure, high temperature and short time are typical parameters of extrusion cooking. The heat transfer medium is water present in the original material and/or added before the process. Because of the short residence time in the extruder (less than 1 min), losses of antioxidants are relatively low. Nevertheless, losses of ascorbic acid and vitamin A up to 50% were reported (Harper, 1979), depending on the extrusion time, temperature and water content. Summarising, losses of antioxidants during processes where water acts as the heat transfer medium are relatively low, because of limited oxygen access, especially in deaerated foods.

15.4 Changes during heating: air as the heat transfer medium

Heat is transferred more slowly by hot air than by hot water because of differences in heat conductivity. Therefore, in most circumstances higher temperatures and longer processing times should be used than is the case with boiling. Hot air reaches the surface of food, which is then changed more intensively than the inner layers where the temperature does not exceed about 100°C. Losses of antioxidants are, of course, also higher on the surface than in the interior of heated food.

During baking, the outer layers of the dough are heated to 120–200°C or even more, while the temperature remains lower than 95–100°C in the inner layers. In the surface layer, the following reactions occur: caramelisation of reducing sugars and ascorbic acid (under these conditions, sucrose is also partially cleaved into reducing sugars); Maillard reactions between reducing

sugars and amino acids or proteins; Strecker degradation of dicarbonylic compounds with amino acids; hydrolysis of ester and glycosides of antioxidants; and lipid oxidation. Phenolic antioxidants are oxidised too, with formation of quinones and their polymers and copolymers. Enzymatic or chemical oxidation of polyphenols is generally responsible for a loss in their antioxidant capacity; however, recent observations suggest that partially oxidised polyphenols can exhibit higher antioxidant activity than that of non-oxidised phenols (Nicoli *et al.*, 2000).

In inner layers, changes are much the same as during boiling. Tocopherols and tocotrienols present in wheat and rye are partially destroyed during baking. In ordinary wheat bread, losses of α -tocopherol amount to about 25%, but in the case of rye bread, prepared by traditional technology, a loss of about 50% was reported (Piironen *et al.*, 1987). Losses of natural antioxidants in coffee brews and tomato puree were also observed (Nicoli *et al.*, 1997).

Losses of antioxidants during roasting are higher than losses during baking as the temperature is higher (up to 250°C), e.g. in the case of coffee roasting or in grilling beef. In the case of beef, natural antioxidants, such as tocopherols, are destroyed only on the surface, and some synergists, such as phospholipids and free amino acids, deteriorate.

The antioxidant effect of aqueous extracts from barley roasted at different temperatures and extracted with water was investigated recently (Duh *et al.*, 2001). All extracts exhibited considerable antioxidant activity, but unroasted samples were more effective and had higher antioxidant properties. This could be partially explained by the changes in catechin, tocopherol and lutein contents during roasting. Antioxidant activities of commonly consumed polyphenolic beverages (coffee, cocoa, and tea) were compared also (Richelle *et al.*, 2001). Under standard cup-serving conditions, the antioxidative activities of all beverages were high. Soluble coffee had the highest antioxidative activity and addition of milk did not affect this. Green coffee beans of robusta coffee had higher antioxidative activity than arabica coffee, but after roasting this difference was not significant.

The Maillard reaction in model and real food systems is known to generate flavour compounds and also compounds possessing anti-mutagenic and antioxidant activity. Decomposition of antioxidants during baking and roasting is thus partially compensated by formation of Maillard products, which also possess antioxidant activity, partially due to their metal chelating capacity. Maillard reactions are very slow below 130°C but, at higher temperatures, their rate rises rapidly. Therefore, the surface of baked and roasted products is dark brown. Reducing sugars react with free amino acids and free amine groups of proteins. An interaction product of glucose and lysine inhibits the peroxidation of lipids (Munari *et al.*, 1995). Dehydroascorbic acid, an oxidation product of ascorbic acid, is essentially a reducing sugar too, and participates in Maillard reactions. A pyrone derivative formed by the reaction of dehydroascorbic acid with the free primary amine group of aspartame shows moderate, but still significant, antioxidant activity in fats (Sakurai *et al.*, 1997).

The end products of Maillard reactions are brown coloured, macromolecular substances called melanoidins. Different intermediary products of melanoidin formation have reducing properties (they react with the Folin–Ciocalteu reagent) and absorb oxygen present in the heated system, thus saving antioxidants. Maillard products, as we saw, possess a metal chelating capacity. They bind iron, copper and other metals into inactive complexes which do not catalyse lipid oxidation. Non-enzymatic browning is not always a positive change during food processing and storage. Pro-oxidants may be formed during the early phases of the Maillard reaction. It is likely that colour changes in the later reaction stages could be related to the overall antioxidant activity of the product. Recent results indicate that colour can be considered as an index of the overall antioxidant properties of foods whenever the mechanisms responsible for the formation of antioxidants and colour follow the same pathway and are strictly known (Manzocco *et al.*, 2001). The activity of Maillard products is stronger in lipid emulsions than in dry systems or in bulk fats (Severini and Lerici, 1995). Maillard products are found to be active as moderate oxidation inhibitors in biscuits, confectionery and sausages (Yamaguchi, 1988) and in cereal extruded products (Yokota *et al.*, 1987).

Drying operations consist in reduction of the water content in foods from 30–70% to only 6–15%. In the original foods, lipid droplets, films, liposomes and lipid membranes are protected against the access of air oxygen by layers of hydrated proteins. The protective layer is destroyed during drying so that lipids and lipidic antioxidants become exposed to free access of air. The oxidation of lipids in dry products is rather rapid, and lipid oxidation products destroy antioxidants. Some antioxidants, such as tocopherols, react with oxygen in dry products too, so that antioxidant losses could be quite considerable. The lipid oxidation and antioxidant losses can be reduced by short drying time and lower pressure. This is because the reduced pressure would require lower drying temperatures, thus allowing the oxidation rate to be reduced.

15.5 Changes during heating: where energy is transferred in waves

Microwave and infrared energy are both transmitted as waves which penetrate food and are converted into heat. Microwave cooking is now the most rapidly expanding meal preparation technology in the world, in households, restaurants and food service plants. The procedure is very short and energy saving. Microwaves induce molecular friction in water molecules to produce heat, while the infrared energy is simply absorbed and converted to heat. Heating by microwaves is determined in part by the water content of the food. In contrast, the extent of heating caused by the application of infrared energy depends on the colour and other characteristics of surface layers. Fats and oils are rapidly oxidised by longer application of microwaves (Lanzón *et al.*, 1997). The heating should thus be stopped after the shortest time necessary.

Tocopherols and other liposoluble antioxidants are partially destroyed during microwave cooking of oilseeds (Shahidi *et al.*, 1997). About 10% are destroyed during the first 6 min of microwave heating, and the losses of tocopherols increase up to 40% during the next 6 min of microwave application (Yoshida and Kajimoto, 1989). The 30-min microwave roasting of sesame seeds resulted in burnt odour and bitter taste, and significant differences in oil quality and fatty acid composition were detected as well. However, after 16 min of roasting no significant differences were found and so the high oxidative stability of sesame oils may be due to synergism between endogenous antioxidants and browning substances produced during the first 15 min of microwave roasting (Yoshida *et al.*, 1995). The use of microwave heating for the reduction of enzymatic browning in mushrooms led to a shorter inactivation time of polyphenol oxidase compared to conventional blanching techniques with hot water treatment (Rodriguez Lopez *et al.*, 1999). However, microwave treatment alone can result in an overtreatment in hot areas and incomplete inactivation of polyphenol oxidase in others (Devece *et al.*, 1999).

The effect of tert. butylated hydroxy toluene (BHT) and natural antioxidants, such as sage and rosemary extracts, was tested during microwave cooking, but the antioxidants were effective only in lard (Sekretár *et al.*, 1999). Their effect was only moderate in sunflower oil, and no effect was observed in rapeseed oil. The potential antioxidant activity of BHA/BHT and citric acid on the oxidation of corn oil had no effect under the high-temperature conditions of microwave heating (Vierira and Regitano d'Arce, 1999). In spite of wide spread use of microwave cooking, the amount of data on changes of lipid and antioxidants during the microwave application is scarce, and more research needs to be done in this area.

15.6 Changes during heating: oil as the heat transfer medium

Frying is a process in which food is heated in contact with hot oil. In the case of shallow frying, the layer of oil in the pan is only 2–20 mm thick, so that food is only partially immersed. The frying oil is not usually used for further frying, but it is used for flavouring the fried meal.

During deep fat frying, fried food floats on the surface of hot frying oil or is completely immersed in it. The layer of frying oil is up to about 200 mm thick in this case. Oil is preheated to 150–200°C, but the temperature decreases to 130–180°C on contact with the food. The frying proceeds at about 150–180°C for 2–10 min, according to the size of the fried material pieces. In some countries, they prefer higher frying temperatures, but they should be avoided because products fried at high temperatures contain carcinogens.

Changes of antioxidants in frying oil are usually very pronounced as it is used repeatedly for frying, sometimes for several days or even weeks. In the case of intermittent frying, hot frying oil is left to cool without further

heating after the operation, and the heating is resumed at the next frying process, usually on the next day. Frying oil usually contains tocopherols and in some cases other antioxidants as well. Some antioxidants, especially BHT and other relatively non-polar synthetic antioxidants or essential oils present in natural antioxidants (which also possess some antioxidant activities), evaporate with water vapour from the frying medium. Therefore, non-volatile antioxidants such as tocopherols or rosemary or sage resins or extracts should be used for the protection of frying oils.

Frying oil contains dissolved oxygen, but that oxygen is consumed before the oil is heated to the frying temperature. Additional oxygen enters only by diffusion from air so that its concentration remains at a very low level unless the frying oil starts foaming. Foam increases the contact area of oil with air. The lipid oxidation proceeds only slowly before foaming occurs but, still, antioxidants are gradually oxidised. Tocopherols present in frying oils are decomposed either directly by molecular oxygen or indirectly by interaction with lipid free radicals, formed by decomposition of lipid hydroperoxides (Kajimoto *et al.*, 1989). Antioxidant free radicals produced by interaction of antioxidants with free lipid radicals can combine with each other or with free antioxidant radicals to produce antioxidant polymers or copolymers.

The tocopherol degradation in frying oils was retarded by addition of spices or their extracts, such as rosemary oleoresins. Carnosol, an active principle present in rosemary, is decomposed faster than tocopherols during frying (Trojáková *et al.*, 2000). Ascorbic acid or ascorbyl palmitate decomposes into a variety of products in parallel with lipid oxidation. Green tea extracts are active antioxidants even under frying conditions, as they are non-volatile. Galocatechins and the respective gallates are more resistant during frying than tea catechins (Kajimoto *et al.*, 1988).

Destruction of antioxidants in frying oil is inhibited by the restricted access of oxygen from air. Air diffusion may be prevented by using floating metal sheets on the surface of frying oil or by adding polysiloxanes to oil before frying. They form a very thin layer of only a few molecules on the surface of frying oil, so that only a few mg/kg are sufficient for the formation of a layer. The diffusion of oxygen through the silicone layer is very slow so that the concentration of dissolved oxygen is kept at a low level. Oxidation reactions thus become much slower, too. The use of polysiloxanes has been approved by authorities for use in frying oils as they are not resorbed and they are considered, therefore, to be safe.

The destruction of antioxidants in fried food is much lower than in frying oil as the contact of food with frying oil is only short. Antioxidants are oxidised only in the surface layers. The deep frying takes only a few minutes so that only the outer layers of deep fried food are heated to 150–180°C, but the inner layers are not oxidised to more than 100°C in such a short time. Therefore, losses of antioxidants in the inner layers are negligible. Many food components, such as proteins, flavonoids or tannins, inhibit the destruction of antioxidants in fried food during the frying operation (Pokorny, 2000).

Oxidation reactions of fried food occur rather intensively on storage as the thin film of frying oil left on the surface of fried food is easily attacked by oxygen from the air. The stabilisation of fried food against oxidation on storage thus offers a more important problem than does oxidation during the frying operation.

15.7 Changes in antioxidants during non-thermal processes

Many food-processing operations do not require heat application. They proceed either at or close to ambient temperature. Smoking and curing meat products or fish are typical methods of preservation. Smoking is a traditional method of preserving foods, since the smoke can have anti-microbial and antioxidant properties. Smoke and smoke condensates contain numerous phenolic and aldehydic compounds, produced by pyrolysis of wood phenolics, glycosides and lignin. During smoking, they are mainly bound to meat proteins but, nevertheless, they do not lose their antioxidant activity, and still increase resistance to oxidation, and protect tocopherols against oxidation by lipid free radicals. Lipoygenases and other oxidoreductases are partially deactivated in the process. Smoking of food products with smoke is being replaced increasingly by the use of smoke aromas, which can be smoke extracts, aromatic smoke preparations, smoke condensation, or liquid smoke. Liquid smoke is generally regarded as a safe product and may reduce some of the health risks associated with smoked foods. Some of the characteristic advantages of smoke aromas are economic and ecological benefits, versatility, consistent quality and anti-oxidant and anti-microbial effects (Guillen *et al.*, 1996).

Curing often precedes smoking. It consists of application of nitrites, nitrates and sodium chloride to protect meat against bacterial spoilage and to prevent discolouration. The advantage of nitric oxide, originating from curing salts, is its ability to inhibit both lipoygenases and cyclooxygenases. They deactivate haemoglobin by converting it to relatively stable nitroxylhaemoglobin. If ascorbic acid is present (it is often added in curing salts), it reduces nitrites into NO, which reacts with ferricytochrome c, forming ferricytochrome c nitroxyl derivatives (Izumi *et al.*, 1989).

Nitrite could be regarded as an antioxidant as it reduces the oxidation of polyenoic fatty acids, but its activity decreases if the product is being cooked for a long time or heated to high temperatures. The antioxidant activity of cooked ground beef or pork was found to be 1.5–3 times better than that of untreated meat on storage at 4°C (Zubillaga and Marker, 1987). However, reaction of nitrites with secondary amines in meat can produce N-nitrosoamines, which have been shown to be carcinogenic in animal experiments. Therefore, alternatives to sodium nitrite, or a considerable reduction in the quantities traditionally used, have been widely investigated (Thiemig *et al.*, 2000; Pegg *et al.*, 2000). Incorporation of ginger extract in the standard curing solution

reduced oxidation and microbial counts and improved the sensory properties of the smoked product (Naveena *et al.*, 2001).

Preparation of emulsified products takes place at room or refrigerated temperatures. No particular damage was observed during the process because of the low temperature and short time. Air bubbles should not be whipped into the emulsion, as air could enhance future oxidation. Fermentation is an anaerobic process, i.e. proceeding either in the absence of oxygen or in the presence of only traces of oxygen. It proceeds either at room temperature or at slightly lower temperatures. Therefore, antioxidants are not damaged during fermentation to any significant degree. Besides, in many fermentation products (for example cheese), microorganisms secrete vitamins and various reducing compounds into the food and improve its nutrition value and antioxidant activity.

15.8 Changes in antioxidants during storage

Storage occurs at ambient or still lower temperatures so that the extent of oxidation and the subsequent antioxidant damage are slow. Nevertheless, after long storage times of several months or even years, they may become quite considerable. The most frequent use of antioxidants is to improve the stability of fats, oils and emulsified fat products. They usually contain natural antioxidants, especially tocopherols, and additional antioxidants are sometimes added, especially to lard. They are mostly stored at 15°C or at even lower temperatures. In our experiments, the content of tocopherols did not substantially decrease during storage for a year in the refrigerator. Pork lard contains no natural antioxidants and, therefore, lard was the first fat to which antioxidants were applied. At the present time, lard is still stabilised mostly by the addition of synthetic antioxidants. Antioxidants are still more stable during the storage of lard than in edible oils, as the polyunsaturated acid content, and thus the initiation rate of oxidation, are only low in lard. Olive oil contains tocopherols and natural antioxidants derived from tyrosol. These antioxidants are also very stable on cold storage because of the low linoleic acid content in olive oil.

Meat products have to be stabilised in some cases, as meat lipids contain no natural antioxidants or only traces of tocopherols. Most muscle foods contain, however, an efficient multi-component antioxidant defence system based on enzymes, but the balance changes adversely on storage. The denaturation of muscle proteins is the main cause of the imbalance as iron may be released from its complexes, catalysing the lipid oxidation. Salting contributes to the negative effects of storage, as it enhances oxidation. Using encapsulated salt eliminates the deleterious effect of sodium chloride.

Positive components in meat are free amino acids and phospholipids, which are active both in lard and in edible oils (Nasner, 1985). The ethanol-

soluble phospholipid fraction is more active than the ethanol-insoluble fraction. A synergism exists between phospholipids and α -tocopherol, particularly in fish (Weng and Gordon, 1993), probably due to a reaction between tocopherol oxidation products and phosphatidylethanolamine. Phospholipids decompose lipid hydroperoxides, converting phosphatidylcholine into trimethylamine oxide, which reduces another molecule of lipid hydroperoxide into a ketone (Ishikawa *et al.*, 1978). The decomposition of phenolic antioxidants is thus reduced by phospholipids.

Mincing, cooking and maturing expose meat products to oxidative stress for a long time so that antioxidants added for lipid protection are slowly destroyed on storage. Onion juice is a powerful antioxidant in meat products, more efficient than garlic juice. Lipid hydroperoxides are reduced to inactive hydroxyl derivatives by reaction with sulphur compounds present in those juices.

Chilling reduces the rate of biochemical and microbiological changes, and extends the shelf life of fresh and processed foods. It causes minimal changes to the sensory characteristics and nutritional properties of foods. However, so-called chilling injury can occur in fruit and vegetables, particularly of tropical and subtropical origin, that are stored below 12°C. The efficacy of the different post-harvest treatments, such as hot-water dipping of mandarins, appears to be related to the induction of enzyme activity during heating and its continuation during cold storage (Sala and Lafuente, 2000). Catalase and superoxide dismutase act as antioxidants that protect the fruit and vegetables against chilling stress. Equally, elevated growing temperatures improve the storage life of cucumbers and reduce chilling injury at a temperature of 10°C (Kang *et al.*, 2002).

On frozen storage, water is crystallised into ice, and air has free access to the lipidic phase. The immobilisation of water to ice and the resulting concentration of dissolved solutes in unfrozen water lower the water activity of food. However, the oxidation of lipids may be quite rapid in spite of the low temperature, and antioxidants present in the food are destroyed. It is advisable to add natural antioxidants, such as spices, into frozen foods or, at least, to apply them on the surface of frozen poultry meat and, especially, fish. Meats have a more flexible fibrous structure, which separates during freezing instead of breaking, but in fruits and vegetables ice crystals may damage the more rigid cell structure. Freezing of apple flesh tissue has been shown to inactivate catalase, an enzyme that protects cells from oxidation by hydrogen peroxide. Freezing caused a 50–90% loss in catalase activity compared with that in fresh apple tissue and, moreover, slower freezing caused a greater loss in activity than rapid freezing (Gong *et al.*, 2000). With raspberries, frozen storage for 12 months resulted in decreases in the content of ellagic acid, the phenolic compound with antioxidant attributes, and vitamin C. The freezing process itself decreased the free radical scavenging capacity, but frozen storage had no significant further effect on it (Ancos *et al.*, 2000). Fried products are another field for antioxidant application. It is possible

either to stabilise frying oil or to apply antioxidants on the surface of fried products after frying. With wine the flavonoids and anthocyanins are sufficiently stable on storage, as it is stored under very low oxygen pressure. The same is true of beer. Nevertheless, after opening the bottle wine should be consumed on the same day as air freely enters the opened bottle and wine phenolics are rapidly oxidised. Very slight oxidation may be advantageous in red wines for sensory reasons. In common with wine, most fruit juices contain plenty of phenolic antioxidants, mainly flavonoids. Natural ascorbic acid is rapidly destroyed on processing and storage, but it is often added after the processing is complete. Flavonoids are more stable.

It is not only lipids but also essential oils which are sensitive to oxidative changes on storage. Sometimes stabilised by synthetic or natural antioxidants, they usually contain substances showing moderate antioxidant activity, but these may be lost by evaporation or oxidised by air oxygen unless more powerful phenolic antioxidants are added.

15.9 Future trends

Synthetic antioxidants are safer, cheaper and purer than natural antioxidants but, nevertheless, the majority of consumers still prefer natural antioxidants. This trend will surely persist in the near future. The mechanisms for the changes of synthetic antioxidants are well known, but the same cannot be stated in the case of natural phenolic antioxidants. They are usually pyrocatechol or pyrogallol derivatives, where the changes during oxidation could be different from those of synthetic antioxidants, which are mostly 1,4-substituted.

The most common natural antioxidants are tocopherols, ascorbic acid and β -carotene (more often synthetic nature-identical compounds than natural products). Their changes were studied in detail in model systems, fats and oils, but experimental evidence is mainly lacking on more complicated systems, such as natural foods and ready dishes. Still less is known on different antioxidants from spices and from essential oils. These data will probably be obtained gradually. Very little is known about synergism of antioxidants in food products other than edible fats and oils or their regeneration from the respective free radicals and quinones. In mixtures, some antioxidants are preferentially destroyed and others are saved. Some data have already been published, but these complex changes should be studied in more detail.

The protection of foods from oxygen is the basic principle upon which antioxidant protective technologies are now based. The contribution of food technology both to food safety and to the maintenance of high nutritional and sensory value should not be underestimated. The mechanisms of antioxidant destruction and the composition of reaction products during miscellaneous technological steps are different, depending on the concentration of free radicals and on oxygen pressure and process temperature. This point of view

needs further research; however, it is clear that monitoring for the retention of antioxidants throughout processing and storage is needed. To do this, it may be necessary either to develop rapid methods of monitoring the survival of the antioxidants themselves, or to measure secondary effect such as, in the case of oils and fats, peroxide values (Lindley, 1998).

Nowadays, consumers would like those antioxidants present in food products not only to stabilise food lipids, but also to be absorbed through the intestinal wall and protect the lipids of blood plasma against oxidation. This effect is relatively evident in the case of tocopherols (which are liposoluble) or ascorbic acid (which is hydrophilic), but much less evidence is available on antioxidants of medium polarity, such as flavonoids, rosemary oleoresins or green or black tea catechins.

Wine phenolics were recently reported as being a very important source of antioxidants. The effect of wine in the Mediterranean diet should not be over-rated, as many more antioxidants are consumed in fruits and other non-fermented foods (Richelle *et al.*, 2001). Other factors, such as ethanol, consumption of olive oil and vegetables, and an easier way of life, are also very influential. Nutritional factors are widely considered to be critical for human health. Overwhelming evidence from epidemiological studies indicates that diets rich in fruit and vegetables are associated with a lower risk of several degenerative diseases. However, the health-promoting capacity of such food is wholly dependent on processing history. This aspect has generally been neglected or scarcely considered in current nutritional and epidemiological studies (Nicoli *et al.*, 1999). Processing is expected to affect the content, activity and availability of bioactive compounds, so the requirement to better understand the role and fate of natural and process-induced antioxidants on both food stability and human health leads to a need for further research.

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Optimising the use of phenolic compounds in foods

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16.1 Introduction

A major challenge for the modern food industry is to make products which combine convenience with freshness and healthy eating. Precooked meat, dried foods and various types of ready-to-eat products, including dishes delivered to senior citizens as part of food service systems, often acquire a stale flavour and lose important nutrients on reheating (Skibsted *et al.*, 1998). The two most important types of chemical reactions responsible for quality loss of processed food have been identified as browning and oxidation (Löfger, 1991). For certain dried food products like soup powders, milk powder and infant formula, browning reactions and lipid oxidation reactions are interrelated and further linked to phase-transitions like lactose crystallisation in dried milk products (Knudsen *et al.*, 2002). Oxidative changes in foods are mainly related to the unsaturated lipids, but also involve proteins and essential nutrients like the vitamins. The different phases in progression of oxidative deterioration in a food system are outlined in Fig. 16.1. Protection of foods against oxidative deterioration depends on a combination of optimisation of processing parameters, use of antioxidants and optimised packaging systems. As for the use of antioxidants, synthetic antioxidants are continuously being replaced by natural antioxidants from plant sources, mainly due to market requirements.

16.1.1 Oxidative deterioration of food

Oxidation is initiated by formation of radicals which may be the result of enzyme catalysed reactions like oxygen activation by xanthine oxidase in

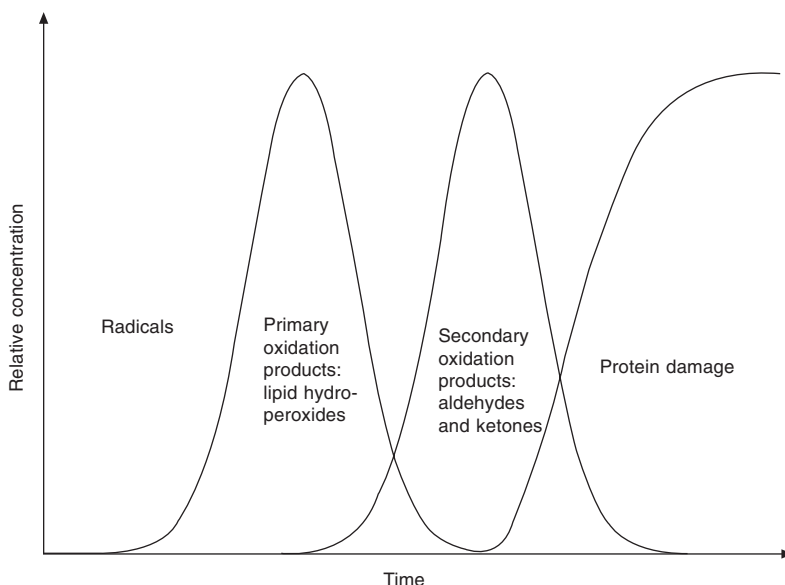


Fig. 16.1 Progression of oxidation in a food system from formation of radicals through primary and secondary lipid oxidation products to protein damage.

milk (Kristensen *et al.*, 2002). Light exposure is also known to induce radical processes in most food products. In milk light absorption thus results initially in protein oxidation followed by lipid oxidation, while in beer free-radical formation has been identified as a primary step in the photodecomposition of isohummulones leading to light struck flavour (Burns *et al.*, 2001). In precooked meat and in meat products, iron catalysed cleavage of preformed peroxides is important for formation of hydroxyl (or alkoxy) radicals initiating oxidative degradation of unsaturated lipids or proteins:



Lipid hydroperoxides are either formed in an autocatalytic process initiated by hydroxyl radicals or they are formed photochemically. Lipid hydroperoxides, known as the primary lipid oxidation products, are tasteless and odourless, but may be cleaved into the so-called secondary lipid oxidation products by heat or by metal ion catalysis. This transformation of hydroperoxides to secondary lipid oxidation products can thus be seen during chill storage of pork (Nielsen *et al.*, 1997). The secondary lipid oxidation products, like hexanal from linoleic acid, are volatile and provide precooked meats, dried milk products and used frying oil with characteristic off-flavours (Shahidi and Pegg, 1994). They may further react with proteins forming fluorescent protein derivatives derived from initially formed Schiff bases (Tappel, 1956).

16.1.2 Classifying natural antioxidants

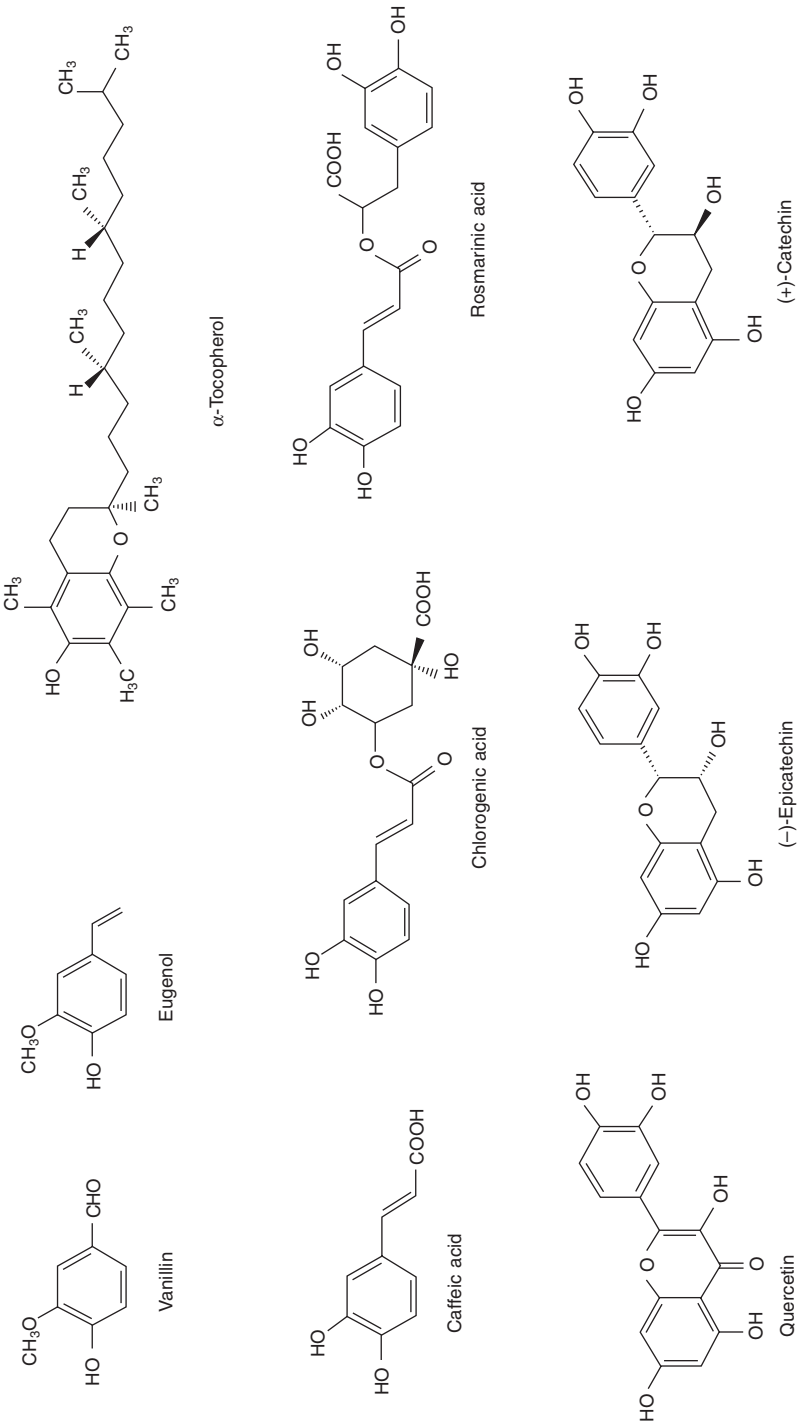
Antioxidants may be active at different stages of the progression of oxidation in food systems. The tocopherols, of which α -tocopherol is vitamin E, are associated with the lipid fraction of food, scavenge radicals and become chain-breaking in the autocatalytic process of lipid oxidation. Ascorbic acid is water-soluble and may deplete oxygen in the product or regenerate α -tocopherol from its one-electron oxidised form in the lipid/water interface, as has been exploited in patented protection systems for lard and marine oils (Löföger, 1991). Metal binders like ethylenediamine tetraacetic acid (EDTA) prevent metal catalysis during initiation of oxidation and have been found to be effective in protecting products like mayonnaise by preventing the formation of radicals in the interphase between water and lipid in the food emulsion (Jacobsen *et al.*, 2001). Carotenoids yield protection against light-induced oxidation processes, as they deactivate singlet-oxygen formed in sensitised processes, or by absorbing harmful light, as was found for dairy products, and probably also by scavenging of free radicals (Hansen and Skibsted, 2000.)

Plant phenolic compounds have antioxidative properties, as has been clearly demonstrated in numerous model systems (Schwarz *et al.*, 2001). It is, however, interesting to note that plant phenols apparently operate as antioxidants by all of the mechanisms described above for the different types of antioxidants. Plant phenols are thus radical scavengers and chain-breaking antioxidants under certain conditions (Bors *et al.*, 1990). Plant phenols may also regenerate other antioxidants and act synergistically with chain-breaking antioxidants under other sets of conditions (Pedrielli and Skibsted, 2002). Polyphenols such as the flavonoids and anthocyanidins have metal chelating properties and bind otherwise redox-active metal ions. Finally plant phenols protect plants against UV-irradiation and light-induced oxidative damage and may have similar effects in food and beverages.

The conditions for which the different antioxidative mechanisms contribute to protection of an actual food product are, however, not well understood. Accordingly, the challenge to the food scientist is to optimise the use of plant extracts, plant waste rich in polyphenols from production of vegetable products, juices or alcoholic beverages, and of herbs and spices for protection of processed food (Hu and Skibsted, 2002a). Moreover, such material of botanical origin may have health benefits not only by keeping the food fresh, but also by an antioxidative effect in the body following absorption.

16.1.3 Nutritive and non-nutritive antioxidants

The chemical formulae for a variety of plant phenols are given in Fig. 16.2, including examples of simpler phenols, such as cinnamic acid derivative, and of tocopherols, flavonoids, flavonoid glycosides and anthocyanidins. The flavonoids include the following subclasses: flavanones (taxifolin), flavones (luteolin), flavonols (quercetin) and flavanols (catechin/epicatechin). The



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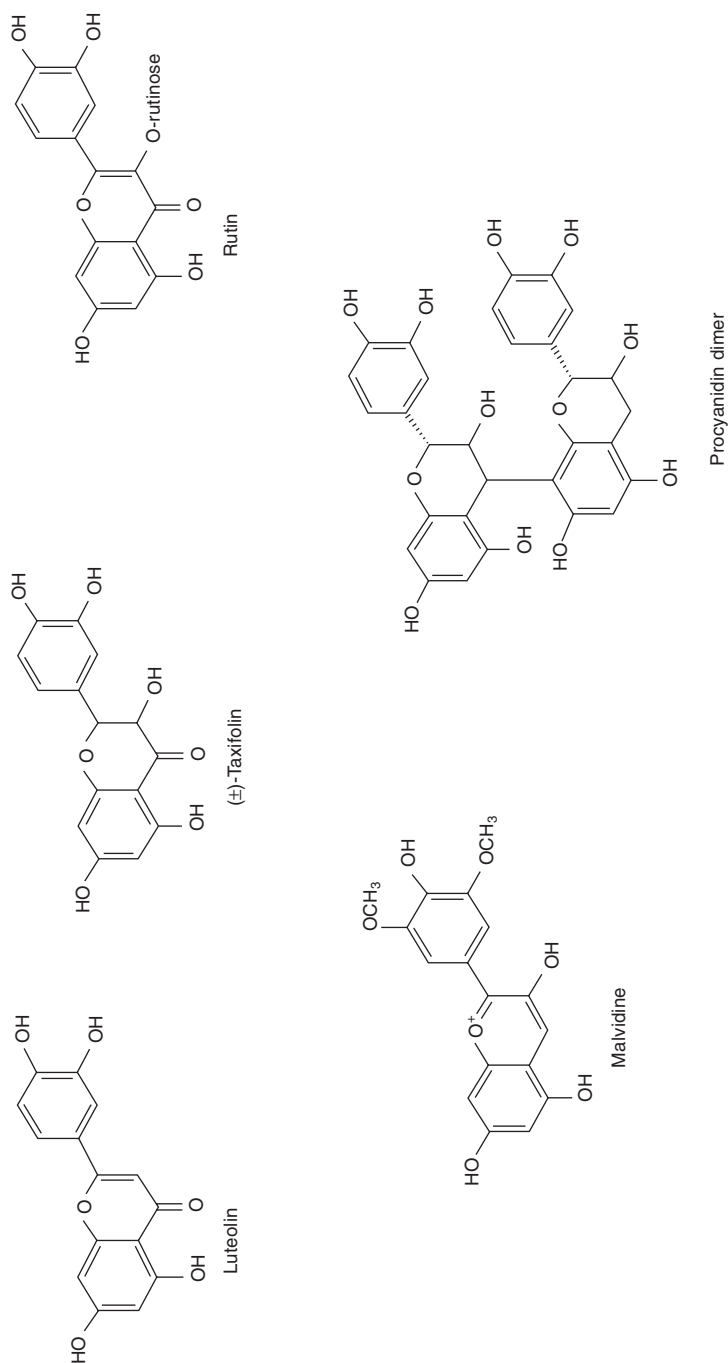


Fig. 16.2 Examples of plant phenols with antioxidant potential in foods.

	Hydrophilic	Hydrophobic
Nutritive	Ascorbate	α -tocopherol
Non-nutritive	Polyphenols	Carotenoids

Fig. 16.3 Classification of antioxidants according to solubility and nutritive value.

flavonoids and the anthocyanidins and proanthocyanins are all 1,3-diphenyl propane derivatives, which, together with the cinnamic acid derivatives, are the most widespread non-nutritive antioxidants.

Natural antioxidants may be classified according to their nutritive value or according to their solubility. The hydrophobic vitamin E and the hydrophilic vitamin C are thus important both as nutrients and as antioxidants. The non-nutritive antioxidants may similarly be divided into lipid-soluble and water-soluble antioxidants, as shown in Fig. 16.3, which will also form the basis for a discussion of exploitation of combinations of antioxidants in order to improve protective effects.

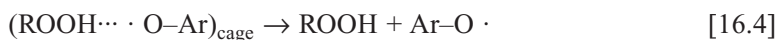
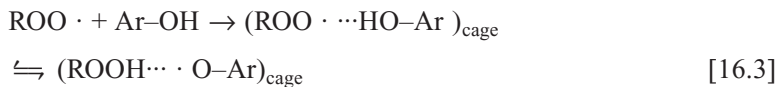
16.2 Analysing antioxidant activity in food

Epidemiological studies have shown an inverse correlation between the intake of fresh fruit and vegetables and the risk of cardiovascular diseases and certain forms of cancer (Hertzog *et al.*, 1993; Steinmetz and Potter, 1996; Terry *et al.*, 2001). These effects have been attributed to the antioxidant content of fruits and vegetables, although it has been difficult to show a direct positive effect on markers of oxidative status after dietary intervention (Young *et al.*, 2002). In contrast, positive antioxidant effects of plant extracts rich in polyphenols have clearly been demonstrated in a large variety of foods and beverages using various methods for detection of lipid and protein oxidation (Nissen *et al.*, 2002). It has often been assumed that the basic antioxidant mechanism of plant phenols is the same in the living organism following intake as it is in foods rich in such antioxidants or enriched with plant extracts. The discussion of antioxidant mechanism will be rather general in order to cover both aspects, although most examples will be related to the better documented effects in model systems and in food and beverages.

16.2.1 Mechanism of antioxidant

Among the plant phenols, the flavonoids and the anthocyanidins, belonging to the 1,3-diphenylpropanes, have been studied in most detail, mainly because of their potential health benefits. With more than 4,000 different flavonoids known, systematic studies of the effects of variation in molecular structure on physico-chemical properties of importance for antioxidative effects have also been possible (Jovanovic *et al.*, 1994; Seeram and Nair, 2002). Flavonoids were originally found not to behave as efficiently as the classic phenolic antioxidants like α -tocopherol and synthetic phenolic antioxidants in donating

a hydrogen atom to a lipid peroxy radical, the chain-carrying species in lipid autoxidation (Roginsky *et al.*, 1996). Both the inhibition rate constant and the number of radicals quenched by each flavonoid showed a strong dependence on the antioxidant concentration, an observation not in agreement with the classic antioxidant mechanism. Solvent effects are, however, important and hydrogen-accepting 'water-like' solvents induce dramatic decreases in the hydrogen-atom donating capability of the phenols (Valgimigli *et al.*, 1995). The inhibition reaction in the autocatalytic lipid oxidation by a phenolic antioxidant occurs in two steps:



and the initial complex formation of eq. 16.3. may be hindered due to a preferential formation of a hydrogen bonded complex between the flavonoid, Ar-OH, and solvent molecule (Foti and Ruberto, 2001). Such an effect was clearly seen for the flavonol quercetin and the flavanol epicatechin, flavonoids which are both widespread in the plant kingdom (Pedrielli *et al.*, 2001a).

The solvent effect between chlorobenzene and *tert*-butyl alcohol for the inhibition reaction corresponding to the net reaction of eqs 16.3 and 16.4 was a factor of 20 for the flavonoids compared to a factor of 4 for α -tocopherol, with the rate constant being slower in the 'water-like' solvent *tert*-butyl alcohol than in the non-hydrogen-bonding solvent chlorobenzene, see Table 16.1. In contrast to α -tocopherol, the flavonoids will only be retarders of lipid oxidation without a distinct lag-phase when the phenol is in contact with water or a 'water-like' solvent. Notably, certain flavonoids like quercetin are almost as efficient as α -tocopherol in solvents without hydrogen-bonding capabilities. Specific solvent effects in the micro-environment of the flavonoid may accordingly provide the rationale for the variation seen for the antioxidative capacity of the same flavonoids. Flavonoids occur primarily as glycosides in plants, and in foods these water-soluble forms are not expected to be efficient chain-breaking antioxidants due to the hydrogen-donor accepting capability of water. In aqueous environments, another mechanism may, however, be important, since electron-transfer becomes facilitated through stabilisation of an ion-pair (Foti and Ruberto, 2001):



Under aqueous conditions, flavonoids and their glycosides will also reduce oxidants other than peroxy radicals and may have a role in protecting membranal systems against pro-oxidants such as metal ions and activated oxygen species in the aqueous phase. Rate constants for reduction of superoxide anion show flavonoids to be more efficient than the water-soluble vitamin E analogue trolox (Jovanovic *et al.*, 1994), see Table 16.1.

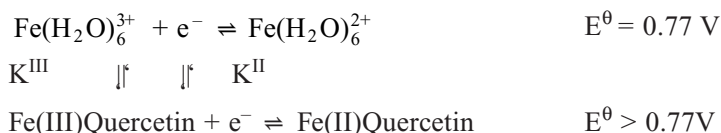
Table 16.1 Thermodynamic and kinetic parameters for plant phenols for their antioxidant capacity and antioxidant activity

	E^{θ} (V) aqueous solution ^a	E^{θ} (V) dimethyl- formamid ^b	k_{inh} (l·mol ⁻¹ ·s ⁻¹) t-butyl alcohol ^c	k_{inh} (l·mol ⁻¹ ·s ⁻¹) chlorobenzene ^d	$k_{O_2^{\cdot-}}$ (l·mol ⁻¹ ·s ⁻¹) aqueous solution ^e	k_{ferry} (l·mol ⁻¹ ·s ⁻¹) aqueous solution ^f
Caffeic acid	0.36					65 ^k
Chlorogenic acid	0.37					216 ^g
α-tocopherol	–	0.93	6.3·10 ⁵ . ^d	3.5·10 ⁶ . ^d		
Trolox	0.31	–			5.8·10 ³	20
Quercetin	0.29	1.07	2.1·10 ⁴	4.3·10 ⁵	4.7·10 ⁴	279
Catechin	0.36	1.16	1.5·10 ⁴	4.4·10 ⁵ . ^h	1.8·10 ⁴	
Epicatechin	–	–	1.7·10 ⁴	4.2·10 ⁵		20
Epigallocatechin gallate	0.43 ⁱ				7.3·10 ⁵ . ^j	1170 ^j
Taxifolin	0.37	1.20				18
Luteolin	0.41	1.15				63
Rutin	0.40				5.1·10 ⁴	23
Apigenin	0.71					31
Ascorbate	0.22					16

^apH = 7.4 at 25°C (Jørgensen and Skibsted, 1998); ^b25°C (Jørgensen *et al.*, 1999), ^c50°C (Pedrielli *et al.*, 2001a, Pedrielli *et al.*, 2001b); ^dcalculated from data in Valmignoli *et al.*, (1999) and in Niki *et al.*, (1984); ^epH = 10 and 20°C (Jovanovic *et al.*, 1994); ^fpH = 7.4 and 10°C, except otherwise noted (Jørgensen and Skibsted, 1998); ^gpH = 7.4 and 25°C (Carlsen *et al.*, 2000); ^h(Pedrielli and Skibsted, 2002); ⁱpH = 7 and 10°C (Jovanovic *et al.*, 1995); ^jpH = 7.4 and 25°C (Hu and Skibsted, 2002b); ^kpH = 7 and 37°C (Castellucio *et al.*, 1995).

Flavonoids have also been identified as metal chelators, and metal binding should prevent metal-ion catalysed formation of radicals (Cheng and Breen, 2000). The copper(II) complex of quercetin is, however, rather weak (Pusz and Kopacz, 1992; Mahgoub and Hudson, 1985), and the binding of iron has been difficult to characterise quantitatively (Cheng and Breen, 2000). It should, however, be noted that chelation of iron by any flavonoid will create a new redox-couple with a standard potential different from $E^\theta = 0.77$ V, the value for the $\text{Fe}(\text{H}_2\text{O})_6^{3+}/\text{Fe}(\text{H}_2\text{O})_6^{2+}$ couple. For flavonoids which bind iron(II) stronger than iron(III), the iron(II) forms become less reducing, and flavonoids like quercetin may prevent the Fenton reaction by such a mechanism (Ferrali *et al.*, 1997):

Scheme 1



Iron(II) complexes of flavonoids like quercetin could, however, be involved in the reduction of peroxides:



but the hydroxyl radical may react directly with quercetin complexed to iron rather than escaping the encounter complex. The reaction of eq. 16.6, is not a chain-breaking reaction and probably has little influence on the protection against oxidation.

Most flavonoids have E^θ values smaller than E^θ for the $\text{Fe}(\text{H}_2\text{O})_6^{3+}/\text{Fe}(\text{H}_2\text{O})_6^{2+}$ couple (see Table 16.1) and could reduce iron(III) to iron(II), in effect being active in the Fenton reaction (Hardwick *et al.*, 1988). The complicated interaction between iron and flavonoids, as outlined in Fig. 16.4, and the effect of this interaction on generations of radicals and on radical quenching deserves further clarification in order to optimise the use of flavonoids as antioxidants and to prevent pro-oxidative effects. The most reducing agents are in general also the most efficient radical scavengers, and the balance between antioxidative effect based on radical scavenging and pro-oxidative effect based on iron activation is very delicate. The most reducing flavonoids like quercetin may even autoxidise, i.e. reduce molecular oxygen to superoxide in a process also catalysed by iron:



in effect initiating radical reactions, since the phenoxyl radical, quercetin $^\cdot$, is more reducing than quercetin (Canada *et al.*, 1990). For anthocyanins, oxidation of ascorbate catalysed by copper(II) was inhibited, probably by formation of a mixed ligand ascorbate/anthocyanin copper complex (Sarma *et al.*, 1997). Ascorbate and wine polyphenolics were also found to interact synergistically against copper(II) induced oxidation of low-density lipoproteins (Ivanov *et al.*, 2001).

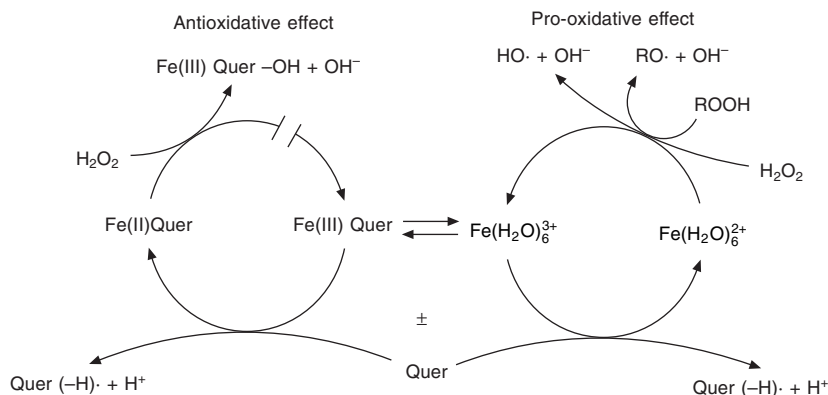


Fig. 16.4 Interaction between quercetin (Quer) and iron and the balance between pro-oxidative and antioxidative effects. Quercetin may reduce $\text{Fe}(\text{H}_2\text{O})_6^{3+}$ to yield $\text{Fe}(\text{H}_2\text{O})_6^{2+}$ active in the Fenton region forming hydroxyl radicals ($\cdot\text{OH}$) or alkoxyl radicals ($\cdot\text{OR}$), in effect being pro-oxidative. In contrast, quercetin may form a complex with iron(II), inactive in reducing H_2O_2 to $\cdot\text{OH}$, but rather oxidised in the quercetin ligand, in effect being antioxidative. Quer(-H)· is the phenoxyl radical.

16.2.2 Antioxidant hierarchies

The electron transfer mechanism for antioxidant activity corresponding to eq. 16.5 makes the standard reduction potentials of interest for evaluation of antioxidative activity. The standard reduction potential of the phenoxyl radical of several flavonoids has been determined and forms the basis for correlation of rate of electron transfer for various oxidants from the flavonoid (Jovanovic *et al.*, 1997; Jørgensen and Skibsted, 1998). The standard reduction potentials have also been used to establish antioxidant hierarchies.

Both thermodynamic and kinetic factors are of importance for antioxidant capacity. The antioxidant has to be located in the right position at the right time in order to prevent oxidative damage to vital cell components and will need to be regenerated from the one-electron oxidised form in a recycling process:



The reaction of eq. 16.9 will regenerate the antioxidant $\text{Ar}_1\text{-OH}$ at the expense of the antioxidant $\text{Ar}_2\text{-OH}$. Despite the fact that such regeneration reactions are not simple electron transfer reactions, the rate of reactions like that of eq. 16.9 has been correlated with the E^θ values for the respective $\text{Ar}\text{-O}\cdot$. Thermodynamic and kinetic effects have not been clearly separated for such hierarchies, but for a number of flavonoids the following 'pecking order' was established in dimethyl formamid (DMF) by a combination of electrolysis for generating the α -tocopherol and the flavonoid phenoxyl radicals and electron spin resonance (ESR) spectroscopy for detection of these radicals (Jørgensen *et al.*, 1999):

α -tocopherol > quercetin > catechin > taxifolin > luteolin

As will be seen from Table 16.1, this ordering of regeneration, i.e. α -tocopherol may regenerate quercetin, which may regenerate catechin, etc., follows the reducing power of the flavonoids according to the standard reduction potentials in DMF, except for luteolin. According to the value of E^0 , luteolin should be placed higher in the antioxidant hierarchy than is actually observed to be the case. The standard reduction potentials depend on the solvent, and values are available also for the aqueous solutions determined by pulse radiolysis or by cyclic voltametry, (CV) (Jovanovic *et al.*, 1994; Jørgensen and Skibsted, 1998). It should, however, be noted that potentials determined by CV are based on reversible electrode processes only in a few cases, and should rather be classified as half-wave potentials. $E_{1/2}$ may differ significantly from the 'true' E^0 value since $E_{1/2}$ depends on the actual electrode kinetics.

The standard reduction potentials determined in aqueous solution give hierarchies slightly different from the antioxidant hierarchy established in DMF. For the potential determined by pulse radiolysis the ordering according to tendency of regeneration is (Jovanovic *et al.*, 1994):

asc > quercetin > trolox > taxifolin > catechin

and for the potentials determined by CV (Table 16.1):

asc > quercetin > trolox > catechin > taxifolin > luteolin

For aqueous solutions, ascorbate can be included in the hierarchy, while α -tocopherol has to be replaced by its water-soluble analogue trolox, which is often assumed to have the same standard reduction potential. The ordering of the antioxidants based on the two different determinations of E^0 in water is rather similar, and it should be noted that ascorbate is the antioxidant which will regenerate the other antioxidants, with the ascorbate itself ending up being oxidised. In contrast to what was observed for DMF, the ordering in water predicts that quercetin could regenerate α -tocopherol from its oxidised form.

16.2.3 Phase distribution of antioxidants

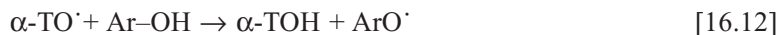
There has been some evidence of a higher antioxidant effect when both flavonoids and α -tocopherol are present in systems like LDL, low-density lipoproteins (Jia *et al.*, 1998; Zhu *et al.*, 1999). LDL will incorporate α -tocopherol, while flavonoids will be present on the outside in the aqueous surroundings. A similar distribution is to be expected for oil-in-water emulsion type foods. In the aqueous environment, the rate of the inhibition reaction for the flavonoid is low due to hydrogen bonding and the flavonoid will not behave as a chain-breaking antioxidant. Likewise, in beer, none of the polyphenols present in barley showed any protective effect on radical processes involved in beer staling, which is an oxidative process (Andersen *et al.*, 2000). The polyphenols have, however, been found to act synergistically

with α -tocopherol, and quercetin and the two epimeric catechins have now been studied in some detail (Pedrielli and Skibsted, 2002).

In the 'water-like' solvent *tert*-butyl alcohol, α -tocopherol was found to prevent lipid oxidation, showing a distinct lag-phase for oxygen consumption. This was in contrast to quercetin or epicatechin, which were only weak retarders of lipid oxidation without any clear antioxidative effect. Quercetin or epicatechin, when combined with α -tocopherol, increased the lag-phase for oxygen consumption as seen for α -tocopherol alone. The stoichiometric factor for α -tocopherol, α -TOH, as chain-breaking antioxidant has the value $n = 2$ according to the well-established mechanism:



and in a mixture of α -tocopherol and one of the flavonoids in equal concentrations values of $n = 1.0$ and $n = 0.8$ were found for quercetin and epicatechin, respectively (Pedrielli and Skibsted, 2002). This effect of the presence of quercetin or epicatechin on the antioxidative effect of α -tocopherol can be classified as synergistic and is only understood if it is assumed that α -tocopherol is regenerated by quercetin or epicatechin (Ar-OH). The regeneration reaction



is apparently not affected by hydrogen bonding to the same degree as the direct inhibition reaction of eq. 16.3 for the flavonoid. The observation that n is smaller than 2 for quercetin and epicatechin, however, shows that the efficiency of the regeneration reaction of eq. 16.12 is less than unity. This synergism between α -tocopherol and flavonoids, depending on regeneration of the more effective antioxidant, could be important at water/lipid interphases as outlined in Fig. 16.5. A further regeneration of quercetin by ascorbate is suggested in Fig. 16.5 in agreement with the ordering of the standard reduction potentials (Table 16.1). Although speculative, such a mechanism would explain the effects of catechin seen for LDL (Zhu *et al.*, 1999).

Flavonoids are chain-breaking antioxidants in 'lipid-like' solvents like chlorobenzene, although the $k(\text{inh})$ is smaller than for α -tocopherol and the lag-phase accordingly less evident. For peroxidating lipids in chlorobenzene the clear lag-phase for α -tocopherol became longer when quercetin or catechin were present. The effect appears to be additive and a regeneration of α -tocopherol by quercetin or catechin in this 'lipid-like' solvent should rather be termed a co-antioxidative effect (Pedrielli and Skibsted, 2002).

The solubility of antioxidants determines their phase distribution in foods. It has been observed that compared to lipid-soluble antioxidants water-soluble antioxidants like ascorbate yield better protection to strongly lipophilic food systems like pure oils. In contrast, antioxidants soluble in lipids like the tocopherols yield better protection to oil-in-water emulsions when compared to water-soluble antioxidants (Porter, 1993). The explanation offered for this

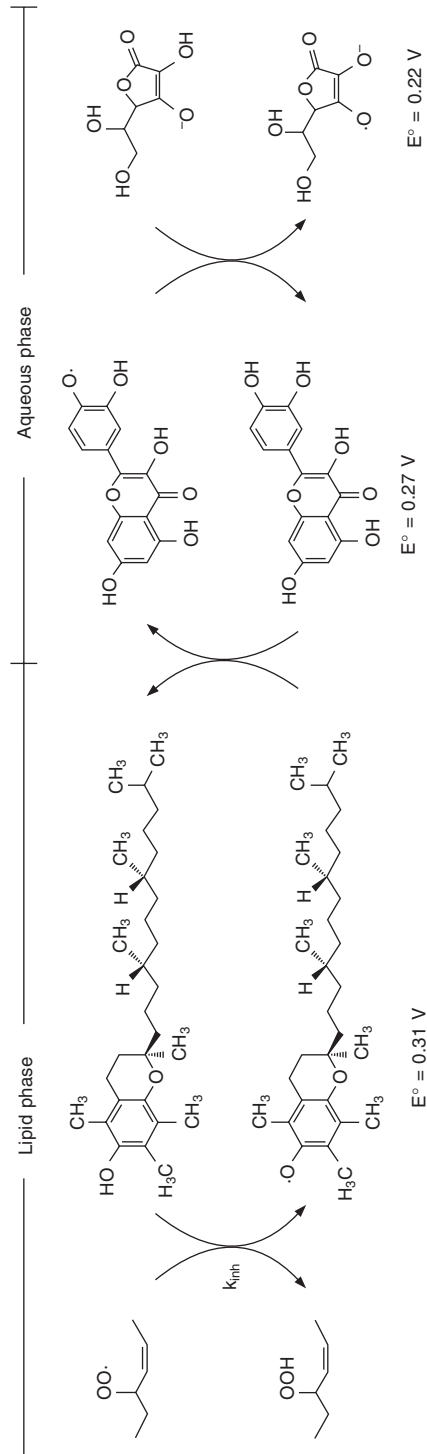


Fig. 16.5 Synergistic regeneration of α -tocopherol by quercetin at a lipid-water interface. α -tocopherol is reacting with a lipid peroxy radical in a chain-breaking reaction. According to the standard reduction potential, the phenoxyl radical of quercetin can further be regenerated by ascorbate.

so-called 'polar paradox' is that initiation of lipid oxidation occurs on the interphase between lipid and water in oil-in-water emulsions like mayonnaise where hydrophobic antioxidants will also concentrate, and on the interphase between air and lipid in oils where hydrophilic antioxidants will concentrate. A similar effect has been seen for antioxidants from spices like rosemary.

16.2.4 Structure activity relationships

Radicals are often formed by enzymes or by metal catalysis in the water phase of a food system, attacking the membrane lipids or the interphase in an emulsion (Jacobsen *et al.*, 2001). Control of radical formation is accordingly important for the oxidative stability of such multi-phase food systems. A well-defined pro-oxidant of relevance for meat products is the hypervalent heme-pigment ferrylmyoglobin, MbFe(IV)=O, which is known to initiate lipid and protein oxidation (Skibsted *et al.*, 1998). Different polyphenols deactivate ferryl-myoglobin with varying stoichiometry depending on structure, but most often according to a 1:2 ferryl/flavonoid stoichiometry:



The rate constant for the reaction of eq. 16.13 $k(\text{ferryl})$ has been determined for an increasing number of flavonoids and other plant phenols (Table 16.1). It was concluded that the reactions occur by an outer sphere electron transfer mechanism with a high enthalpy of activation and positive entropy of activation, corresponding to a relatively fast reaction with a high temperature dependence (Jørgensen and Skibsted, 1998). It is interesting to compare the rate for superoxide anion reduction by flavonoids with that of ferrylmyoglobin, since E^0 has the value 0.94 V for $\text{O}_2/\text{H}_2\text{O}_2$ and 0.85 V for MbFe(IV)=O/MbFe(III), respectively. Despite the very similar E^0 value for the two pro-oxidants, and accordingly a very similar driving force for deactivation, the rate is rather different with superoxide reacting faster (Table 16.1). Moreover, reduction of the superoxide radical by flavonoids has low values for enthalpies of activation, i.e. shows little temperature dependence, and has negative entropies of activation. Based on these differences it was concluded that flavonoids deactivate activated heme pigments like MbFe(IV)=O by an outer sphere electron transfer with an electron jumping from the flavonoid to the heme protein. Superoxides in contrast establish more firm contact with the flavonoid prior to electron transfer as reflected in the negative entropy of activation.

The less specific binding of flavonoids to ferrylmyoglobin is in agreement with establishment of LFERs, linear free energy relationships, i.e. $\ln k(\text{ferryl})$ depends linearly on E^0 , for reduction of MbFe(IV)=O by flavonoids within series of flavonones and flavonols (Jørgensen and Skibsted, 1998). The relevance of such LFERs, as demonstrated for MbFe(IV)=O and plant phenols, draws further support from the observation that the same sequence, i.e. chlorogenic ~ caffeic > ferulic > coumaric acid, is seen for reaction of the phenols with

MbFe(IV)=O and with lipid peroxy radicals (Castelluccio *et al.*, 1995). It may accordingly be concluded that the most relevant single parameter for predicting the antioxidative activity of a 'new' plant phenol would be the standard reduction potential, E^{θ} .

For most of the reactions in which flavonoids deactivate free radicals or other pro-oxidants, conjugation throughout the three rings of the flavonoid skeleton seems to be the most important single structural factor for providing high antioxidative activity, in combination with the presence of a catechol structure (Jovanovic *et al.*, 1994, Jovanovic *et al.*, 1995; Cao *et al.*, 1997; Bors *et al.*, 1990). Quercetin (Table 16.1) and myricetin both have these structural features and are among the most reducing and the most reactive flavonoids, see. Fig. 16.6. The extremely good antioxidative activity of the flavon-3-ols like quercetin and myricetin has been explained using *ab initio* calculations by intramolecular hydrogen bonding in the phenoxyl radical (van Acker *et al.*, 1996). In summary, flavonoids with the three structural requirements outlined in Fig. 16.6, are the most effective antioxidants (Rice-Evans *et al.*, 1996).

Specific effects are, however, noted such as epigallocatechin gallate from green tea which has the highest rate constant among flavonoids both for reaction with superoxide and with ferrylmyoglobin, although it is only moderately reducing (Table 16.1). Studies of scavenging of other radicals by catechins have likewise pointed out that the presence of a gallate group at position 3 plays a most important role for a high scavenging activity (Guo *et al.*, 1999). Also apigenin from parsley, which is even less reducing, shows a surprisingly high rate of deactivating MbFe(IV)=O (Table 16.1).

Anthocyanins and anthocyanidins, compounds present with high structural diversity in fruits and wines, showed a pattern as antioxidants different from that of the tea catechins with respect to the effect of substituents. In a liposomal model system induced peroxidation was inhibited increasingly by anthocyanins/anthocyanidins with an increasing number of hydroxyl groups in the B-ring (Fig. 16.6), while the opposite was seen for the catechins (Seeram and Nair, 2002). For anthocyanidins, the presence of a 3-hydroxy group is important

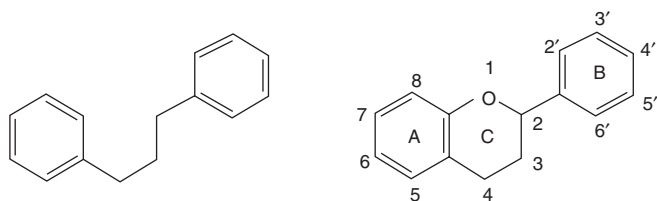


Fig. 16.6 The 1,3-diphenyl propane skeleton of flavonoids and the numbering system for flavonoids. Three structural features optimise the radical scavenging properties of a flavonoid: (i) an *ortho*-dihydroxy structure of the B-ring (catechol); (ii) 2,3 double bond in conjugation with a 4-oxo group; (iii) 3- and 5-hydroxy groups (Bors and Saran, 1987).

Quercetin (Fig. 16.1) fulfils these three requirements (Rice-Evans *et al.*, 1996).

for the antioxidative capacity, and glycosylation of the 3-hydroxy group decreases the antioxidative capacity considerably. The anthocyanidins are, however, strongly reducing and may act pro-oxidative like ascorbate.

16.3 Antioxidant interaction in food models

16.3.1 Analysis of phenols in foods

Knowledge of the identity of phenolic compounds in food facilitates the analysis and discussion of potential antioxidant effects. Thus studies of phenolic compounds as antioxidants in food should usually be accompanied by the identification and quantification of the phenols. Reversed-phase HPLC combined with UV-VIS or electrochemical detection is the most common method for quantification of individual flavonoids and phenolic acids in foods (Merken and Beecher, 2000; Mattila and Kumpulainen, 2002), whereas HPLC combined with mass spectrometry has been used for identification of phenolic compounds (Justesen *et al.*, 1998). Normal-phase HPLC combined with mass spectrometry has been used to identify monomeric and dimeric proanthocyanidins (Lazarus *et al.*, 1999). Flavonoids are usually quantified as aglycones by HPLC, and samples containing flavonoid glycosides are therefore hydrolysed before analysis (Nuutila *et al.*, 2002).

The use of HPLC for quantification of phenols is often limited to a single class of phenolics and then often only to low-molecular weight compounds that are available as standards. It is, therefore, often necessary to use colorimetric assays such as the Folin–Ciocalteu assay which rely on the reducing ability of phenols to quantify the amount of total phenolics in a sample (Waterman and Mole, 1994; Singleton *et al.*, 1999; Schofield *et al.*, 2001). The degree of condensation of polyphenols can be quantified by colorimetric assays such as the acid–butanol assay and the vanillin assay (Waterman and Mole, 1994; Schofield *et al.*, 2001).

16.3.2 Studying the actions of antioxidants in food

The conclusions about the role phenol plays as an antioxidant in real food systems are often reached by comparing the oxidative behaviour of food samples with different contents of phenolic compounds. The variations in phenolics are usually obtained by using products made from different raw materials (e.g. malts containing different levels of polyphenols for production of beer (Andersen *et al.*, 2000)). However, using different raw materials not only affects the levels of phenols, but also affects the levels of transition metals and enzymes which can have profound effects on the oxidative behaviour of the finished product. It is, therefore, often advantageous to study the oxidative behaviour of samples derived from a single batch of production where the level of phenols has either been increased by addition or decreased

by removal (e.g. by fining) (Siebert and Lynn, 1997; Andersen *et al.*, 2000; Andersen and Skibsted, 2001).

A number of methods are available for following the oxidative behaviour of food samples. The consumption of oxygen and the ESR detection of radicals, either directly or indirectly by spin trapping, can be used to follow the initial steps during oxidation (Andersen and Skibsted, 2002). The formation of primary oxidation products, such as hydroperoxides and conjugated dienes, and secondary oxidation products (carbohydrides, carbonyl compounds and acids) in the case of lipid oxidation, can be quantified by several standard chemical and physical analytical methods (Armstrong, 1998; Horwitz, 2000).

It is preferable that the action of phenolic antioxidants should be studied during realistic processing and storage of food; however, the oxidative changes during storage are often too small or too slow to be detected within a convenient timeframe. It is, therefore, often necessary to accelerate the testing of the oxidative behaviour by subjecting the food system to high temperatures, adding transition metals, radical initiators such as peroxides or azo compounds, or by irradiation with intense UV or visible light (Frankel and Meyer, 2000). The accelerated testing can potentially distort the action of antioxidants. Heating will, for example, overestimate the role of reactions with high activation energies, and disturb the distribution of components such as polyphenols between the different phases in complex foods. Accelerated testing has been found to be able to predict the flavour stability of beer (Uchida *et al.*, 1996).

16.3.3 Antioxidant assays

The use of real food systems for detailed studies of antioxidants is complicated by a large number of factors which are often unknown or cannot be controlled due to the complex nature of foods. Using simplified model systems, which mimic the main features of a given food system, or antioxidant assays for quantifying the antioxidant action, can be very helpful in clarifying the action of potential antioxidants (Aruoma, 1996; Møller *et al.*, 1999; Prior and Cao, 1999; Frankel and Meyer, 2000). The extrapolation of conclusions based on the behaviour of model systems or antioxidant assays to real complex food systems should generally be done with great care, and should ideally be based on results from more than one model system or assay (Frankel and Meyer, 2000).

The terminology describing the action of antioxidants is unfortunately not clear. Terms such as 'antioxidant power', 'antioxidant effectiveness', 'antioxidant ability', 'antioxidant activity', and 'antioxidant capacity' are often used interchangeably and without discrimination. Here we use the term 'antioxidant activity' as meaning a measure of the rate of antioxidant action, and the term 'antioxidant capacity' as meaning a measure of the extent of antioxidant action, i.e. the amount of radicals or intermediates and products produced during oxidation that are quenched by a given antioxidant. Thus antioxidant activity is related to the kinetics of the antioxidant action and antioxidant capacity to the stoichiometry.

Quantification of antioxidant action usually relies on the reducing ability of antioxidants, measured either by electron transfer, reaction [16.15], or by hydrogen atom transfer reactions, reaction [16.16]:



Electrochemical measurement of redox potentials gives direct information about the ability of antioxidants to donate electrons (Buettner, 1993; Hagerman *et al.*, 1998; Jørgensen and Skibsted, 1998). The electron transfer antioxidant capacity of antioxidants is commonly quantified by the TEAC assay and the FRAP assay. The TEAC assay (TEAC = trolox equivalent antioxidant capacity) relies on the reduction of the coloured cation radical of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) (ABTS⁺), and the antioxidant capacity is quantified as amount (mM) of the water soluble-vitamin E analogue trolox that produces the same effect as the test sample (Re *et al.*, 1999; Frankel and Meyer, 2000). For flavonoids with high antioxidative capacity, a stoichiometric factor far larger than 2 is, however, being found in simple solutions of the flavonoid in the TEAC assay; an observation which is difficult to understand (Rice-Evans *et al.*, 1996). The FRAP assay (FRAP = ferric reducing antioxidant power) measures antioxidant capacity by the reduction of the ferric 2,4,6-tripyridyl-s-triazine complex to the blue ferrous complex (Benzie and Strain, 1999).

The reduction of the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH[·]) has been used to assess the efficiency of antioxidants in beverages (Larrauri *et al.*, 1999; Porto *et al.*, 2000), vegetable oils (Espin *et al.*, 2000) and of pure phenolic compounds (Madsen *et al.*, 2000), reaction [16.17]:



DPPH[·] has an intense absorption maximum around 520 nm (Yordanov and Christova, 1997), and antioxidant capacity and activity measured by the reduction of DPPH[·] are easily quantified by VIS-spectroscopy (Brand-Williams *et al.*, 1995; Bondet *et al.*, 1997; Espin *et al.*, 2000). The stable radicals Fremy's salt (potassium nitrosodisulphonate) and galvinoxyl (2,6-di-tert-butyl- α -(3,5-di-tert-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-p-tolyloxy radical) have been used in a similar manner but with ESR detection, which can be used with samples that are not optically transparent (Gardner *et al.*, 1998).

The reactivity of antioxidants towards short-lived radicals, such as hydroxyl and peroxy, can be tested by the ORAC assay (ORAC = oxygen radical absorbing capacity) (Cao *et al.*, 1997). This assay furthermore allows the testing of pro-oxidant behaviour. The assay is based on quenching the fluorescence from the protein β -phycoerythrin in the presence of radicals (Cao *et al.*, 1993). The activity of antioxidants in retarding lipid oxidation in heterogenous systems can be assessed by measuring the oxygen consumption in linoleic acid oil-in-water emulsions or in methyl linoleate emulsions where

the oxidation is initiated by hypervalent myoglobin species (Møller *et al.*, 1999; Madsen *et al.*, 2000).

16.3.4 HPLC and on-line antioxidant assays

An interesting development is the combination of HPLC and on-line measurement of reducing capacity or antioxidative activity. This approach allows both direct identification of antioxidative species in complex foods and quantification of the contribution to the overall antioxidative capacity in the absence of synergistic and antagonistic effects. Major advantages are less sample handling and the ability to run large series of samples in an automated process.

The on-line measurement of reducing capacity can be performed with either a single or a series of electrochemical detectors, and linear correlations have been demonstrated between total antioxidative activities determined by the electrochemical detection and those determined by DPPH \cdot reduction or by the ORAC assay (Guo *et al.*, 1997; Peyrat-Maillard *et al.*, 2000). The reducing capacity must also be quantified by post-column reactions, either with DPPH \cdot or by the reduction of phosphomolybdenum complexes followed by UV-VIS-detection (Bandoniene and Murkovic, 2002; Cardenosa *et al.*, 2002). A combination of HPLC and semi-automatic ORAC analysis has also been described (Caldwell, 2001).

16.4 Polyphenols in processed food

Herbs and spices have been used by mankind since prehistoric time to preserve foods. Around the Mediterranean oregano and rosemary were widely used and in the Nordic countries thyme was added to sausages and lard. The modern development in the use of such plant material for protecting processed food against oxidation started half a century ago. The rich use of a variety of spices, mainly from the plant family *Labiatae*, has been reviewed and presented in tabulated form by Madsen and Bertelsen (1995). In the mid 1990s, around 10% of the antioxidant used by the European food and feed industry was based on spices (Krishnakumar and Gordon, 1996). Other substances, such as tea, coffee and waste from the manufacture of various vegetable products and beverages, are now being considered as sources for phenolic compounds to prevent or retard oxidation processes in food and to replace synthetic antioxidants. Some of these sources have the obvious advantage that they will not dominate the flavour of the food as do some species like clove. For each plant material, extraction conditions need to be optimised with respect to solvent polarity and physical conditions (Wettasinghe and Shahidi, 1999; Schwarz *et al.*, 2001). In agreement with the high antioxidant activity of the tea catechins found in model systems, green tea is among the more promising sources of natural antioxidants (Huang and Frankel, 1997). A few examples

from recent studies will illustrate some of the developments in the exploitation of various sources of antioxidants for protection of processed foods, see Table 16.2.

16.4.1 Green tea

Tang *et al.* found that various raw and cooked meat and fish products had a significantly higher antioxidant capacity when treated with tea catechins than they did when treated with α -tocopherol (Tang *et al.*, 2001a, b, c; 2002). Part of the antioxidative effect may be due to protection or regeneration of α -tocopherol already present in the meat or in the fish muscles. A high affinity of the tea catechins for lipid bilayers of muscles together with the radical scavenging activity was suggested as providing the explanation for the high antioxidant activity (Tang *et al.*, 2001b). For chicken, feed supplemented with tea catechins gave later protection of α -tocopherol in the meat during frozen storage and reduced lipid oxidation (Tang *et al.*, 2002). The tea catechins showed a pro-oxidative effect in corn oil-in-water emulsions, but a significant antioxidant effect in liposomes, a difference which also could be understood on the basis of the high affinity of the catechin to liposome surfaces (Huang and Frankel, 1997). In another study, green tea extracts have shown a better antioxidant capacity than rosemary extracts in canola oil, pork lard and chicken fat heated to 100°C (Chen *et al.*, 1998).

16.4.2 Tart cherry

Very promising results have been obtained by the addition of tart cherry tissue to ground beef patties (Britt *et al.*, 1998). Secondary lipid oxidation products for raw patties treated with cherry tissue were reduced by up to 80% after storage for nine days at 4°C while for cooked patties, stored for 4 days also at 4°C, the reduction was almost 90% compared to control patties without addition of cherry tissue. For the cooked patties the concentration of cholesterol oxidation products was reduced by up to 92% by the addition of tart cherry tissue prior to cooking, with a comparable reduction in the level of mutagenic amines in the cooked product. This significant reduction in oxidation products seems to be caused by the flavonoids found in tart cherry, consisting mainly of anthocyanidins and flavon-3-ols, although hydroxycinnamic acids derivatives are also present (Wang *et al.*, 1997).

16.4.3 Spices

Based on a review of the numerous studies of a great variety of food products clove, rosemary and sage were concluded generally to be among the most potent antioxidative spices (Madsen and Bertelsen, 1995). In studies comparing different spices and extracts thereof, clove seems to have the largest antioxidant potential in oil-in-water emulsions while rosemary and sage have the largest

Table 16.2 Examples of recent developments in use of plant materials or extract of plant material for protection of processed food against oxidation

Source	Antioxidative component	Type of food	Comments	References
Green tea	Tea catechins (300 ppm is typically required)	Raw minced beef, pork, poultry and fish	Effect up to four times that of α -tocopherol	Tang <i>et al.</i> , 2001c
		Cooked red meat, poultry and fish	Inhibits pro-oxidative effect of added NaCl	Tang <i>et al.</i> , 2001b
		Frozen chicken meat	Protection of α -tocopherol in muscles when added to chicken feed	Tang <i>et al.</i> , 2002
Olive oil	Elenolic acid derivatives bonded to tyrosol are main components (400 ppm provides protection, in low concentration prooxidative)	Cooked tuna in brine	Less protection of cooked tuna in oil	Medina <i>et al.</i> , 1999
Wine and by-products	Cinnamic acid derivatives, anthocyanins and flavanols dominate (10 to 20 μ M gallic acid equivalents)	Oil-in-water emulsion (dressing model)	Red wine yields better protection, but phenols in white and rosé wine seem more efficient on a molar basis	Sánchez-Moreno <i>et al.</i> , 2000
	Cinnamic acid derivatives, anthocyanins and flavonols dominate	Raw and cooked beef patties	Reduces secondary lipid oxidation and cholesterol oxidation products up to 90%	Britt <i>et al.</i> , 1998
Tart cherries	Rosemary (1000 ppm of extract with 0.92 mmol/g total phenols)	Dried chicken meat for soup powder (up to 1000 ppm is acceptable sensorially)	Rosemary extract gave better protection than extracts of tea, grape skin or coffee	Nissen <i>et al.</i> , 2000
Spices	Rosemary (200 ppm of extract with 0.92 mmol/g total phenol)	Potato flakes for mashed potatoes (up to 200 ppm is acceptable sensorially)	Rosemary extract gave better protection than extracts of green tea, grape skin or coffee	Nissen <i>et al.</i> , 2002

antioxidative effect in lard (Chipault *et al.*, 1955; Palitzsch *et al.*, 1969; Bishov *et al.*, 1977; Farag *et al.*, 1989; Chang *et al.*, 1977). Rosemary and sage, in combination with citric acid, seem to improve the oxidative stability of frying oil even more than each of the two spices alone (Jaswir *et al.*, 2000). Extraction of antioxidants from spices depends on the polarity of the solvent. Rosemary and sage mainly contain the polar components, rosmarinic acid, carnosol and carnosolic acid of which rosmarinic acid is the most water-soluble, and extracts seem more effective in bulk oil systems compared to oil-in-water emulsions, due to affinity for the lipid–air interface. Clove contains the monophenols eugenol and gallic acid but, because of the characteristic strong flavour of eugenol, it has certain limitations as a food additive. Oregano extracts also are effective against lipid oxidation in lard and oils (Economou *et al.*, 1991). Oregano contains rosmarinic acid and various flavonoids with high antioxidative activity, but with large variation among different sub-species. Dittany, a Cretan mountain herb closely related to oregano, thus has a high content of water-extractable antioxidants. (Møller *et al.*, 1999). Different cultivars of hops with different phenol profiles also yield different protection of beer (Lermusieau *et al.*, 2001). It is most likely that extracts will continue to be preferred for purified compounds like rosmarinic acid for legal reasons since the purified compounds will have to go through a rigorous toxicological testing.

16.4.4 Olive oil

Olive oil is renowned for its high oxidative stability and contains polyphenols with high antioxidative capacity. Phenolic substances extracted from extra virgin olive oil have been shown to be effective in protecting minced tuna cooked and stored in brine against oxidation, but to yield less protection of the same product cooked and stored in refined olive oil (Medina *et al.*, 1999). The higher antioxidative activity in the aqueous system was explained by the greater affinity of the phenol of the extract towards the polar interphase between water and the fish oil. Isolated phenols, especially myricetin, tannic acid and ellagic acid, showed similar effect on lipid oxidation in steam cooked fish (Ramanathan and Das, 1992, 1993).

16.4.5 Grape pomace

Wines are known to have higher radical scavenging activity than the juices from the grapes the wines are produced from (Sánchez-Moreno *et al.*, 1999), and wine also shows antioxidative capacity when tested in corn oil-in-water emulsions (Sánchez-Moreno *et al.*, 2000). There is, however, strong variation in the antioxidative capacity of wines with red wines having the highest. By-products from wine production, such as grape seed extract, grape skin extract and grape pomace, have great potential as food additives and as dietary

supplements (Shrikhande, 2000). Grape pomace in particular seems to be promising as a new source of antioxidants (Saura-Calixto, 1998).

16.4.6 Waste from vegetable products

Evening primrose meal is an example of a by-product with a great potential as a source of natural antioxidants as it has proved effective in meat systems (Wettasinghe and Shahidi, 1999). Other waste products like hulls from a variety of grains have also been considered. The large production in China of peeled rhizomes of edible lotus gives large quantities of waste and the extract of the rhizome knots especially has a high content of polyphenols of great potential for use as food additive (Hu and Skibsted, 2002a).

16.5 Bioavailability of plant phenols

Enrichment of processed food with plant material or plant extracts rich in polyphenols has two aspects in relation to human nutrition and human health. Food protected against oxidation has better keeping quality and will stay healthy longer since formation of toxic oxidation products, like cholesterol oxides, is being prevented (Britt *et al.*, 1998). The other aspect is the beneficial effects of the intake of polyphenols on human health. Both of these aspects are, however, related to the availability of the phenolic substances.

16.5.1 Optimising phenol content

Specification and quantification of phenols in plant food and plant extracts depend on development of specific analysis (see Section 16.3.1). The availability in food for protective reactions rather depends on specific assays while the bioavailability depends on human studies. As for the bioavailability of a specific flavonoid, urinary excretion of apigenin was found to be a useful biomarker for absorption of flavonoids from parsley (Nielsen *et al.*, 1999). Catechins from green tea extracts were likewise excreted into urine with a half-life of less than two hours, and only short-term effects on plasma antioxidant capacity were seen (Young *et al.*, 2002). The overall effect of a 10-week period without dietary fruits and vegetables prior to the intake of meat patties with green tea extracts was, however, a decrease in oxidative damage to DNA, blood proteins and lipids, underlining the general lack of knowledge of the mechanisms behind the beneficial effects of fruits and vegetables (Young *et al.*, 2002). While many studies of the content of phenolics in wine have appeared, including quantification of catechins, procyanidin, dimers, simple phenolic acids and flavonoids such as malvidine-3-glycoside, few data seem to exist on the absorption and metabolism of phenolics from wine (Teissedre and Landrault, 2000). Absorption of catechin and procyanidins from chocolate have been shown to increase serum antioxidative capacity (Ying *et al.*, 2002).

16.5.2 Glycosides versus aglycols

Flavonoids and anthocyanidins occur in plants mainly as glycosides, but seem to be absorbed in the intestine following hydrolysis to the aglycones (Kühnau, 1976). Individual differences seem, however, to have been detected for absorption of the glycosides (Paganga and Rice-Evans, 1997). Glycosilation of flavonoids at the 3-hydroxy group normally decreases the antioxidative activity due to the reduction of the number of phenolic groups as seen for quercetin/rutin (Jørgensen and Skibsted, 1998). The effect of glycosilation of the 7-hydroxy group seems not to have been investigated. For the anthocyanins and anthocyanidins, high pH as in the intestine will transform the flavylium cation as present under acidic conditions into carbinol pseudo-bases and quinoidal bases, which appear to be the forms being absorbed from the gut into the blood, with an even higher antioxidative capacity (Lapidot *et al.*, 1999). Such effects should also be considered when using wines or wine by-products for protection of processed foods.

16.5.3 Effect of polymerisation

Polyphenols are ubiquitous in all plant organs where they are found as monomers or in polymerised forms (Schofield *et al.*, 2001). In addition to the beneficial effect of polyphenols, they also bind minerals and precipitate proteins and carbohydrates, in effect reducing the nutritive value of foods. Polyphenols have been classified for nutritional purposes into extractable and non-extractable types (Bravo, 1998). Extractable polyphenols are low- and intermediate-weight phenolics while non-extractable polyphenols have high molecular weight and are insoluble in normal solvents.

Polymerisation of polyphenols is seen in a variety of beverages like beer and wine. In beer, the transformation of low molecular weight phenols into insoluble polymers is seen as haze (Andersen and Skibsted, 2001). Such polymerisation, also observed in alcoholic bitters based on various herbs, is induced by oxidation of soluble phenols and will accordingly lower the antioxidative capacity (Refsgaard *et al.*, 1996). The primary step in such polymerisation reactions, which also involve proteins and sugars, is seen in Fig. 16.7. For bitters, the formation of precipitate also decreases the astringency of the product. Enzymatic browning of plant products will lower the phenol content and accordingly the antioxidative capacity, a well-known example being green tea/black tea.

16.6 Future trends

Plants modified to have higher content of polyphenols for use by the food industry or for production of dietary supplements will become available together with polyphenol mixtures produced by cell cultures. However, in order to benefit from such production of optimised mixtures for food use, a

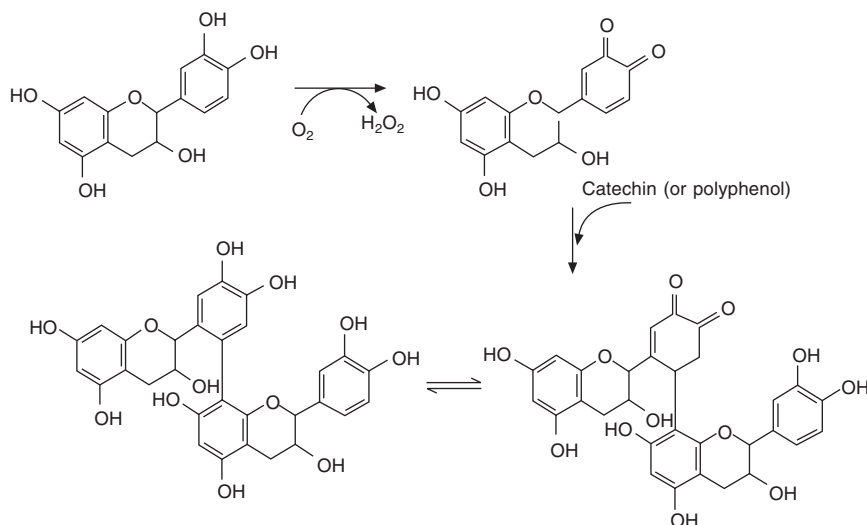


Fig. 16.7 Proposed mechanism for polymerisation of polyphenols in aromatic bitters (Refsgaard *et al.*, 1996).

better understanding of antioxidant synergism will be needed. In Fig. 16.3, antioxidants are classified into four groups and, while the synergistic interaction between the tocopherols and ascorbate is well understood for food systems (Löfliger, 1991), the interaction between the polyphenols and the carotenoids especially has not been investigated in any detail. Antioxidant hierarchies, as known for flavonoids (Section 16.3.2), have also been established for carotenoids with inclusion of the tocopherols (Mortensen and Skibsted, 1997). In order to combine the hierarchies of the flavonoids and the carotenoids, the thermodynamics and kinetics of their interaction need to be investigated. The synergism between polyphenols and α -tocopherols, now being confirmed in some food systems, can be described as an effect of regeneration of the more active by the less active antioxidant. The synergism between ascorbate and the tocopherols rather depends on the phase distribution of the two types of antioxidants. When a better understanding of the mechanism behind antioxidant synergism is available, protective systems based on such an understanding will probably be developed. The use of green tea extract in meat systems may present a break-through in exploitation of synergism between nutrient and non-nutrient antioxidants (Tang *et al.*, 2002, Hu and Skibsted, 2002b). The use of plant material and plant extracts as food ingredients will not only involve antioxidative effects. A perspective of a 'green revolution' for the food ingredients industry is to modify plants to produce molecules which combine emulsifying and/or thickening effects with antioxidative effects and anti-microbial effects.

16.7 Sources of further information and advice

A number of handbooks and monographs are available with detailed descriptions of a variety of plant products and their use (Shahidi and Naczki, 1995). From a more practical point of view, an interlaboratory comparison between six university and industry laboratories of 17 extracts of spices, teas, coffees, and grape skin and of tomato peel slurry established within the framework of an EU sponsored programme, would be of interest (Schwarz *et al.*, 2001). In this collaboration, detailed chemical analysis of the content of different phenolic compounds is compared with six antioxidant assays for the 17 extracts including different extraction procedures.

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Phytochemical products: rice bran

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17.1 Introduction

The exciting discovery of the twenty-first century, of how to unlock the human genome mapping, has illuminated the very blueprint of life. This phenomenal breakthrough in medical science heralds a new era of hope for the treatment of genetic diseases. Other similarly outstanding scientific achievements are equally exciting and offer the potential of exploring scientific means of treating chronic diseases. The discovery of a potential disease-fighting component, 'IP6' (inositol hexaphosphate), from a natural source, rice bran, offers a safe and promising future for chronic disease prevention ('First International Symposium on Disease prevention by IP6 and other Rice Components', June 1998, Kyoto, Japan). With the advent of sophisticated instrumentation techniques and novel biomedical technologies, scientists have unlocked the bioactive phytonutrients of rice bran and demonstrated its role in several physiological and pharmacological aspects of disease prevention and health maintenance.

Rice is consumed in its polished form throughout the modern world and is a staple food in many countries. Rice bran is the outer layer of brown rice, obtained as a by-product of the rice milling industry. The process of polishing brown rice removes the bran and the germ of the rice kernel, which is known as rice bran and which constitutes 10% of the paddy. Raw rice bran rapidly becomes rancid within hours of processing due to an active enzyme called lipase that is released on milling. Lipase promotes the hydrolysis of the fat in the bran into glycerol and free fatty acids rendering the bran rancid (Juliano, 1985; Saunders, 1986). Raw rice bran was a wasted by-product until recently, due to the fact that free fatty acids build-up causes the bran to lose its

sensory qualities, such as taste and flavor, making it unfit for utilization as an animal feed or food for humans. Recently, a unique non-chemical stabilization technology has been developed (RiceX Company's proprietary technology) resulting in the manufacture of Stabilized Rice Bran (SRB).

The hydrolytic, oxidative, microbiological and nutrient stability of SRB have been studied by established AOAC (Association of Official Analytical Chemists) methods. Free fatty acids and hexanals are the accepted yard sticks for measuring the hydrolytic and oxidative stability of rice bran (Fritsch and Gale, 1977). Shelf-life studies of SRB at ambient temperature and under accelerated conditions (Shin *et al.*, 1997; Reddy Sastry and Rukmini, 1997) indicated that it is stable for more than one year when stored at ambient temperature. SRB offers stability of both shelf life and nutrients and is safer than raw bran in terms of its microbiological purity. It is a valuable nutraceutical product and an excellent dietary supplement for maintaining good health and preventing disease (Rukmini, 2000).

SRB contains high-quality protein, oil, dietary fiber, polysaccharides, fat-soluble phytochemicals (plant derived bioactive compounds) and other bran nutrients. Rice bran and germ are the richest natural sources of B complex vitamins as well as E vitamins, polyphenols, several antioxidants and minerals. It is now available in the commercial food ingredient market as a safe and effective functional food and dietary supplement.

SRB is further processed non-chemically by pre-digestion with an enzyme (RiceX Company's proprietary technology) separating the water-insoluble fiber from the water-soluble nutrients. These products are separated and dried into powders which are utilized as nutraceutical products. This process renders the nutrients of the water-insoluble and water-soluble fractions of SRB more concentrated and bioavailable than is the case with those from stabilized rice bran itself. The water-soluble product of SRB concentrates all of the non-starchy water-soluble polysaccharides, soluble fiber, vitamins, minerals, phytonutrients and antioxidants of rice bran. It is denser in nutrients than SRB itself and demonstrates excellent biological effects in maintaining health. The water-insoluble product is a non-bloating hypoallergenic nutritional fiber. It contains 42% insoluble fiber, 20% protein, 13% fat and is rich in minerals and antioxidants. These products demonstrate clinical efficacy in chronic metabolic disorders such as diabetes and cardiovascular disease (Rukmini *et al.*, 2000). Rice bran was accorded a GRAS (Generally Regarded and Accepted as Safe) status and is safe for human and animal consumption.

Rice bran is a storehouse of unique bioactive compounds. The human body is a remarkable self-regenerating system. Hippocrates, the father of medicine, wrote that 'Disease does not occur unexpectedly. It is the result of constant violation of nature's laws. Accumulation of such violations results in diseases'. The possible involvement of all the bioactives of rice bran indicates a holistic approach to helping the body to regenerate to a normal state from a disease state. Holistic or alternative medicine does not treat or cure a disease but fights symptoms, makes the body handle the challenges

presented by the environment and helps it to heal itself, thus addressing the actual root of the problem. Rice bran products belong to this category, supporting the holistic approach to treatment. Conventional medicine may be regarded as a solution for crisis management.

17.2 Phytonutrients in rice bran

Nutrient analysis of stabilized rice bran and its derivatives indicates that it is a good source of protein, dietary fiber and carbohydrates, in addition to several valuable phytonutrients, antioxidants, vitamins and minerals (Table 17.1). SRB and its water-soluble and water-insoluble derivatives contain all the nutrients at different levels. They are gluten and lactose free and do not give rise to any food allergy.

Rice bran has 20% **fat** and that fat is extracted to obtain Rice Bran Oil (RBO), which is another value added product of rice bran. RBO has been demonstrated by several clinical studies (Raghuram *et al.*, 1989; Sugano and Tsuji, 1997; Rukmini and Raghuram, 1991) to be a nutrient-rich oil for cooking and salad dressing. Most of the earlier studies of rice bran were carried out with RBO since raw rice bran does not have a stable shelf life. RBO retains all of the fat-soluble vitamins, antioxidants and phytonutrients of rice bran at high concentrations. The fatty acid composition of RBO (Table 17.2a) indicates that it is low in saturated fat (18%), moderately high in monounsaturated fat (42%) and optimal in polyunsaturated fat (40%). The polyunsaturated fat contains 38% omega-6 fatty acids and 2% omega-3 fatty acids. Rice bran oil also has a higher unsaponifiable fraction of RBO (4%) than any other vegetable oil. The unsaponifiable fraction is rich in fat-soluble phytonutrients such as tocopherols, tocotrienols, γ oryzanol, phytosterols, ferulic acid and squalene (Table 17.2b). RBO in the diet is a healthy source of vegetable oil (Orthoefer, 1996) and has been reported to reduce total cholesterol, low-density lipoprotein cholesterol (LDL-C) and triglycerides and to increase high-density lipoprotein cholesterol (HDL-C) levels in the blood (Raghuram *et al.*, 1989; Nicolosi *et al.*, 1993; Lichtenstein *et al.*, 1994; Hegsted and Kousik 1994). RBO has been demonstrated as having anti-atherogenic properties (Seetharamiah and Chandra Sekhara 1989; 1990), improving diabetic neuropathy and skin nutrition and controlling blood pressure (Morita, 1986; Nakazawa *et al.*, 1977). Several tailor-made products from RBO are now on the market. A tocotrienol concentrate from rice oil is sold as capsules and a tocotrienol-rich fraction of rice bran oil called 'Nutriene'® is also available. These are useful as dietary supplements for specific health conditions. Another product developed from RBO is a concentrate of the unsaponifiable fraction containing the total fat-soluble antioxidant of rice bran, tocopherol, tocotrienols, γ oryzanol and phytosterols at a much higher concentration (Reddy Sastry *et al.*, 1999). As a dietary supplement, this product offers potential benefits for hypercholesterolemia, diabetes and hypertension.

Table 17.1 Nutrient composition of rice bran products

Nutrients Macronutrients (%)	Stabilized rice bran	Water-soluble derivative of stabilized rice bran	Water-insoluble derivative of stabilized rice bran
Protein	14.5	7.5	20.5
Fat	20.50	26.50	13.50
Total dietary fiber	29.00 (soluble fiber 2–6%)	3.00 (all soluble Fiber)	42.00 (soluble fiber 0–1%)
Carbohydrates (available)	22.00	54.5	0.50
Ash	8.00	5.00	10.00
Moisture	6.00	3.00	3.50
<i>Micronutrients</i>			
<i>(mg/100 g)</i>			
<i>Water-soluble vitamins</i>			
<i>B-vitamins</i>			
Thiamin	2.65	3.64	2.00
Riboflavin	0.28	0.46	0.19
Niacin	46.87	76.6	30.55
Pantothenic acid	3.98	5.82	1.90
Vitamin B6	3.17	5.81	1.67
Biotin	0.014	0.015	0.011
Inositol	149.60	149.00	192.30
<i>Fat-soluble vitamins</i>			
<i>Vitamin E</i>			
Tocopherols and Tocotrienols	25.61	18.00	3.73
Total carotenoids (mcg/100 g)	130.00	46.57	34.53
γ -oryzanol (mg/100 g)	245.15	248.10	174.10
Total phytosterols (mg/100 g)	302.00	385.00	317.20
<i>Minerals (mg/100 g)</i>			
Sodium	8.00	15.75	16.00
Calcium	39.70	8.33	92.50
Potassium	1573.00	1562.00	1670.00
Phosphorous	1591.00	763.00	2330.00
Zinc	5.50	1.80	9.40

The **protein** in rice bran is complete in all of the essential amino acids (Table 17.3), the most limiting of which are threonine and isoleucine (Wang *et al.*, 1999). The protein efficiency ratio (PER) of rice bran protein is 2.0 (Prakash, 1996; Shih and Daigle, 2000). It is a hypoallergenic protein, as it does not contain the 16kDalton protein which causes allergic reactions (Burks and Helm, 1994; Helm and Burks 1996; Hettiarachchy, 1998). The arginine/lysine ratio of rice bran protein has been shown to enhance insulin production and help regulate cholesterol levels (Sugano *et al.*, 1984).

Table 17.2a Fatty acid profile of rice bran oil and peanut oil (%)

Fatty acid profile (%)	RBO	Peanut oil
Palmitic 16:0	16.0	15.0
Stearic 18:0	2.0	3.1
Oleic 18:1	42.0	42.6
Linoleic 18:2	38.5	35.9
Linolenic 18:3	1.5	nil
Arachidic 20:0	nil	2.2
Behenic 22:0	nil	1.0
Total saturated fatty acids	18.0	21.3
Monounsaturates	42.0	42.6
Polyunsaturates	40.0	35.9
γ -oryzanol (ppm)	14 000–15 000	nil
Total T + T3 (ppm)	1200–1500	nil
Unsaponifiable fraction of rice bran oil		4.2%

Table 17.2b Composition of RBO unasaponifiables

Phytosterols	43%
γ -oryzanol	38%
Hydrocarbons	18%
T + T3	1%

Table 17.3 Rice bran–amino acid analysis (HPLC)*

Amino acid profile	%
Aspartic acid	1.36
Threonine	0.36
Serine	0.97
Glutamic acid	1.90
Proline	0.63
Glycine	0.80
Alanine	0.96
Valine	0.56
Methionine	0.25
Isoleucine	0.34
Leucine	0.91
Tyrosine	0.44
Phenylalanine	0.55
Histidine	0.38
Lysine	0.68
Arginine	1.08
Cysteine	0.32
Tryptophan	0.21
Ammonia	0.20

*High Pressure Liquid Chromatography.

The **dietary fiber** of rice bran is nutritionally superior to other fibers available in the market. Most of these contain only soluble fiber up to 60–65% and are devoid of insoluble fiber, phytochemicals or antioxidants. Large amount of soluble fiber in the diet produce gases such as methane and carbon dioxide on fermentation in the gut, and these gases produce bloating and abdominal distention resulting in a constant feeling of discomfort (Life Science News Letter, 1999). Rice bran dietary fiber has a combination of both soluble and insoluble fibers. It also contains 20% protein besides fat, phytochemicals and antioxidants which together are beneficial for health maintenance and disease prevention (Saura-Calixto, 1998). Rice bran fiber is non-bloating and has demonstrated favorable results in the form of increased fecal bulk, producing short-chain fatty acids such as propionic and butyric acids as fermentation products (Folino *et al.*, 1995), and maintaining acidic colonic pH. This low pH promotes bifidobacterial proliferation, resulting in improved colon health. The high butyrate content and low pH help in maintaining a healthy colonic epithelium, promoting intestinal cell growth and enhancing water and mineral absorption. Butyrate, the preferred fuel of the colonocyte, is not produced by mammalian tissues but is only available as the result of the fermentation process (Folino *et al.*, 1995).

Rice bran fiber has fructo-oligosaccharides – a pre-biotic that helps friendly bacteria to proliferate in the gastrointestinal environment and improves intestinal and colon health (Tomlin and Read, 1988). Recent studies in humans (Kahlon and Chow, 1997) have revealed that rice bran fiber not only normalizes bowel function, but also helps in conditions such as irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and Crohn's disease, and lowers the lipid levels. Rice bran fiber has been shown to significantly reduce renal stones (Jahnen *et al.*, 1992). It is a good source of fiber in weight loss programs and therapeutic fiber diets for diabetics and heart patients. Fiber diets prevent cancer of the colon and large bowel, control obesity and improve bowel function.

Recent scientific studies have clearly established the nutritional role of dietary fiber in several health conditions (Jalili *et al.*, 2000), such as hypercholesterolemia (Topping *et al.*, 1990), diabetes (Chandalia *et al.*, 2000) and bowel function. It is also effective in preventing urinary stones (Ohkawa *et al.*, 1984). The Food and Nutrition Board (Institute of Medicine Report, 2001) recommended a daily allowance for fiber of 25–30 g, though it is not an essential nutrient. Rice bran fiber offers a good source of non-bloating dietary fiber and is marketed as RiceMucil® by NutraStar Company in the USA.

The **carbohydrates** in rice bran are made up of the cell wall components such as polysaccharides, oligosaccharides, hemicelluloses, starch and some sugars. The health benefits of rice bran polysaccharides and hemicelluloses are discussed under phytochemicals of rice bran below. It is lactose-free and gluten-free. The water-soluble non-starchy polysaccharides, oligosaccharides and hemicelluloses are concentrated in the water-soluble fraction of rice

bran along with B-complex vitamins, minerals and other water-soluble phytochemicals. Polysaccharides have a specific conformational structure which induces the secretory proteins, elevating the anti-inflammatory cytokines and inhibiting the pro-inflammatory cytokines, and they are also immunoprotective (Kelly, 1999; Tzianabos, 2000). The water extract of rice bran has been demonstrated as being effective in reducing blood glucose levels and improving the immune function (Hikino *et al.*, 1988). The water extract of defatted rice bran has been shown to suppress visceral fat accumulation in rats (Tsutsumi *et al.*, 2000).

There are more than 100 **antioxidants** identified in rice bran and its products. Antioxidants are substances capable of inhibiting oxidation. In chronic diseases, inflammatory response cells produce reactive oxygen species. These free radicals cause DNA damage, and this may lead to gene modifications that might be carcinogenic. The antioxidant defense mechanism in a biological system plays a major role in the prevention of disease and the maintenance of health (Lin *et al.*, 1995). Rice bran antioxidants exert their antioxidant activity effectively through several mechanisms at the molecular level (Rukmini, 2000). These antioxidants also stimulate the endogenous antioxidant enzymes to respond to meet the challenges of biological free radical damage (Manorama, 1993; Rukmini and Kalpagam, 1985). A short synopsis of the bioactive phytonutrients of rice bran and their various health effects is given in the Table 17.4. The positive role of these specific phytonutrients of rice bran in several health problems is briefly discussed below.

17.3 Phytonutrients with particular health benefits

Phytochemicals have little nutritional value and do not get absorbed in the body, but they seem to turn on certain switches in the biochemical mechanisms, which signal the beneficial pathways to maintain health, and to turn off the switches which proceed to adverse biochemical pathways. Rice bran products have demonstrated significant benefits as nutritional therapies in diabetes, hyperlipidemia, cancer, fatty liver, hypercalcuria and heart disease. There is experimental and clinical evidence for the beneficial health effects of the following bioactives of rice bran:

- B-complex vitamins;
- vitamin E complex (tocopherols and tocotrienols);
- γ -oryzanol;
- plant sterols and their glucosides;
- inositol and inositol hexaphosphate (IP6);
- ferulic acid and related polyphenols;
- squalene;
- water-soluble non-starchy polysaccharides and hemicelluloses.

Table 17.4 Rice bran phytochemicals and health benefits

Rice bran phytochemicals	Health benefits
	Cardiovascular disease management
Tocotrienols	Inhibition of HMGCoA reductase and hypolipidemic effect (Qureshi and Qureshi 1992; Tomeo and Geller 1995; Parker <i>et al.</i> , 1993). Inhibition of phospholipase A2, thromboxane B4 and enhancement of prostacyclin production (Raederstorff <i>et al.</i> , 2002; Qureshi and Qureshi, 1992). Antioxidant effect (Xu <i>et al.</i> , 2001).
Tocopherols	Inhibition of LDL-C oxidation (Tappel, 1997; Raederstorff <i>et al.</i> , 2002). Vitamin E activity (Meydani, 1995). Antioxidant activity (Packer, 1995). Improves immune responses (Ozer <i>et al.</i> , 1995).
Gamma oryzanol	Inhibition of platelet aggregation (Rong <i>et al.</i> , 1997). Inhibition of aortic streaks (Seetharamaiah and Chandrasekhara 1990; Rong <i>et al.</i> , 1997). Hypolipidemic effect (Yoshino <i>et al.</i> , 1989; Seetharamiah and Chandrasekhara, 1990; Nicolosi <i>et al.</i> , 1993; Rukmini and Raghuram, 1991). Reduction of triglycerides, elevation of HDL-C (Yoshino <i>et al.</i> , 1989). Anti-inflammatory effect (Xu <i>et al.</i> , 2001) Antioxidant activity (Fukushi, 1996).
Fiber	Hypolipidemic effect (Story J A and Kritchevsky, 1976; Anderson <i>et al.</i> , 1990; Normand <i>et al.</i> , 1987; Gerhard and Gallo., 1998).
Fat	Immune complexes enhancement (Nicolosi <i>et al.</i> , 1993). Reduction of serum cholesterol and LDL-C; elevation of HDL-C (Seetharamiah and Chandrasekhara, 1989; Raghuram <i>et al.</i> , 1989; Rukmini and Raghuram, 1991; Lichtenstein <i>et al.</i> , 1994; Sugano and Tsuji, 1997). Hypolipidemic effect (Lichtenstein <i>et al.</i> , 1994).
Phytosterols	Hypolipidemic effect (Lees and Lees, 1976; Weststrate and Meijer, 1998; Moreau <i>et al.</i> , 1999). Improves immune function (Vanderhaeghe and Bouic., 2000).
Polyphenols	Hypolipidemic effect (Kikuzaki <i>et al.</i> , 2002; Butterfield <i>et al.</i> , 2002). Antioxidant effect (Graf, 1992). Anti-inflammatory effect (Akihisa <i>et al.</i> , 1997). Regulates blood pressure (Ichiro <i>et al.</i> , 2002).
Antioxidants	Inhibition of LDL-C oxidation and elevation of immune function (Lin <i>et al.</i> , 1995; Rukmini, 2000).

Table 17.4 *cont'd*

Rice bran phytochemicals	Health benefits
	<p>The rice bran phytochemicals and antioxidants have a profound influence on the induction of antioxidant enzymes at the cellular level and control lipid peroxidation at the cellular level.</p> <p>Diabetes management</p>
B-complex vitamins	<p>Regulation of fasting serum glucose (Jacob and Swendseild, 1996).</p> <p>Improvement of glucose utilization (Leklem., 1998).</p> <p>Non-starchy polysaccharide Improving the immune function and increased insulin release from the pancreas (Masayoshi <i>et al.</i>, 1987).</p> <p>Increased insulin receptor sites (Urberg and Zemel, 1987).</p> <p>Regulation of fasting serum glucose (Qureshi <i>et al.</i>, 2002).</p> <p>Improves peripheral neuropathy (Davis <i>et al.</i>, 1976).</p>
Gamma oryzanol	<p>Immune complex enhancement, Beta-cells activation and Increased insulin production (Bruni, 1988).</p> <p>Neuro-regulatory effect (Nakazawa <i>et al.</i>, 1977; Bruni, 1988; Hiraga <i>et al.</i>, 1993).</p>
Protein	<p>Regulation of fasting serum glucose, improvement of glucose utilization, improvement in energy, Beta-cells activation and enhancement of insulin production (Sugano <i>et al.</i>, 1984).</p>
Carbohydrates	<p>Fatigue reduction and energy enhancement.</p>
Polysaccharides	<p>Non-starchy polysaccharides improve the immune function and improve insulin synthesis (Masayoshi <i>et al.</i>, 1987).</p>
Hemicelluloses	<p>Increased peripheral utilization of glucose, increased insulin receptors and improved glucose absorption. (Hikino <i>et al.</i>, 1988; Hikino and Hayashi <i>et al.</i>, 1989; Masayoshi, 1987).</p>
Fat	<p>Fatigue and energy improvement.</p> <p>Omega-3 fatty acids and increased prostacyclin synthesis.</p>
Fiber	<p>Regulation of fasting serum glucose and improvement in glucose utilization (Jenkins, 1978; Wolver and Jenkins, 1993; Chandalia <i>et al.</i>, 2000).</p> <p>Blood glucose and serum lipid lowering effects in humans with diabetes (McPeak <i>et al.</i>, 2001; Rukmini <i>et al.</i>, 2002; Qureshi <i>et al.</i>, 2002).</p>
Tocopherols and tocotrienols	<p>Improvement of peripheral insulin utilization, beta-cells activation and increased insulin production.</p> <p>Prevention of diabetic complications such as retinopathy, nephropathy, cardiomyopathy.</p>
Antioxidants	<p>Prevention of glycation and glycoxidation.</p>

Table 17.4 *cont'd*

Rice bran phytochemicals	Health benefits
	Prevention of diabetic complications such as retinopathy, cardiomyopathy, nephropathy. Neuro-regulatory effect (Nakazawa <i>et al.</i> , 1977; Bruni, 1988; Hiraga <i>et al.</i> , 1993).
	Cancer Management
Ferulic acid, γ -oryzanol	Anti-mutagenic and anti-carcinogenic effect: elevation of phase II microsomal enzymes and inhibition of phase I enzymes (Manorama, 1993). Anti-mutagenic and anti-carcinogenic effect (Rukmini and Kalpagam 1985; Tamagawa <i>et al.</i> , 1992; Tsushimoto <i>et al.</i> , 1991).
Inositol, IP6	Anti-carcinogenic effect in cancer of several organs (Shamsuddin, 1995, 1998; Shamsuddin, 1997).
Fiber	Prevention of colon and large bowel cancer (Cummings 1992).
Tocotrienols	Anti-cancer effect (Nesaretnam <i>et al.</i> , 1998) Human mammary and large intestinal cancer inhibition (Nesaretnam <i>et al.</i> , 1998).
Antioxidants	Prevention of colon and large bowel cancer (Cummings 1992).
Phytosterols	Anti-cancer effect: protection from most common cancers such as colon, breast, and prostate cancer (Awad and Fink, 2000). Anti-cancer agent (Awad and Fink, 2000).
Lipoprotein fraction	Apoptosis of cultured human endometrial adenocarcinoma cells and inhibition of cell proliferation (Miyoshi <i>et al.</i> , 2001; Akeshita <i>et al.</i> , 1992). Chemoprevention and suppression of cancer progression (Shamsuddin, 1995).
Polysaccharides	Chemoprevention and immunocompetence effect of rice bran saccharides (Nakamura, 1992; Miyoshi <i>et al.</i> , 2001).
Polyphenols	Chemoprevention (Hudson <i>et al.</i> , 2000).
Antioxidants	The rice bran phytochemicals and antioxidants induce several antioxidant enzymes at the cellular level which protects the DNA and other cellular constituents from damage.
	Liver disorders management
Inositol, IP6 and phytates	Controls liver cirrhosis, improves liver cell regeneration and helps in effective liver detoxification (Colodny and Hoffman, 1998).
B-complex vitamins	Helps to improve liver cirrhosis and helps in liver detoxification.

Table 17.4 *cont'd*

Rice bran phytochemicals	Health benefits
Tocotrienols, gamma	Controls liver cirrhosis and helps in effective liver detoxification (Bruni, 1988).
Oryzanol	
Phosphatidyl choline	Protectant against liver damage (Kidd, 1996).
Antioxidants	Antioxidant enzymes prevent lipid peroxidation and helps protecting the liver cells from damage.
	Other health benefits
γ -oryzanol	Estrous cycle regulation and endocrine system improvement (Ichikawa, 1974). Capillary blood circulation improvement (Kamimura <i>et al.</i> , 1964). Neuro-regulatory action (Bruni, 1988). Anabolic steroid replacement and lean body mass Increase (Cockeril and Bucci, 1987). Cosmetic applications and skin nutrition (Sugano, 1979; Noboru and Yusho, 1997). Improves skin nutrition and protects from UV radiation (Morita, 1986; Nakazawa <i>et al.</i> , 1977).
IP6 and phytates	Kidney and gall bladder stone (calcium stones) management, hypercalcuria prevention (Ohkawa <i>et al.</i> 1984). Gastro-intestinal and colon health (Folino <i>et al.</i> , 1995).

17.3.1 B-complex vitamins

Rice bran is the richest natural source of B-complex vitamins. Considerable amounts of thiamin (B1), riboflavin (B2), niacin (B3), pantothenic acid (B5) and pyridoxin (B6) are available in rice bran (Table 17.1). Thiamin (B1) is central to carbohydrate metabolism and kreb's cycle function. Niacin (B3) also plays a key role in carbohydrate metabolism for the synthesis of GTF (Glucose Tolerance Factor). As a pre-cursor to NAD (nicotinamide adenine dinucleotide-oxidized form), it is an important metabolite concerned with intracellular energy production. It prevents the depletion of NAD in the pancreatic beta cells. It also promotes healthy cholesterol levels not only by decreasing LDL-C but also by improving HDL-C. It is the safest nutritional approach to normalizing cholesterol levels. Pyridoxine (B6) helps to regulate blood glucose levels, prevents peripheral neuropathy in diabetics and improves the immune function.

17.3.2 Tocopherols (T) and tocotrienols (T3)

Tocopherols and tocotrienols belong to the vitamin E family of compounds, which are potent antioxidants. The four isomers of tocotrienols (α -T3, β -T3, γ -T3, δ -T3) are structurally related to their corresponding homologues of tocopherols (α -T, β -T, γ -T, δ -T), but differ in their side-chain in that T3

isomers contain three double bonds (Qureshi and Qureshi, 1992). These structural differences bring diverse biological effects. α -T is a powerful free radical chain-breaking antioxidant (Packer, 1995) and a most potent essential fat-soluble vitamin E isomer (Tappel, 1997). α -T inhibits biological oxidation such as LDL-C oxidation and protein glycoxidation as well as oxidation in foods. It enhances immune responses and offers a high level of protection against cardiotoxicity (Ozer *et al.*, 1995). Human supplementation studies indicate bio-absorption of T and T3 from the intestinal tract into the chylomicron fraction, and the subsequent reappearance in lipoprotein fractions. This suggests the involvement of a specific tocopherol binding protein, which regulates vitamin E metabolism in the hepatocytes (Meydani, 1995).

Tocotrienols are found in the highest concentrations in rice bran and in lesser amounts in palm and barley. T3 have been shown to exert a stronger anti-tumor action (Nesaretnam *et al.*, 1998). Tocotrienols inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity post-transcriptionally and regulate endogenous synthesis of cholesterol (Parker *et al.*, 1993). Elevated plasma cholesterol levels are a major risk factor for coronary heart disease. Tocotrienols in the diet lower total cholesterol, LDL-C and apolipoprotein-B and reduce the risk of coronary heart disease (Qureshi *et al.*, 1997; Raederstorff *et al.*, 2002). A novel T3 fraction known as T25 has been demonstrated as modulating cardiovascular disease risk parameters in human subjects (Qureshi *et al.*, 1997; Qureshi *et al.*, 2001). The antioxidant activity of T, T3, and γ -oryzanol against cholesterol oxidation has recently been demonstrated (Xu *et al.*, 2001). T3 in the diet inhibit cell proliferation by the inhibition of protein kinase activity and provide stability to cellular constituents (Ozer *et al.*, 1995). T3 also prevent DNA damage and inhibit tumor formation (Guthrie *et al.*, 1997).

17.3.3 γ -Oryzanol

γ -Oryzanol is a large group of plant sterol esters of ferulic acid that have positive metabolic effects in the human system. Nearly ten components of γ -oryzanol were identified as a mixture of ferulic acid esters of plant sterols and triterpene alcohols (Xu and Godber, 1999), having powerful antioxidant activity and occurring naturally only in rice bran. Ferulic acid is a derivative of 3,4-dihydroxy cinnamic acid, which is abundantly found in rice bran. γ -Oryzanol has been demonstrated as being a potent hypolipidemic agent (Yoshino *et al.*, 1989; Lichtenstein *et al.*, 1994). It has also been shown to inhibit platelet aggregation and to have an anti-atherogenic effect (Seetharamiah and Chandrasekhara, 1990; Rong *et al.*, 1997). These authors showed that addition of 0.5% γ -oryzanol to cholesterol-enriched experimental diets in rats was effective in lowering triglycerides, LDL-C and very low-density lipoprotein cholesterol (VLDL-C), increasing HDL-C levels, and also reducing cholesterol levels in the liver. γ -Oryzanol inhibits LDL-C oxidation and is a potent lipotropic agent (Xu *et al.*, 2001). The antioxidant activity of γ -oryzanol

has also been demonstrated by the inhibition of lipid peroxidation in the retina (Fukushi, 1996).

γ -Oryzanol has also been shown to quench UV irradiation, and this property has given rise to a new series of cosmetic products useful as sunscreen and suntan lotions and creams (Noboru and Yusho, 1997). In addition, in the cosmetic nutraceuticals γ -oryzanol is used in anti-dandruff shampoos (Sugano, 1979). γ -Oryzanol is a potent neuro-regulator, acting on the autonomic nervous system (Nakazawa *et al.*, 1977). It influences the hypothalamus, activating growth hormone production (Ieiri *et al.*, 1982) and maintaining estrogen balance in post-menopausal women (Ichikawa, 1974), and, in addition, acts as an anabolic steroid, improving lean body mass (Cockeril and Bucci, 1987; Bruni, 1988). It also improves the capillary blood circulation in the extremities (Kamimura *et al.*, 1964). γ -Oryzanol was demonstrated to have anti-mutagenic and anti-cancer properties (Rukmini and Kalpagam, 1985; Tamagava *et al.*, 1992; Tsushimoto *et al.*, 1999). Recently Akihisa *et al.* (2000) reported its anti-inflammatory effect. γ -Oryzanol is one of the major bioactive components of rice bran and is present at 1.5–2.0%.

17.3.4 Plant sterols and their glucosides

Plant sterols and their glycosides are derived from plants, are structurally similar to cholesterol and are abundantly found in rice bran. There are 27 different sterols identified in rice bran (Juliano, 1985), including β -sitosterol and its glucoside β -sitosterolin, as well as campesterol, stigmasterol, branosterol and stigmastanol. The hypocholesterolemic effect of plant sterols has been reported by several authors (Westrate and Meijer *et al.*, 1998; Moreau *et al.*, 1999). Recent reports indicate that these phytosterols, together with their glucosides (sterolins), are powerful immune enhancing and cholesterol reducing components (Bouic *et al.*, 1996). Although these compounds have no nutritional value, they provide excellent health benefits. Phytosterols have a similar structure to cholesterol; they compete with the uptake of dietary cholesterol thereby facilitating its excretion from the body. Phytosterols trap the bile pigments and bile acids that are the end products of cholesterol metabolism and prevent their re-conversion to cholesterol.

The sterols combined with their glucosides (sterolins) have a strong immune enhancing effect, but the sterols on their own have been shown to be much less effective (Vanderhaeghe and Bouic, 2000). This combination has been used to treat benign prostate hyperplasia (BPH), resulting in significant improvement in symptoms and urinary flow parameters, thus demonstrating the effectiveness of β -sitosterol (Klippel *et al.*, 1997). The impact of β -sitosterol and sterolins on several diseases such as HIV, tuberculosis, exercise induced stress, rheumatoid arthritis, asthma, cervical cancer and hepatitis C has been studied by Bouic *et al.* (1996). The improvement in the immune function resulting from the ingestion of phytosterols and sterolins has been demonstrated. A strong immune function helps to improve health and prevent infections (Bouic *et al.*, 1996).

Phytosterols have been demonstrated as being anti-cancer components in the diet (Awad and Fink 2000). Scientific studies indicate that phytosterols may offer protection against colon, breast and prostate cancers (Vanderhaeghe and Bouic, 2000). The possible mechanisms, as reported by these authors, include the effect of phytosterols on membrane structure and function and on the signal transduction pathways that regulate tumor growth and apoptosis. The rice bran derived phytosterol-cycloartenol-ferulic acid ester on the central nervous system has been studied by Hiraga *et al.* (1993).

17.3.5 IP6 (Inositol hexaphosphate)

Myo-inositol is one of the most biologically active forms of inositol. It exists in several isomeric forms, the most common being the constituent of phospholipids in biological cell membranes. It also occurs as free inositol and as inositol hexaphosphate (IP6) also known as phytate which is a major source from food. Rice bran is one of the richest sources of IP6 as well as free inositol. Inositol is considered to belong to the B-complex vitamins. It is released in the gastrointestinal tract of humans and animals by the dephosphorylation of IP6 (phytate) by the intestinal enzyme phytase. Phytase also releases intermediate products as inositol triphosphate and inositol pentaphosphate. Inositol triphosphate in cellular membrane functions as an important intra- and intercellular messenger, that merits its value as a nutritional therapy for cancer.

The First International Symposium on 'Disease Prevention by IP6 and other Rice Bran Components.' was conducted in Kyoto, Japan on June 8–9, 1998. Scientists from all over the globe gathered and presented their research findings on the effect of IP6 and other rice bran components on several aspects of health. The brain storming sessions of nearly 35 presentations demonstrated that IP6 is a chemopreventive agent as both a cancer inhibitor and a cancer suppressor in mammary gland, colon and lung cancer (Shamsuddin *et al.*, 1997).

The molecular basis of the anti-cancer effect is by the metabolism of IP6 to lower inositol phosphates (IP5–IP1) which become incorporated into membrane-associated lipids and function as secondary cellular messengers by blocking the enzyme(s) affecting cell proliferation. One such enzyme is PI-3 kinase, which plays a key role in the signal transduction and cell transformation triggered by a growth factor or tumor promotor. This may be one of the mechanisms by which IP6 acts as a cancer blocking agent. IP6 *in vitro* has been demonstrated to inhibit PI-3 kinase activity (Huang *et al.*, 1997). Phosphatidylinositol plays an important role in maintaining cell membrane integrity and cell function. During cell stimulation by hormones or growth factors phosphatidylinositol is converted into inositol triphosphate (IP3). A major discovery is that IP3 plays a key role as a second messenger by controlling the cellular process through the release of calcium signals bound to receptors within the smooth endoplasmic reticulum (Colodny and

Hoffman, 1998). Calcium in turn activates various enzymes, thereby regulating diverse cellular processes. A nuclear inositol pathway was described at the Symposium, with signal transduction components located and acting in the nucleus. The signaling pathway appears to be important in switching cell programming from proliferation to differentiation (Shamsuddin, 1995). Thus IP6 appears to be acting both as a blocking agent (preventing cancer) as well as a suppressor agent (arresting the progression of cancer).

Other papers in the Symposium deal with the antioxidant and hypolipidemic effects of IP6, its chelating effects in heavy metal toxicity, inhibition of renal stones and other beneficial effects such as inhibition of platelet aggregation, inhibition of inflammatory responses (Shamsuddin, 1998). The lipid lowering effect and anti-neoplastic effect of IP6 were extensively reviewed (Jariwalla, 1999). Hence, IP6 is a valuable component of rice bran in preventing disease and maintaining health. IP6 is present at 1.8–2% in rice bran.

17.3.6 Polyphenols

Recent scientific investigations of natural polyphenols have demonstrated their powerful antioxidant property (Niki *et al.*, 1995). Several classes of polyphenols have been chemically identified. Some of these are grape polyphenols, tea polyphenols, soy polyphenols, oligomeric proanthocyanidines (OPA) and other natural polyphenols of the flavone class. Rice bran polyphenols are different from the above in that they are p-hydroxy cinnamic acid derivatives such as p-coumaric acid, ferulic acid and p-sinapic acid. Tricin, a flavone derivative, has also been isolated from rice bran.

Studies by Hudson *et al.*, (2000) have demonstrated the presence of eight polyphenols in rice bran by using high-pressure liquid chromatography. They are protocatechuic acid, p-coumaric acid, ferulic acid, sinapic acid, vanillic acid, caffeic acid, which is a methoxycinnamic acid derivative, and triclin. The effect of these polyphenols on cell viability and on the colony-forming ability of human-derived MDA MB 468 and HBL 100 breast cells, colon-derived SW 480 and human colonic epithelial cells was assessed. These authors concluded that rice bran polyphenols have putative cancer chemopreventive properties.

The antioxidant property of ferulic acid and related compounds from rice bran was reported by Kikuzaki *et al.*, (2002). Their results indicated that these compounds elicit their antioxidant function through radical scavenging activity and their affinity with lipid substrates. Another recent study reported by Butterfield *et al.*, (2002) demonstrated that ferulic acid offers antioxidant protection against hydroxyl and peroxy radical oxidation in synaptosomal and neuronal cell culture systems *in vitro*. The effect of ferulic acid on blood pressure (BP) was investigated in spontaneously hypertensive rats (SHR). After oral administration of ferulic acid the systolic blood pressure (SBP) decreased in a dose-dependent manner. There was a significant correlation between plasma ferulic acid and changes in the SBP of the tail artery, suggesting

that the absorbed ferulic acid reduces the BP. Intravenous administration of ferulic acid at lower doses caused the hypotensive effect. The depressor effect of intravenous ferulic acid was significantly attenuated by pretreatment of SHR with NO synthase inhibitors. These data suggest that the hypotensive effect of ferulic acid is associated with NO-mediated vasodilatation (Ichiro *et al.*, 2002). Ferulic acid has a UV quenching effect and is used in several sunscreen lotions and creams (Morita, 1986). Ferulic acid, being a strong membrane antioxidant, has a significant protective effect against the free radicals produced by anaerobic exercise which cause decreased endurance, muscle fatigue and decreased performance (Graf, 1992). Ferulic acid esters were demonstrated to have anti-inflammatory properties (Akihisa *et al.*, 2000).

17.3.7 Squalene

Squalene is an isoprenoid compound that is found in large quantities in shark liver and in smaller quantities in olives, rice bran and wheat germ. It is a bactericidal compound and an antioxidant and it aids in skin nutrition. Several cosmetic applications of rice bran and rice bran oil arise from the biological effects of their squalene, vitamin E and γ -oryzanol content. Since these compounds are fat-soluble, rice bran oil is used for all these preparations.

17.3.8 Polysaccharides

Rice bran polysaccharides are the cell wall components and consist of water-soluble and water-insoluble polysaccharides. The latter is also known as insoluble dietary fiber and has been discussed in the earlier part of this chapter. The water-soluble non-starchy polysaccharides include hemicelluloses, α -cellulose and pectin. Rice bran does not contain 1-3 or 1-4 β -glucan but has α -glucan. These polysaccharides are non-nutritional phytochemicals, which are not digested in the gastrointestinal tract and do not impart any calories to the diet, but which appear to elicit excellent physiological properties in maintaining health and preventing diseases. An arabinogalactan was isolated from water-soluble hemicelluloses of bran polysaccharides and has been demonstrated to have anti-tumor properties in gastrointestinal carcinoma (Akeshita *et al.*, 1992) and colon cancer (Cummings, 1992). Rice bran hemicelluloses have been demonstrated to have a significant effect in increasing the peripheral blood lymphocytes and enhancing the immune function (Takenaka and Itoyama, 1993).

Rice bran hemicelluloses reduce thymus atrophy in rats (Takenaka, 1992). Hikino *et al.* (1988) isolated and purified four glycan fractions from rice bran hemicelluloses and named them as Oryzabrans A, B, C, and D. All these glycans were shown to improve the peripheral utilization of insulin, resulting in significant hypoglycemic activity (Hikino *et al.*, 1988; Hikino and Hayashi, 1989). Several investigations into rice bran polysaccharides and hemicelluloses (Masayoshi *et al.*, 1987) have indicated a strong improvement in the immune

function together with anti-cancer and anti-diabetic effects (Tzianabos, 2000). Rice bran products also naturally contain minor amounts of alpha lipoic acid, several antioxidant enzymes, minerals such as magnesium and potassium, and trace minerals, all of which have nutritional significance.

17.4 Functional benefits: cancer

The above scientific information on rice bran phytochemicals indicates that a multitude of mechanisms are operating at the cellular level to bring about specific health effects. Several health benefits of rice bran appear to be the result of the synergistic function of the many phytochemicals, antioxidants, vitamins and minerals which operates through a specific immune response. Their role in the biochemical mechanisms at the cellular level which result in major health effects is shown in Fig. 17.1. A short overview summarizing the effect of the various phytochemicals on major health issues such as cancer, immune function, cardiovascular disease, diabetes, altered liver function and gastrointestinal and colon disease will be given below.

Although whole grains in the diet have been identified as significant contributors to cancer prevention, a report of the committee from the National Academy of Science on Diet, Nutrition and Cancer in 1983 concluded that the relevance of these diets needs further evaluation and greater recognition. However, US dietary guidelines (Adams and Engstrom, 2000) include whole grains as part of a healthy diet. Case-control cancer studies with cereal fibers and whole grain cereal foods have offered adequate proof of protection against colorectal cancers, gastric cancers and possibly breast, endometrial and prostate cancers (McIntosh, 2001). Most of these grains and fibers have phytonutrient profiles which are more or less similar to that of rice bran. In addition, there are some unique phytonutrients identified in rice bran that are not present in other cereal brans (Table 17.5). A survey of recent literature on rice bran phytonutrients reveals a strong body of evidence relating to their role in the prevention of cancers as well as degenerative diseases such as coronary heart disease and diabetes mellitus Type 1 and Type 2.

Cancer is a result of free radical damage to DNA and protein in various organs of the body. Cancer formation occurs in three stages: an initiation stage, a latent progression stage and a tumor formation stage. Wattenberg, the father of chemoprevention, who devoted his life to researching phytochemicals and chemoprevention, said that natural products inhibit cancer by two major mechanisms: a) as blocking agents; and b) as suppressing agents. By blocking the DNA and protein attack by an active carcinogen, cancer can be prevented at the initiation stage itself. Even after the formation of the DNA adducts during the latent period, cancer can be arrested by the suppressor mechanism, with these adducts being suppressed and prevented from further proliferation.

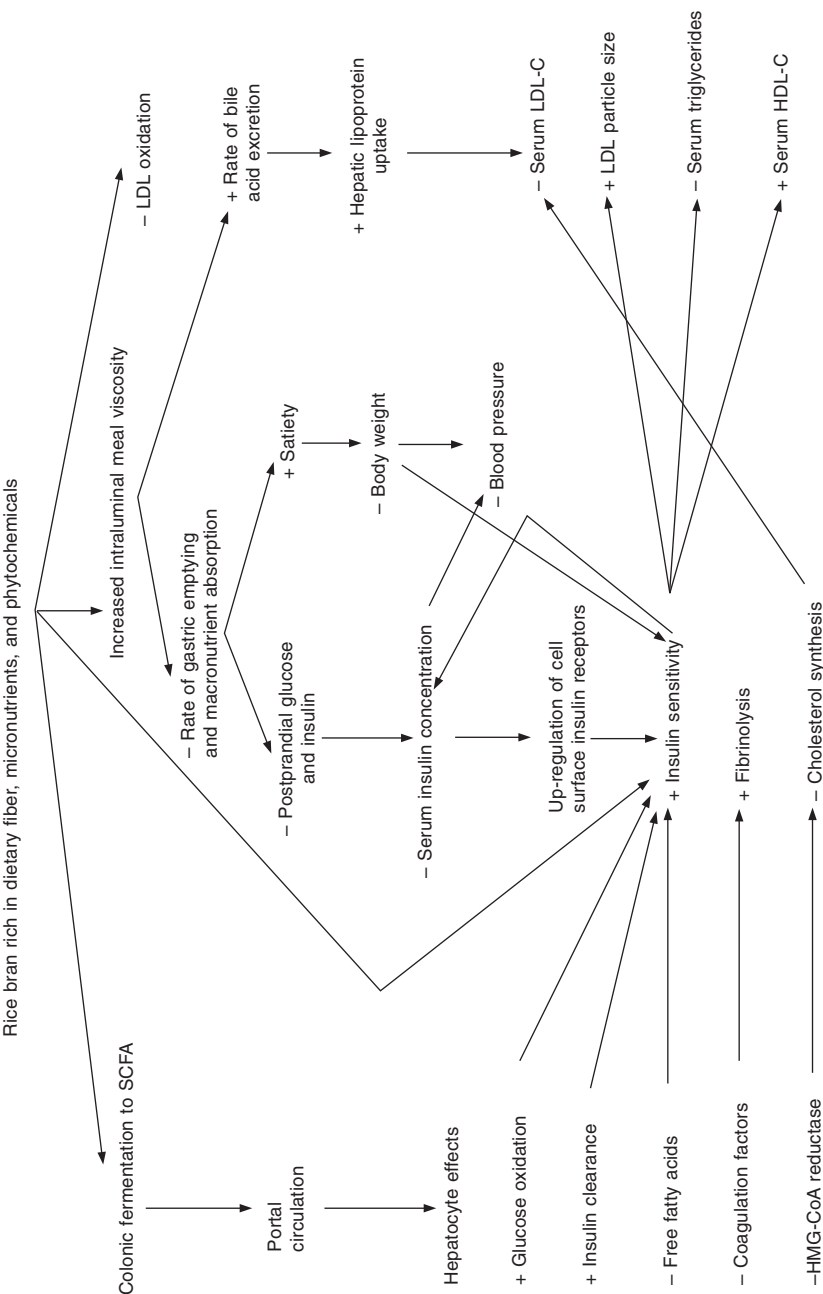


Fig. 17.1 The functional benefits of rice bran.

Table 17.5 Comparison of phytonutrients and antioxidants of rice bran with corn bran, oat bran and wheat bran

Nutrients (values/100 g)	Rice bran	Corn bran	Oat bran	Wheat bran
Calories	330.00	129.00	345.00	192.00
Moisture(g)	6.00	8.88	8.98	12.20
Protein(g)	14.5 ¹	6.50	17.06	14.71
Ash(g)	8.00	2.17	3.08	5.17
Total carbohydrate(g)	51.00	80.78	63.98	64.14
Total fat(g)	20.50²	2.02	1.33	3.78
Saturated fat (%)	3.70	0.32	1.33	0.77
Total dietary fiber (g)	29.00	70.27	15.60	42.52
Soluble fiber(g)	4.00	10.93	4.97	3.11
Vitamin A (IU)	0.00	24.75	12.37	0.00
Vitamin C (mg)	0.00	0.00	4.29	0.00
Vitamin E,tocols (mg)	25.61	0.00	0.00	0.00
Vitamin B complex				
Thiamin (mg)	2.65	0.12	0.97	0.55
Niacin (mg)	46.87	2.18	1.60	16.49
Riboflavin (mg)	0.28	0.43	0.32	0.49
Pantothenic acid (mg)	3.98	0.00	0.00	0.00
Vitamin B6 (mg)	3.17	0.00	0.00	0.00
Total sugars (g)	8.00 ³	0.50	1.96	2.50
γ-Oryzanol (mg)	245.15	0.00	0.00	0.00
Phytosterols (mg)	302.00	ND	ND	ND
Potassium (mg)	1573.00	236.75	655.35	1025.42
Sodium (mg)	8.00	11.43	8.42	7.50
Magnesium (mg)	727.00	0.00	0.00	0.00
Calcium(mg)	40.00	13.33	69.58	108.72
Iron (mg)	7.70	1.70	5.29	15.29
Manganese(mg)	25.60	ND	ND	ND
Phosphorous(mg)	1591.00	ND	ND	ND
Inositol (mg)	1496.00	ND	ND	ND
Zinc(mg)	5.50	ND	ND	ND

¹ Hypoallergenic protein.² Good quality fat with omega-3 and omega-6 fatty acids are essential to health. These fat concentrates are the fat-soluble vitamins and antioxidants.³ No lactose.**Bold letters** represent some unique antioxidants and some in high concentrations.

ND: Not determined.

Ferulic acid increases the liver microsomal phase 2 detoxifying enzymes several fold and inhibits the phase 1 carcinogen activating enzymes (Manorama,1993). The carcinogen entering the body cannot even reach the DNA as it is not activated to a reactive metabolite to attack it or the vital part of the cell, and it is simultaneously eliminated by the detoxification mechanism. As the result of this blocking mechanism cancer formation is prevented. The suppressor mechanism acts on the already formed DNA adducts, preventing further proliferation and suppressing the progression of cancer. The signal transduction pathway created by T3, IP6 and other phytonutrients appears to

switch the cell programming from a proliferation to a differentiation pathway, which can reverse cancer progression. IP6 has been shown to act as both a blocking agent and a suppressing agent. Data on IP6 treatment of breast cancer, lung cancer, colon cancer and cancer of several other organs (Shamsuddin, 1995) indicate a strong chemopreventive effect with the IP6 functioning as both a blocking and a suppressive agent. Rice bran polyphenols have been shown to have a chemopreventive effect (Hudson *et al.*, 2000).

Tocotrienols are another group of phytochemicals of rice bran which have a chemopreventive effect and have been demonstrated to inhibit breast cancer (Nesaretnam *et al.*, 1998). The polysaccharides of rice bran contain α -glucan, the anti-tumor effect of which has been demonstrated by its inhibition of gastrointestinal carcinogenesis (Akeshta *et al.*, 1992). Rice bran agglutinin has been shown to induce apoptosis of cancer cells by the mechanism of cell cycle dysregulation (Miyoshi *et al.*, 2001).

In addition to these compounds, rice bran contains potent antioxidants that prevent the free radical attack at several stages of carcinogenesis. Some of these phytochemicals, such as phytosterols, get incorporated into cell membranes and improve cell integrity and the fluidity of the cell membrane. This prevents the carcinogens from entering the cell. An anti-tumor polysaccharide from rice bran has been isolated (Ito *et al.*, 1985) and has been demonstrated as having anti-cancer and immune system enhancing properties (Nakamura, 1992). Another anti-tumor lipoprotein fraction of rice bran has been demonstrated to induce apoptosis and growth inhibition of cultured human endometrial adenocarcinoma cells (Fan *et al.*, 2000). There may be several as yet unknown mechanisms operating, but with this scientific background rice bran merits its use as an effective natural chemopreventive product.

17.5 Functional benefits: cardiovascular disease and diabetes

Cardiovascular disease (CVD) is characterized by the involvement of the heart and allied vascular system. High cholesterol, associated lipid abnormalities and high blood pressure are recognized as the major risk factors of CVD. There have been several animal experiments and clinical studies using rice bran and rice bran oil, which have demonstrated a hypocholesterolemic effect (Raghuram *et al.*, 1989; Rukmini and Raghuram, 1991; Sugano and Tsuji, 1997). The mechanisms involved are briefly summarized.

Tocotrienols present in rice bran inhibit the liver microsomal enzyme HMGCoA reductase (Qureshi and Qureshi, 1992), the key enzyme involved in the endogenous synthesis of cholesterol, and this helps to lower the circulating cholesterol. Inhibition of another enzyme, ACAT (Acyl coenzyme A; acyl transferase), by γ -oryzanol results in lowered LDL-C synthesis and enrichment

of HDL-C and increased cholesterol excretion. The enzyme inhibitions by these unique rice bran phytonutrients, tocotrienols and γ -oryzanol, help to reduce the elevated cholesterol and other lipid parameters, thereby reducing the risk of CVD. In a separate animal experiment, γ -oryzanol was shown to inhibit platelet aggregation (Seetharamiah and Chandrasekhara, 1988) and dissolve the aortic streaks (Seetharamiah and Chandrasekhara, 1990; Rong *et al.*, 1997); the water-soluble polysaccharides as well as γ -oryzanol inhibit leucotriene production. These properties of rice bran result in the altered macrophage function which is responsible for reducing the chemotaxis and preventing atherogenic lesions. Tocotrienols are naturally present only in rice bran and palm oil and the latter contains a lower level than is found in rice bran oil. γ -Oryzanol is a unique natural antioxidant occurring only in rice bran.

Phytosterols of rice bran also contribute to the cholesterol lowering effect by complexing with cholesterol and excreting the cholesterol metabolites which are otherwise reabsorbed into the liver. The rice bran fiber offers a protective effect during cholesterol metabolism and reduces the circulating cholesterol levels (Gerhardt and Gallo, 1998). The antioxidants of rice bran effectively prevent the free radical damage and inhibit oxidation of LDL-C and other vital components thus reducing the risk of CVD. The protein and amino acids in rice bran are also beneficial and the arginine/lysine ratio in particular is a favorable parameter for cardiovascular health (Sugano *et al.*, 1984). The quality of the fat, with omega-6 and omega-3 fatty acids and fat-soluble vitamins, and the phytonutrients of rice bran also contribute to positive cardiovascular health. Niacin and niacinamide are abundantly available in rice bran and help to keep lipid levels under control. A recent clinical study (Qureshi *et al.*, 2002) with rice bran derivatives in 57 diabetic subjects with lipid abnormalities also confirmed the results of earlier studies. Rice bran phytonutrients are effective nutraceutical products which reduce the risk of CVD in humans.

Diabetes is a metabolic disorder where glucose metabolism in the body is impaired. Type 1 diabetes is an early onset disease in which the pancreatic cells lose the function of insulin secretion either by genetic disposition or by a viral attack. Type 2 diabetes is a late onset disease developed due to insufficient insulin secretion or insulin resistance resulting in impaired glucose metabolism.

Rice bran is the richest natural source of the B-complex vitamins niacin and niacinamide which are important for intracellular energy production and which regulate blood sugar levels in diabetics (Urberg and Zemel, 1987). Pyridoxine has a role in gluconeogenesis (Davis *et al.*, 1976) and prevents peripheral neuropathy in diabetics (Jones and Gonzalez, 1978). Thiamin, riboflavin and biotin are also required as cofactors in the glycolytic pathway. Rice bran hemicelluloses have been shown to reduce high blood sugar (Masayoshi *et al.*, 1987; Hikino *et al.*, 1988). Tocopherols (vitamin E) and α -lipoic acid also improve glucose metabolism as they have been shown to inhibit glycosylation in diabetic patients (Ceriello *et al.*, 1991).

It has also been demonstrated that a novel polysaccharide from rice bran has a glucose lowering effect (Hikino and Hayashi, 1989). Dietary rice bran has been shown to improve glycemic responses in rats with streptozotocin induced diabetes (Lai *et al.*, 2001). Rice bran is rich in magnesium, phosphorous and potassium which are required for proper glucose metabolism. The protein and the omega-6 and omega-3 fatty acids also contribute to the regulation of glucose metabolism. The results of a clinical study in Type 1 and Type 2 diabetic subjects indicate significant reduction of hyperglycemia ($P < 0.01$).

Rice bran in the diet improves pancreatic insulin production (McPeak *et al.*, 2001) and decreases hepatic glucose levels. It does this through increased glucose uptake and increased muscle glycogen synthesis. Increased insulin receptor activity may also be a reason for glucose modulation in diabetics. Rice bran in the diet appears to improve insulin utilization resulting in decreased fasting glucose levels. This appears to be the result of the synergistic function of the above discussed specific rice bran phytonutrients, antioxidants, vitamins and minerals in diabetic subjects. Therefore, this natural product can be used as a nutritional therapy in Type 1 and Type 2 diabetic subjects to help regulate glucose metabolism.

17.6 Functional benefits: immune function

The immune system is a complex function made up of a remarkably integrated network of cells that protects the host in times of infection and is finely tuned to be able to respond quickly when needed. It has many control mechanisms and is the key to combatting disease and ensuring health. A strong genetic constitution preserves and controls these mechanisms; should they fail the response goes out of control, resulting in serious degenerative disorders such as autoimmune problems, cancers and allergies. When we provide the body with the right food and supplements its resistance to disease starts to build up, and a capacity either partial or complete to fight disease can be achieved.

Rice bran is endowed with several potent antioxidants which primarily control the free radical formation resulting from biological oxidations (Rukmini, 2000). In addition to these antioxidants rice bran has strong immune system enhancing compounds, namely phytosterols and sterolins, water-soluble polysaccharides, minerals and trace minerals such as magnesium, selenium, zinc, vitamin E, reduced glutathione, B6, omega-3 fatty acids and several other phytonutrients. The polysaccharides are potent signaling factors for the anti-inflammatory cytokine cascade and also inhibit phospholipase A2, influencing the eicosanoid pathway (Tzianabos, 2000). Cytokines are secretory proteins that regulate hematopoiesis, inflammatory responses and immune functions. They also inhibit leucotriene (LTB4) production and enhance prostacycline levels. Some of these nutrients of rice bran are COX-2 inhibitors, which are potent mediators of inflammation and which help suppress allergic

manifestation and are also beneficial for autoimmune diseases such as rheumatoid arthritis.

The sterols and sterolins in rice bran are potent immunomodulators. The best response was obtained with a 100:1 sterol/sterolin mixture that demonstrated T-cell proliferation from 20% to 920% and active cell antigens after four weeks in human subjects (Bouic *et al.*, 1996). Another *in vitro* experimental study with sterol/sterolins, demonstrated a significant increase in cytokinines, interleukin-2 and γ -interferon between 17% and 41% in addition to an increase in natural killer cell activity. These experiments (Bouic *et al.*, 1996) prove that sterol/sterolins are potent immunomodulators with important implications for the treatment of immune dysfunction. Rice bran products are excellent dietary supplements for the improvement of immune function. It is probable that the effects of rice bran on diabetes, CVD and cancer all result from improved immune function.

17.7 Functional benefits: liver, gastrointestinal and colonic health

The liver is the warehouse of the human body where everything from the enzyme complexes and the co-factors to all the biochemical pathways is available. Phosphatidyl choline (PC) is the integral part of the liver cell membrane and rice bran is rich (2.0–2.5%) in it. It is required for liver cell regeneration. It is incorporated into the parenchymal cells which control the membrane-based structure and function of the liver, thereby protecting it from several abnormalities such as fatty liver, liver cirrhosis, viral hepatitis, iron toxicity and liver cancer. Phosphatidyl choline, vitamin B, vitamin E, inositol, coenzyme Q10, more than 100 antioxidants, polyphenols like tricin and ferulic acid in rice bran provide additional protection to the liver. It is a magic bullet for liver diseases. Lysophosphatidylcholine is present in rice bran products in high concentrations and has been demonstrated as having hepato-protective action, preventing liver damage and regenerating liver cells (Kidd, 1996).

The fiber of rice bran products, especially the RiceMucil® is helpful in maintaining normal gastrointestinal and colon health (Tomlin and Read, 1988). It helps in bowel regularity. Patients with irritable bowel syndrome, inflammatory bowel disease and colitis get excellent relief with RiceMucil®. As has been mentioned in the earlier part of this chapter, the fiber of rice bran is non-bloating and lactose free, and the acidic environment the fiber creates during the fermentation of undigested food improves colon health and induces all the healthy enzymes and friendly bacteria to proliferate (Folino *et al.*, 1995; Life Sciences News Letter, 1999). It has been scientifically demonstrated to have an excellent nutritional support for gut and colon health.

Other health effects such as neuro-regulation (Butterfield *et al.*, 2002, Bruni 1988), skin nutrition, thyroid regulatory function and regulation of blood pressure have also been reported with rice bran. However, more detailed studies are required to establish these results.

17.8 Conclusions

Natural products have been noted for their potential health benefits from time immemorial and are the basis of Ayurveda, an ancient Indian medical practice (Bushkin and Bushkin, 2002). However, the potential benefits of several natural products reside in one or two active ingredients. For example green tea stands for polyphenols, soy for soy estrogens, broccoli for isothiocyanates and grape seed for polyphenols. The beauty of rice bran is that there are more than 100 antioxidants, several categories of bioactive phytonutrients, such as IP6, polyphenols, phytosterols, tocotrienols, γ -oryzanol, B vitamins, minerals and trace minerals in addition to fat, protein, fiber, polysaccharides and other nutrients. These phytonutrients and antioxidants of rice bran are believed to act at the cellular level, and their synergistic function is responsible for the positive health benefits.

Although rice bran is still a wasted product all over the world, recent scientific studies have recognized its potential health benefits. This is a unique, nutrient-dense natural product which offers health benefits for a series of ailments. It is a food pharmacy worth considering not only for general health maintenance but also as a dietary supplement for serious health conditions. With the advent of unique stabilization technology rice bran, an under-utilized waste product, has now been made available as a highly nutritious, health-promoting food for humans.

The dramatic shift in consumer understanding and readiness to accept functional foods for health care means that clinically efficacious rice bran products can be introduced to the functional food market. Efficient marketing strategies through healthcare professionals need to be developed if rice bran products are to reach their target population.

17.9 Acknowledgements

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