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The biochemistry and medical significance of the flavonoids

Bent H. Havsteen*

Department of Biochemistry, University of Kiel, Olshausenstrasse 40, D-24098 Kiel, Germany

Abstract

Flavonoids are plant pigments that are synthesised from phenylalanine, generally display marvelous colors known from flower petals, mostly emit brilliant fluorescence when they are excited by UV light, and are ubiquitous to green plant cells. The flavonoids are used by botanists for taxonomical classification. They regulate plant growth by inhibition of the exocytosis of the auxin indolyl acetic acid, as well as by induction of gene expression, and they influence other biological cells in numerous ways. Flavonoids inhibit or kill many bacterial strains, inhibit important viral enzymes, such as reverse transcriptase and protease, and destroy some pathogenic protozoans. Yet, their toxicity to animal cells is low. Flavonoids are major functional components of many herbal and insect preparations for medical use, e.g., propolis (bee's glue) and honey, which have been used since ancient times. The daily intake of flavonoids with normal food, especially fruit and vegetables, is 1–2 g. Modern authorised physicians are increasing their use of pure flavonoids to treat many important common diseases, due to their proven ability to inhibit specific enzymes, to simulate some hormones and neurotransmitters, and to scavenge free radicals.

Keywords: Flavonoids; Benzopyrones; Heat shock proteins; Gene expression; Enzyme inhibition

Abbreviations: Aβ, β-amyloid; AC, adenylate cyclase; ACTH, adrenocorticotrophic hormone; AD, Alzheimer's disease; AIDS, acquired immunodeficiency syndrome; APC, antigen-presenting cell; cAMP, cyclic AMP; CAT, chloramphenicol acetyltransferase; cGMP, cyclic GMP; CoA, coenzyme A; COX, cyclo-oxygenase; CSF, colony stimulating factor; DAG, diacylglycerol; ER, estrogen receptor; FA, fatty acid; GABA, γ-aminobutyric acid; GC-MS, gas chromatography-mass spectrometry; GSH, glutathione; HIV, human immunodeficiency virus; HMG, 3-hydroxy-3-methyl-glutaryl; HSE, heat shock regulatory element; HSF, heat shock factor; HSP, heat shock protein; HTLV, human T-lymphocyte-associated virus; IAA, indolyl acetic acid; ICE, interconverting enzyme; IFN, interferon; Ig, immunoglobulin; IL, interleukin; LDL, low-density lipoprotein; MHC, major histocompatibility complex; NK-T-Ly, natural killer T-lymphocyte; NO, nitric oxide; PDE, phosphodiesterase; PG, prostaglandin; PGI_2, prostacyclin; PIL, phosphatidylinositol lipase; PKC, protein kinase C; PL, phospholipase; PRR, proton relaxation rate; Pyr-P, pyridoxal phosphate; R, receptor; RA, rheumatoid arthritis; SIV, Simian immunodeficiency virus; SOD, superoxide dismutase; THF, tetrahydrofolate; TIMP, tissue inhibitor of matrix metalloproteinase; TNF, tumor necrosis factor; Tx, thromboxane; XO, xanthine oxidase.

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* Current Address: Abildgaardsvej 49, DK-2830 Virum, Denmark. Tel.: +49-0431-880-3214.
E-mail address: benthavs@worldonline.dk (B.H. Havsteen).

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1. Preface

Humans have gathered food and medical herbs ever since their arrival on earth. We were guided then by instinct, followed by experience, and more recently, also by rational thought. For millions of years, mankind has fared quite well using this approach, but after the development of science and technology, many people felt that the current state of affairs was quite satisfactory and, hence, they failed to support research and education adequately. Yet, the activities of humans on this clod evidently interact effectively with other evolving systems of nature, with consequences that may become very harmful to higher life soon. Therefore, it is time to examine more closely what we are eating, how diseases can be treated more rationally, and how we can more effectively conserve our natural resources. Although the analyses of such problems at the moment are neither sufficiently diversified nor adequately penetrant, the feeling that such work is urgent has become widespread (Geissman, 1963; Harborne, 1988a, 1988b; Harnaj, 1975; Dixon et al., 1998; Montanari et al., 1998); many living species of all biological kingdoms become extinct before their significance to the ecology has been ascertained. Reasons for this are based on the laws of nature and the increasingly aggressive and thoughtless exploitation of nature by humans. One of our natural resources is the plants in remote forests, some of which undoubtedly contain compounds of potential medical use. The first medical treatment was performed with natural products, and later the pharmaceutical sciences developed from these roots. Practitioners of lay medicine still use herbs in lone localities, where scientifically trained medical staff is not readily available, or where the latter have lost the confidence of the patients. The lay medical practitioners rely on experience handed down through the generations and on common sense. Although such persons may cause a few medical accidents, which might also happen to medical doctors, especially of past generations, in some cases, the lay treatment can be effective and, therefore, deserves an examination with the methods of modern science.

The flavonoids appear to have played a major role in the successful medical treatments of ancient times, and their use has persevered up to now. The recent interest in the properties of the flavonoids has several converging explanations.

(1) Since flavonoids are pigments, which are ubiquitous to green plant cells and are highly diversified, as well as easily separable with modern chromatographic equipment, botanists have long used the pattern of occurrence of these compounds for taxonomical studies. This approach is a substitute for full sequencing of the genome and only an indirect reflection of the hereditary traits, but the procedure is quick, easy, and useful.

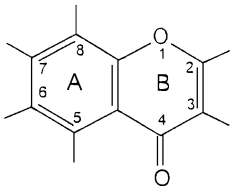


Fig. 1. Structure of benzo-γ-pyrone. Note the numbering of the atoms of the ring structure, which is essential to the nomenclature of the derivatives. Examples: pelargonidin, R = H; R′ = OH, R″ = OH; cyanidin, R = OH; R′ = OH, R″ = H; delphinidin, R = OH; R′ = OH, R″ = OH; peonidin, R = OCH_3; R′ = OH, R″ = H; and malvidin, R = OCH_3; R′ = OH, R″ = OCH_3.

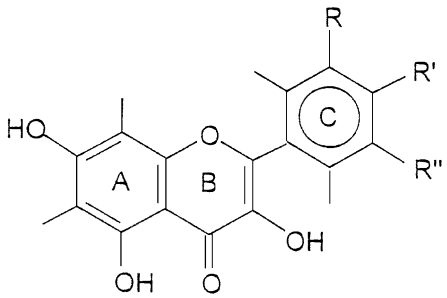


Fig. 3. Structure of flavonoles. Examples: kaempherol, R = H; R′ = OH; quercetin, R = OH; R′ = OH.

(2) Another reason for the increasing interest in the flavonoids is that the pharmaceutical industry, true to its tradition, is always searching for new medical herbs, the functional compounds of which can serve as a starting point for the development of optimal derivatives. During such scanning procedures, flavonoids possessing interesting properties were discovered.
(3) A third reason for the growing activity in the field of flavonoid biochemistry is the persistent claim by many lay medical practitioners of the beneficial effects of treatment with natural products, which proved to be rich in flavonoids. Some biochemists from scientifically recognized laboratories felt compelled to text some of the seemingly exaggerated claims made by laymen and confirmed the existence of many interesting effects of the flavonoids (e.g., Havsteen, 1983).

During the past 2–3 decades, the literature on flavonoids in highly rated scientific journals has swelled enormously. More than 1000 substantial articles have been recorded. Accordingly, the need for reviews and monographs on the subject has to be satisfied. So far, only a few such publications have appeared. Those that emerged mainly dealt with the isolation, identification, and synthesis of the flavonoids, whereas the physiological properties, with a few notable exceptions (Das, 1989; Bentsáth et al., 1936; Kubota et al., 1992), were neglected. Since flavonoids are produced by plants, the existing reviews mainly deal with the role of these compounds in plant physiology. From a medical point of view, the treatment of the effects of flavonoids on animal biochemistry, therefore, is due. The author hopes that this review will contribute to the fulfillment of this need.

2. Introduction

The flavonoids are members of a class of natural compounds that recently has been the subject of considerable scientific and therapeutic interest. The flavonoids are ubiquitous to green plant cells and, therefore, could be expected to participate in the photosynthetic process (Mukohata et al., 1978). However, so far, no evidence of a direct involvement of these compounds in photosynthesis has been found. In contrast, detailed evidence of the role of flavonoids in gene regulation and growth metabolism is known. The mutagenic role of flavonoids is of particular interest to botanical taxonomists and a reminder to medical practitioners of the potential dangers of the consumption of natural products. Nutritionists estimate the average intake of flavonoids by humans on a normal diet is 1–2 g per day (see Table 3 and de Vries et al., 1997). Such a high consumption of relatively unknown compounds is a good reason for contemplations about a revision of the research effort in the fields of toxicology and nutrition, since so far, much attention has been given to highly toxic compounds in low concentration, but little attention has been given to the massive intake of weak toxins. However, in spite of the substantial daily exposure of our bodies to flavonoids, the fact that this state of affairs has existed since the arrival of

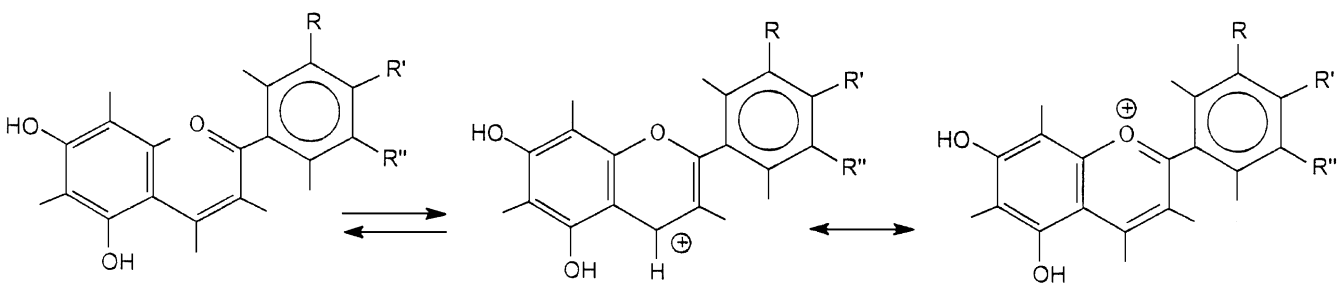


Fig. 2. Structure, tautomerism, and mesomerism of anthocyanidines.

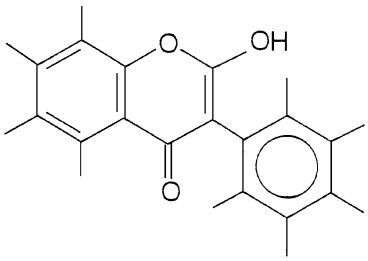


Fig. 4. Structure of isoflavonoles.

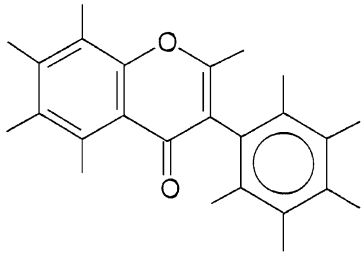


Fig. 6. Structure of isoflavones.

mankind seems to indicate that there is no reason for great alarm. On the other hand, we need to improve our knowledge of the effects of the food we eat. The evidence given below shows that they are far from trivial. Detailed books on flavonoids have been published, which impress by their comprehensiveness in the description of the structures, procedures of isolation, and approaches to the organic synthesis of flavonoids. However, the wealth of detail is likely to deter readers seeking clarity, basic principles, and applications. Hence, there seems to be a need for a review with a different emphasis.

3. The chemistry of flavonoids

3.1. Structure and nomenclature

The term flavonoids is a collective noun for plant pigments, mostly derived from benzo-γ-pyrone, which is synonymous with chromone (Hassig et al., 1999; Harborne, 1964, 1967; Croft, 1998) (Fig. 1).

The group comprises anthocyanidines, hydroxyl-4-dihydroflavonoles; anthocyanides, glycosides of anthocyanidines (Fig. 2); flavonoles, 2-phenyl-3-hydroxy-chromones (Fig. 3); iso-flavonoles, 3-phenyl-2-hydroxy-chromones (Fig. 4); flavones, 2-phenyl-chromones (Fig. 5); iso-flavones, 3-phenyl-chromones (Fig. 6); flavanes 2-phenyl-3-dihydro-chromones, 2-phenyl-flavanones (Fig. 7); iso-flavones, 3-phenyl-2-dihydro-chromones (Fig. 8); flavanols, 2-phenyl-3-hydro-3-hydroxy-chromones (catechins) (Fig. 9); iso-flavanols, 2-hydro-2-hydroxy-3-phenyl-chromones (Fig. 10); flavanes, 2-phenyl-di-hydro-benzo-γ-pyranes (Fig. 11); iso-flavanes, 3-phenyl-di-hydro-γ-benzo-pyranes (Fig. 12); aurones, benzo-furones (Fig. 13); and coumarins, benzo-γ-pyron derivatives (Fig. 14).

Reviews are found in Fruton and Simmonds (1959), Cody et al. (1986a, 1986b), and Lahann and Purucker (1975). Separate genes control the production of 4′-hydroxylated aglycones (e.g., pelargonidin, apigenin, and kaempferol) and of 3′,4′-dihydroxylated aglycones (e.g., cyanidin, luteolin, and quercetin) (Jörgensen & Geissman, 1955; Geissman & Harborne, 1955; Geissman, 1962). The number and position of hydroxyl groups attached to the A-ring are also controlled by different genes, and the nature and position of the carbohydrate units in the glycosides are determined by still other genetic factors.

The color production is one of the most explored areas in the study of the genetics of higher plants (Laurence & Price, 1940; Brouillard & Cheminat, 1988). The biosynthesis of the plant pigments has been reviewed by Seshadri (1951) and Peach (1955). Examples of the chemical synthesis of flavonoids are given by Baker and Robinson (1928), Dunne et al. (1950), Mozingo and Atkins (1938), as well as by Tatsuda (1947).

3.2. The oxidation-reduction potential of flavonoids

The flavonoids are phenolic compounds and, therefore, are prone to oxidation to quinones. The process, which can be accompanied with a ring opening at C_1, which occurs in

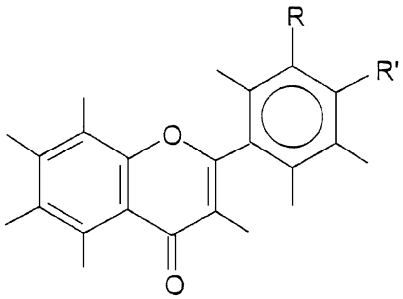


Fig. 5. Structure of flavones. Examples: orysin, R = H; R′ = H; apigenin, R = H; R′ = OH; luteolin, R = OH; R′ = OH.

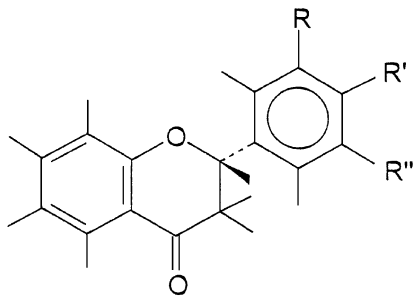


Fig. 7. Structure of flavanones. Examples: naringenin, R = H; R′ = OH, R″ = OH; eriodictyol, R = OH; R′ = OH, R″ = OH; liquiritin, R = H; R′ = OH, R″ = OH.

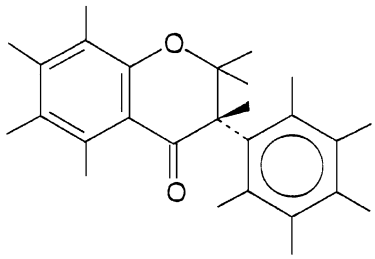


Fig. 8. Structure of isoflavanones.

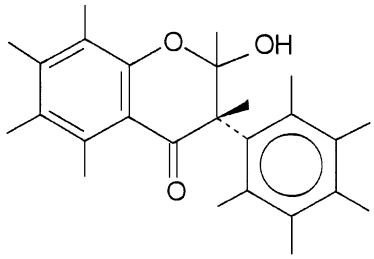


Fig. 10. Structure of isoflavanols.

the case of the anthocyanidines, easily proceeds in UV light, especially if heavy metal ions are also present. Since flavonoids are capable of protecting unsaturated fatty acids (FAs) in membranes as well as ascorbate against oxidation, certain brackets of their physiological oxidation-reduction potentials can be estimated (Zloch & Ginter, 1979; Zloch & Sidlova, 1977; Bors et al., 1997; Cai et al., 1999; Jörgensen et al., 1998). A guideline is provided in Table 1.

The existence of a great variety of related flavonoids suggests that the associated oxidation-reduction potentials somewhat differ (Xu & Liu, 1981). Since a large number of different flavonoids usually coexist in plant cells, in the transport system of the plant sap, and in plant products, a spectrum of electron transfer catalysts would be expected, which could accelerate physiological oxidation systems. A similar system is known from the respiratory chain and from experimental chemical reaction systems. This might be an important physiological function of the flavonoids, and may be a significant factor in their claimed and, in some cases, proven beneficial influence on our health.

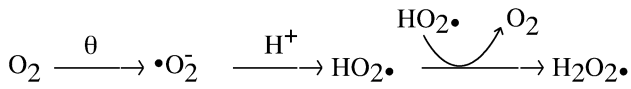
3.3. Acid-base properties

Flavonoids are phenolic compounds. The pK values of a large number of similar nonflavonoid substances are known. These values, which are very sensitive to the nature and position of neighbouring groups, usually lie in the pH range of 8–10.5. Examples are given in Table 2.

So far, only a few direct measurements of the pK values of flavonoids have been published. The state of ionisation of the flavonoid phenolic groups greatly influences the light absorption (color) and fluorescence spectra of these substances and, hence, the conditions for a qualitative or quantitative analysis (Peinado & Florinda, 1988; Briggs & Colebrook, 1962; Calman, 1972). This is due to prototropic tautomery. The phenomenon, which probably is responsible for flower and fruit pigmentation, is exemplified below for anthocyanidin (Stewart et al., 1975).

3.3.1. The tautomery of anthocyanin

The basic forms of anthocyanin are denoted by A^- and the conjugate acidic ones are denoted by A. The subindices refer to the position of the keto groups. The flavylium ion is marked with AH^+ and the corresponding hydroxylated forms with B_2 and B_4, respectively, where the subindices 2 and 4 refer to the position of the introduced hydroxyl group. The enols B_2 and B_4 are converted to the keto forms C_E and C_Z by tautomery. The latter forms are interconvertible by geometric isomery about the double bond in the bridge connecting the two phenolic rings. The pK'_a - values of the proton equilibria:



range from 3.50 in *Zebrina pendula* anthocyanin (Bruillard, 1981) to 4.85 by 4′-methoxy-4-methyl-7-hydroxyflavylium chloride (Bruillard, 1982). Note the high acidity, which is due to the extensive resonance stabilisation over numerous mesomeric forms. A proton can be dissociated from any of the hydroxyl groups at C-4′, C-5, or C-7. These groups are much more acidic than the corresponding hydroxyls, e.g., in flavones and flavonoles. All known natural anthocyanins

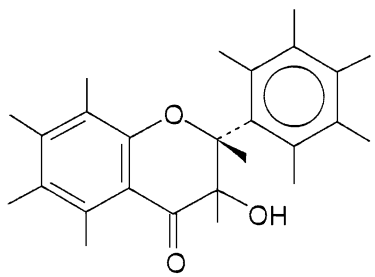


Fig. 9. Structure of flavanols.

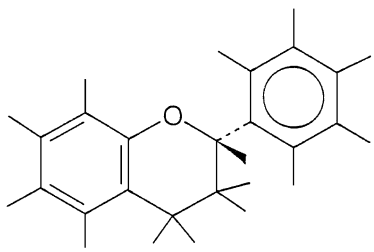


Fig. 11. Structure of flavanes.

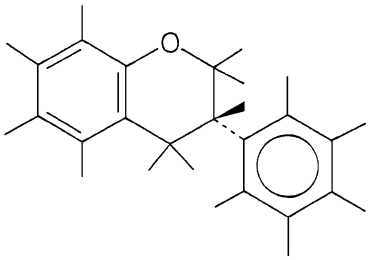


Fig. 12. Structure of isoflavanes.

possess a free hydroxyl group in one of the positions 4′, 5, or 7, and thus, are capable of forming a quinoidal base, which is believed to be of vital importance to flower pigmentation. If two phenolic hydroxyl groups are present in the cation, proton dissociation occurs at pH > 6 (Bruillard, 1982). Since high pH values have been measured in some petal vacuoles, the anionic quinoidal bases must contribute to the flower coloration.

Natural anthocyanin flavylium actions are often rapidly and completely hydrated to colorless carbinol pseudobases at pH 3–6. The hydration preferably takes place at position 2 (Cheminat & Brouillard, 1986). The presence of a glycoside at position 3 suppresses the hydration, which in that case requires a higher pH value (4–5). The acidity constant of the hydration equilibrium is invariably greater than that of phenolic hydroxyl groups. Hence, the colorless carbinol B_2 prevails in the weakly acidic pH range. At room temperature and slightly acidic pH, the chalcone C_E is rapidly formed from the pseudo base carbinol B_2 (Bruillard & Delaponte, 1977; Bruillard, 1981), but in natural anthocyanins, only small amounts of the open tautomer have been observed.

When a flavylium salt is dissolved in slightly acidic or neutral aqueous solution, the neutral and/or ionized quinoidal bases appear immediately. However, the more common 3-glycosides and 3,5-diglycosides convert more slowly to the more stable, weakly colored carbinol and chalcone pseudobases. Consequently, biochemical reactions in the vacuoles must suppress the hydration to ensure the coloration. Yet, colorless pseudobases have been observed in vivo in plants (Harborne, 1967). Hydration of the flavylium cation, which causes decoloration, may be prevented by formation of a complex between this ion and other substances, e.g., quercitrin. This phenomenon is called copigmenting (Robinson & Robinson, 1931). The stability constant of the cyanin-quercitrin complex is $\sim 2 \times 10^3$ M^{-1}, which diminishes the apparent hydration constant from 10^{-2} to 7×10^{-4} M (Bruillard et al., 1982). Most natural anthocyanins form complexes with copigments (Asen et al., 1972). The latter are often polyphenols (Chen & Hrazdina, 1981). Apparently, the copigments form coplanar complexes, thus protecting both sides of the flavylium ring from attacking water molecules. Such complexes can also form by intramolecular rearrangements. An example of a flavonoid that is capable of such a conformational change is platyconin (Saito et al., 1971). Another example is the main pigment "Heavenly Blue." The latter, which possesses a peonidin aglycone with six glycosyl groups and three caffeic acid moieties, has an unusually high color stability due to its ability of protective isomery (Goto et al., 1986).

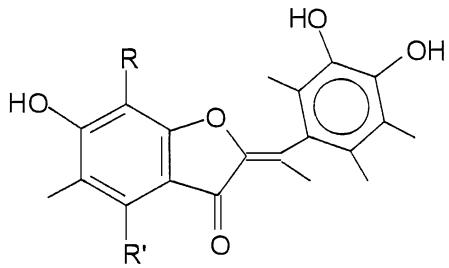


Fig. 13. Structure of aurones. Examples: aurensidin, R = H; R′ = OH; sulfuretin, R = H; R′ = H; marinetin, R = OH; R′ = OH.

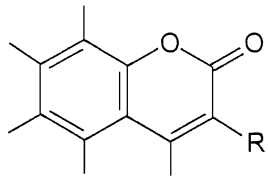


Fig. 14. Structure of coumarins.

The pH values of crude extracts of flower, fruit, and leaf tissues vary from 2.8 to 6.2 (Shibato et al., 1949). In young epidermal flower cells, a pH value between 2.5 and 7.5 is found (Stewart et al., 1975). The vacuolar pH value in epidermal petal cells of the rose "Better Times" changed from 3.70–4.15 in fresh leaves to 4.40–4.50 in 3-day-old cut petals (Asen et al., 1971). Simultaneously, the color changed from red to blue. In the "Heavenly Blue" flower, the pH of reddish-purple buds changed from 6.5 to 7.5, as

Table 1
Physiological oxidation-reduction potential (pH 7.0, 30 °C)

Reaction	E'_o volt	Reference
$H_2O \rightarrow 1/2\ O_2 + 2H^+ + 2\theta$	0.81	Fruton & Simmonds, 1959
Horseradish peroxidase	−0.27	Harbury, 1953, 1957
Glutathione $2GSH \rightarrow GSSG + 2H^+ + 2\theta$	~0.10	Harbury, 1953, 1957
Hemoglobin → methaemoglobin	0.14	Harbury, 1953, 1957
Myoglobinmetmyoglobin	0.05	Harbury, 1953, 1957
Cytochrome $c(Fe^{2+}) \rightarrow$ cytochrome $c(Fe^{3+}) + \theta$	0.26	Harbury, 1953, 1957
Ascorbate → dehydroascorbate + 2θ	0.058	Harbury, 1953, 1957
Catechol → o-quinone + 2θ	~0.33	
Dehydrolipoate → lipoate	~−0.4	Harbury, 1953, 1957
Flavine nucleotides	−0.22	Harbury, 1953, 1957
Pyridine nucleotides	−0.32	Harbury, 1953, 1957
Succinate → fumarate + $2H^+ + 2\theta$	~0.00	Harbury, 1953, 1957
$H_2 \rightarrow 2H^+ + 2\theta$	−0.42	Harbury, 1953, 1957
Pyocyanine (oxidant)	−0.032	Harbury, 1953, 1957
$H_2O_2 \rightarrow 1/2\ O_2 + H_2O$	~0.68	Harbury, 1953, 1957
Hydroquinone → quinone + 2θ	~0.70	Harbury, 1953, 1957

Table 2
pK values of phenolic compounds

Substance	pK value	Comment	Reference
Phenol	9.95	Thermodynamic value	Ficking et al., 1959
0-Methoxyphenol	9.93		Gawron et al., 1952
0-Hydroxybenzaldehyde	7.95		Gawron et al., 1952
2-Amino-4,5-dimethyl-phenol, HCl	(5.28), 10.4		Sims, 1959
4,5-Dihydroxbenzene-1,3-disulphonic acid	7.66, 12.6		Bjerrum, 1958
5-Hydroxy-2-hydroxy-methyl-γ-pyrone (kojic acid)	9.40		Bjerrum, 1958
Resorcinol	9.15	30°C	Gawron et al., 1952
p-Methoxyphenol	10.16		Gawron et al., 1952
3-Hydroxyantranilic acid	(5.20), 10.09		Sims, 1959
2-Aminophenol, HCl	(4.86), 9.99		Sims, 1959
Trimethylamino-phenol	o7.42, m8, p8		Jencks & Carriuola, 1960
CH_3SO_2-trimethylamino-phenol	m9.33, p7.83		Jencks & Carriuola, 1960
Acetyl-trimethylamino-phenol	m9.13, p8.05		Jencks & Carriuola, 1960
$C_2H_5CO_2$-trimethylamino-phenol	p8.50	Thermodynamic value	Jencks & Carriuola, 1960
$C_3H_5CH_2CO_2$-trimethylamino-phenol	p9.41		Jencks & Carriuola, 1960
HO-trimethylamino-phenol	9.48, m 9.44, p9.96		Jencks & Carriuola, 1960
CH_3-trimethylamino-phenol	o10.28, m10.08, p10.10		Jencks & Carriuola, 1960
CH_3O-trimethylamino-phenol	o9.93, m9.65, p10.20		Jencks & Carriuola, 1960
C_6H_5-trimethylamino-phenol	o9.93, m9.59, p9.51		Jencks & Carriuola, 1960
OCH-trimethylamino-phenol	o6.79, m8.00, p7.66		Jencks & Carriuola, 1960
CH_3O_2C-trimethylamino-phenol	p8.47		Jencks & Carriuola, 1960
n-$C_4H_9O_2C$-trimethylamino-phenol	p8.47		Jencks & Carriuola, 1960
$HOCH_2$-trimethylamino-phenol	o9.92, m9.83, p9.82		Jencks & Carriuola, 1960
C_2H_5-trimethylamino-phenol	o10.2, m9.9, p10.0		Jencks & Carriuola, 1960

(), the pK values of nonphenolic groups; o, ortho position; m, meta-position; p, para-position.

the buds developed to light-blue open flowers (Asen et al., 1977). A decrease in the pH value causes the opposite color change. Therefore, young blue-violet petals of Fuchsia were changed to purple-red as the pH value decreased from 4.8 to 4.2 (Yazaki, 1976). These pH effects may all be explained by the reactions shown in Fig. 15.

3.4. Absorption and fluorescence spectra of flavonoids

Since the colors of the flowers appear to be the major attracting factor for bees and other insects, which, in the course of their foraging activities, inadvertently spread pollen to receptive plants, and since the flavonoids are the most prominent petal pigments, these compounds owe important physiological qualities to their electronic properties. In this case, light absorption is linked to arousal by nervous perception, whereas in another well-known example of a link between electronic properties and physiological function, the hemoproteins, light absorption is connected with the transport of substrates and metabolites (O_2, CO_2, 2,3-diphosphoglycerate, nitric oxide [NO], CO, C_1-fragments, etc.).

Whereas the light absorption and the fluorescence of the flavonoids are of great importance to the analyst (El'-kommos & Maksiutina, 1978; Briggs & Colebrook, 1962; Romanova & Vachalkova, 1999), the plants could gain a particular benefit from a special electronic phenomenon, the charge-transfer complex. This phenomenon, which is recognised by the disappearance of a band in the spectrum of the isolated flavonoid aglycone and the arrival of a new band in the spectrum of a coplanar complex of the aglycone with a suitable aromatic compound, displaces water molecules from the vicinity of the chromophore. The complex is stabilised by the transfer of one or more electrons from one of the aromatic nuclei to the other, by hydrophobic interactions, by prevention of the hydrolysis of the anthocyanidin flavylium ring, and possibly also by hydrogen bonding. A charge transfer can be difficult to detect because the shift of the spectral band can be hidden by other strong transitions. Charge-transfer compounds are, for example, formed by aromatic or unsaturated hydrocarbons (Whelan, 1960).

Such complexes are also called donor-acceptor compounds or π-complexes. The partners in such complexes are attracted to each other by forces that appear to be chemical, but do not act between individual atoms. Hence, they cannot be regarded as valence bonds. An example is the interaction between isobutylene and silver ions, which is responsible for the increased solubility of the former in water in the presence of the latter. This charge-transfer complex may be regarded as a resonance hybrid of the mesomeric forms in Fig. 16.

Accordingly, the silver ion is not bonded to any unique carbon atom, but is linked to the entire unsaturated center. An alternative and equivalent description of the addition compound is based on the molecular-orbital theory.

The representation in Fig. 16 corresponds to mesomeric forms, but the one shown at the extreme left is believed to prevail. The distortion of the orbital is due to the interaction between the positive charge on the silver ion and the π-electrons (Fig. 17).

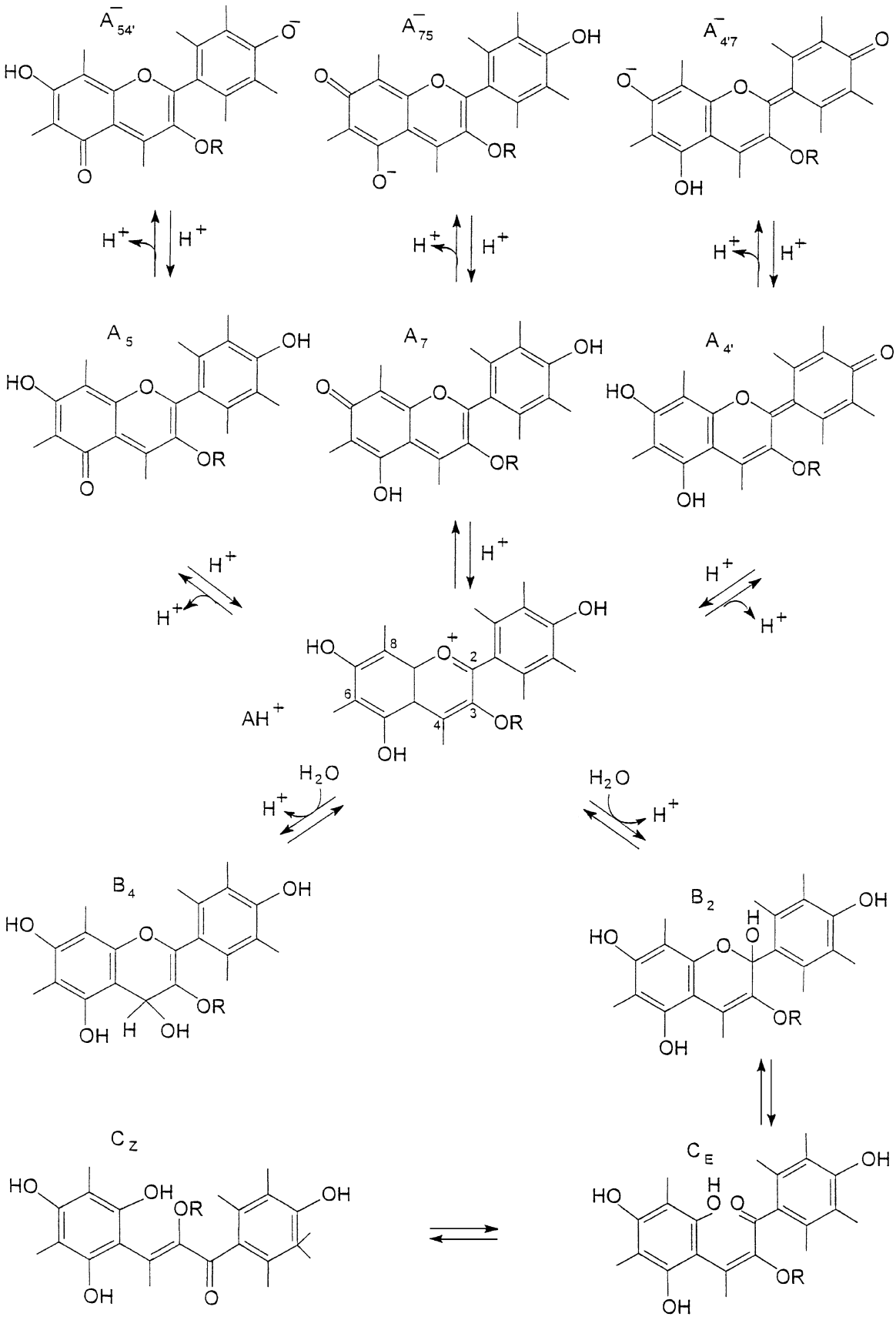


Fig. 15. Tautomerism of flavonoles in fuchsia petals.

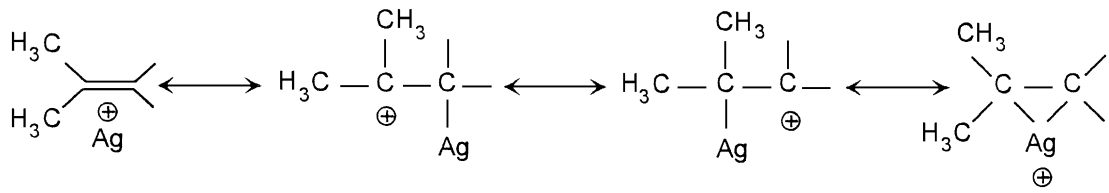


Fig. 16. Mesomeric forms of the isobutylene-Ag^+ complex.

Since the energy is lowered when the electrons are drawn closer to the atomic nuclei by the silver ion, this complex is more stable and soluble in water than the isolated partners. The π-orbitals and not the σ-orbitals of the covalent C-bond are involved in the binding of the metal ion since the former are much more easily displaced than the latter. Thus, a charge is transferred from the double bond, the donor, to the silver ion, the acceptor.

In addition to the silver ion, many other heavy metal ions can bond to π-electrons. Since flavonoids possess many π-electrons and are known to bind heavy metal ions, e.g., Hg^{2+}, with strong affinity, this phenomenon is most likely due to the formation of charge-transfer complexes. Aromatic rings, like those of flavonoids, possess many π-electrons. An example is benzene, which has three π-electrons on each side of the ring. The electron in the least stable orbital is more difficult to identify in benzene than in an alkene, but the problem can be resolved by a quantum-mechanical method. The latter approach shows that the metal ion, e.g., Ag^+, in the complex with benzene is located closer to two of the carbon atoms in the ring than to the remaining four. This result, which is counter-intuitive since a symmetric configuration would be expected, has been confirmed by X-ray crystallography. Consequently, the silver ion binds to one of the virtual double bonds of the Kekulé structure.

When a substance can be considered as a hybrid between two structures, then the resonance results in the formation of two distinct states of the system. The more stable of these states is the ground state, whereas the less stable state may be considered as excited. Since a transition between the two states should be accompanied by the absorption or emission of light, the spectrum of a charge-transfer complex is not a simple super position of the spectra of the components, but should contain a band shift. Such a feature would also be expected in the spectra of the flavonoids after the conformation change of the anthocyanidins and anthocyanins mentioned above, after copigmentation, and after the binding of heavy metal ions to flavonoids. The electronic spectra of flavonoids, therefore, should be a rich source of structural information about this class of natural products. Although the literature contains many spectral parameters of flavonoids (see, e.g., Harborne, 1992; Briggs & Colebrook, 1962), the spectra rarely have been examined in detail. The theory needed for this purpose has been reviewed by Donovan (1969) and Suzuki (1967). A technique that is similar and complementary to light absorption spectrometry, but orders of magnitude more sensitive, is spectrofluorometry. This method also provides additional structural information. However, this technique is more prone to systematic errors than absorption spectrophotometry. Therefore, a study of the theory and correct experimental procedures is advisable. Reviews on this topic have been published by Chen et al. (1969), Foerster (1951), and Hercules (1966). Fluorescence is often used for the identification of flavonoids, e.g., on chromatographic thin-layer plates, and for the semiquantitative estimation of the amount of flavonoids in an extract of plant material, bee products, or dietary components, and of the proportion of individual flavonoids in a mixture. However, the fluorescence can be highly dependent upon the presence of substituents in the aromatic nucleus, and it may be quenched, e.g., by accompanying ions. Therefore, the procedure is only reasonably safe, at least for the purpose of identification, if the aglycones have been separated from the glycosides, etc. by hydrolysis and/or extraction before the chromatographic evaluation.

3.5. Optical activity of flavonoids

The flavonoids are a class of natural product that more impresses by its great variety and the number of its members

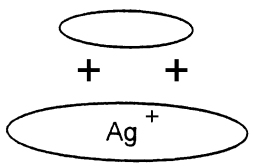


Fig. 17. The molecular orbitals of the two π-electrons in an alkene, which is strongly polarized by a silver ion. The two positive charges between the orbitals reside on the carbon atoms of the double bond.

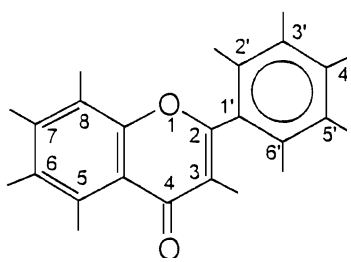


Fig. 18. Numbering of the atoms in the flavonoid aglycone at which a substitution may occur.

than by the complexity of the constituents in the structure. If the positions in the characteristic flavonoid nucleus, at which derivatives most commonly are formed by hydroxylation, methylation, acetylation, glycosylation, isoprenylation, etc. (Fig. 18), then it is possible by simple permutation to estimate the number of individual flavonoids that we can expect to find in nature.

The flavonoids are often hydroxylated in positions 3, 5, 7, 3′, 4′, and 5′. Some of these hydroxyl groups are frequently methylated, acetylated, or sulphated. When glycosides are formed, the glycosidic linkage is normally located in position 3 or 7, and the carbohydrates are commonly L-rhamnose, D-glucose, glucorhamnose, galactose, or arabinose (Kühnau, 1976). Prenylation usually occurs directly at a carbon atom in the aromatic rings, but 0-prenylation has also been found.

These features alone can account for $\sim 3 \times 10^5$ members of the flavonoid class, but the latter also includes a large number of more exotic forms, which have been omitted here for the sake of simplicity. The actual number of flavonoids that have been found so far and for which the structure has been completely elucidated is large, but probably does not exceed 1% of the theoretical number of possible variants. The discovery of a large number of additional, naturally occurring flavonoids, therefore, must be expected. This abundance of variants is further augmented by the chirality of the subunits and their connections. Since many stereoisomers do not differ significantly in their electronic or fluorescence spectra, the optical activity of the species is often a very useful analytical parameter. Incident linearly or circularly polarised electromagnetic waves sensitively interact with the electrons of the substance examined, thus shifting the phase of the former and producing a change in the optical rotation. This effect is wavelength-dependent, but particularly sensitive in the UV range. Accordingly, optical rotatory dispersion spectra or their correlate, circular dichroism spectra, often are very useful to distinguish between stereoisomers, to identify the absolute configuration of the structure, and to recognise centers of chirality. The theory, applications and experimental techniques of optical rotatory dispersion and circular dichroism, have been reviewed by Imahori and Nicola (1973), Djerassi (1960), Tinoco (1970), and Gaffield (1970), as well as by Sears and Beychok (1973).

3.6. Radical scavenging by flavonoids

One of the prominent and medically most useful properties of many flavonoids is their ability to scavenge free radicals (Agarwal & Nagaratnam, 1981; Wang & Zheng, 1992; Robak & Gryglewski, 1988; Gyorgy et al., 1992; van Acker et al., 1995, 1996; Ubeda et al., 1995; Clemetson & Andersen, 1966). These highly reactive species arise in the course of many physiological processes, especially in the respiratory chain and in oxidations catalyzed by oxygenases. These reactions are very common since molecular oxygen is a very good electron acceptor. Its high affinity for electrons provides the thermodynamic driving force, but in contrast to other electron acceptors, dioxygen reacts only slowly in the absence of a catalyst. The transfer of four electrons to dioxygen generates two molecules of water, i.e., a safe product, but partial reduction leads to the formation of highly toxic compounds, e.g., the superoxide anion $\cdot O_2^-$:

$$O_2 + \theta^- \rightarrow \cdot O_2^-$$

This species is, e.g., formed by macrophages in the first line of defence against invading foreign cells or virus particles. This reaction is desirable, but excess superoxide anion must be removed quickly before it has the opportunity to destroy too many essential, unsaturated liquids in the membranes, as well as sulfhydryl groups, e.g., in the active sites of key enzymes.

Normally, the release of partly reduced intermediates in the reaction with dioxygen is prevented. In the case of cytochrome oxidase, this reaction is mediated by metal ions. The dioxygen molecule is suspended at first between the Fe^{2+} ions and the Cu^+ ions of the a_3-Cu_B center in this enzyme (Stryer, 1988). Each metal ion then donates an electron to O_2, thus converting it to a dianion. Subsequently, an electron is donated from the cyt a-Cu_A center, which produces an intermediate ferryl ion. After the uptake of two protons from the medium, a water molecule is formed and the transfer of a second electron leaves a hydroxyl ion bound to Fe^{3+}. A third electron reduces Cu^{2+} to Cu^+, and the uptake of a proton produces a second water molecule (Fig. 19).

If superoxide, e.g., due to denaturation of the enzyme, escapes the heme protein before its full reduction, this free radical starts a chain reaction that may involve nucleic acid bases and many other vital cellular compounds, and results in mutation, metabolic derailment, and possibly cancer. Protonation of the superoxide anion yields the hydroperoxide radical $HO_2\cdot$, which spontaneously reacts with a second of these anions to produce H_2O_2:

$$O_2 \xrightarrow{\theta} \cdot O_2^- \xrightarrow{H^+} HO_2\cdot \xrightarrow[HO_2\cdot]{O_2} H_2O_2\cdot$$

Another common source of free radicals is radiation, e.g., X-rays or γ-rays. The main target is water, due to its ubiquity and high concentration in living organisms. Upon irradiation, this molecule produces hydroxyl radicals ·OH, which, apart from the above mentioned targets, also attacks other free radicals, e.g., NO· and superoxide, thus forming peroxynitrite, H_2O_2, nitrous acid, etc. The production of peroxynitrite is suppressed by flavonoids (Haenen et al., 1997). Naturally, the organism has developed a defence against such toxic substances. An important mechanism is

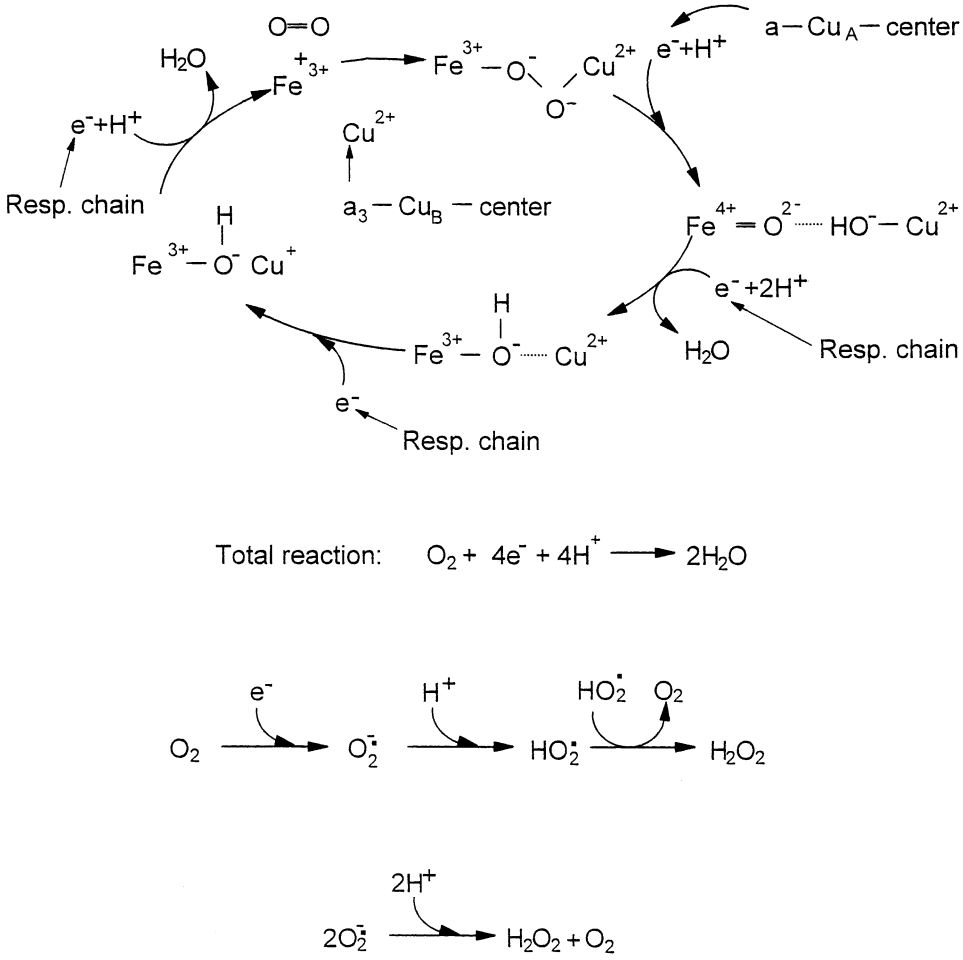


Fig. 19. The four electron reduction of O_2 by cytochrome oxidase. Resp. chain, respiratory chain. Adapted from Stryer (1988).

catalyzed by the enzyme superoxide dismutase (SOD), which converts two superoxide anions to H_2O_2 and O_2:

$$\cdot O_2^- + \cdot O_2^- \xrightarrow[\text{SOD}]{2H^+} H_2O_2 + O_2$$

The active site of the cytosolic, eucaryotic SOD contains a copper ion and a zinc ion coordinated to the imidazole moiety of a histidine residue. The negatively charged superoxide is guided electrostatically to a positively charged catalytic site at the bottom of a crevice. The superoxide anion binds to Cu^{2+} and the guanidino group of an arginine residue. An electron is transferred to Cu^{2+} from $\cdot O_2^-$ to form Cu^+ and O_2. The latter molecule is then released. Then a second superoxide anion enters the cavity to bind to Cu^+, arginine, and H_3O^+. An electron is transferred from Cu^+, and two protons are delivered from the two other binding partners to form H_2O_2 and to regenerate Cu^{2+} (Fig. 20).

SOD is a relatively small enzyme that can be injected into the blood stream without much danger of immunological complications. It is used to scavenge free radicals in the reperfusion phase after ischemic heart stop, e.g., during heart transplantation (Gulati et al., 1992; Fritz-Niggli, 1968). Another molecule, which is used for the same purpose, is ubiquinone, coenzyme Q. This compound is of particular interest since its properties resemble those of the flavonoids. Ubiquinone (Q) is an active participant in the respiratory chain (Fig. 21). Like cytochrome C, ubiquinone is a soluble substance and, hence, diffusible, but unlike cytochrome C, it can also traverse many biological lipid membranes. Therefore, it is easy to administer to support the respiratory chain, as well as the associated oxidative phosphorylation, and to scavenge free radicals.

During the operation of the respiratory chain, electrons flow from iron-sulfur clusters of the NADH-Q reductase to ubiquinone. The latter compound is a quinone derivative with an isoprenoid tail, the length of which in mammals usually is 10 isoprene units (Q_{10}). As in the case of the cytochromes, but different from the pyridine nucleotides and the flavonucleotides, a single electron is transferred to ubiquinone, which reduces it to an intermediate free-radical semiquinone. This intermediate avidly scavenges other free radicals that may be present, and this effect accounts in part for the protective effect of ubiquinone, e.g., against active oxygen species. In the respiratory chain, the semiquinone is

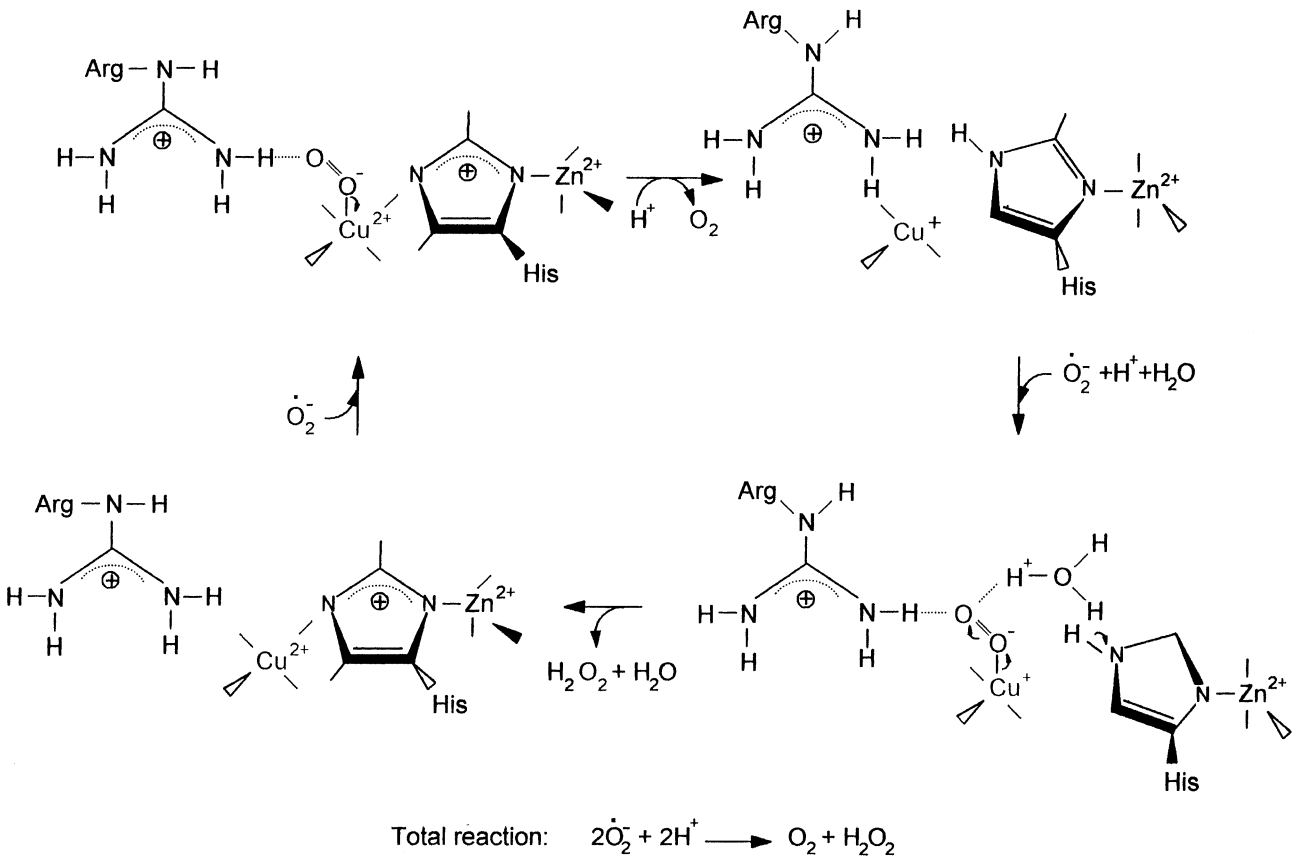


Fig. 20. Catalytic mechanism of superoxide dismutase. Copper cycles between the oxidation numbers +2 and +1 to catalyse the dismutation, whereas Arg141 and His61 serve as binding partners and polarizing agents. Adapted from Tainer et al. (1983).

reduced to ubiquinol (QH_2) by the uptake of a second electron (Figs. 21 and 22).

Ubiquinone is toxicologically quite an innocuous substance, which, according to some advocates, may be consumed orally in quantities of up to 1 g per day as a preventive measure against the damage caused by active oxygen (Folkers et al., 1992). In contrast to ascorbic acid (Vitamin C), which according to another Nobel prize winner, Linus Pauling, should be taken in gram quantities daily to prevent a disease, ubiquinol is not acidic. Neither is its consumption known to be followed by any other undesirable side effects, e.g., acidosis. Therapy of cardiac infarction patients with ubiquinone has been shown to reduce the risk of a recurrence of infarction (Folkers et al., 1992).

It is also interesting that the heart muscle responds to stress by producing more ubiquinone (Suzuki et al., 1992). Ubiquinone is not only a free-radical scavenger, but is also an antioxidant. These two properties are not necessarily closely connected, since the former depends on the presence of an unpaired electron, whereas the latter is determined by the oxidation-reduction potential. This can be seen when the effects of (+)-catechin and flavonoids on the conversion of arachidonic acid to prostaglandin (PG) catalyzed by the PG cyclo-oxygenase (COX) are compared (Baumann et al., 1980; Morrow et al., 1990; Liang et al., 1999). Interestingly, ubiquinone-3 protects human low-density lipoprotein (LDL) against oxidation of its unsaturated FA moieties (Merati et al., 1992). This is a property

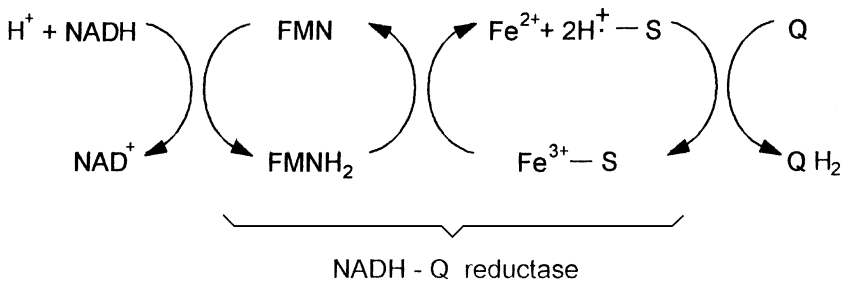


Fig. 21. NADH-Q reductase.

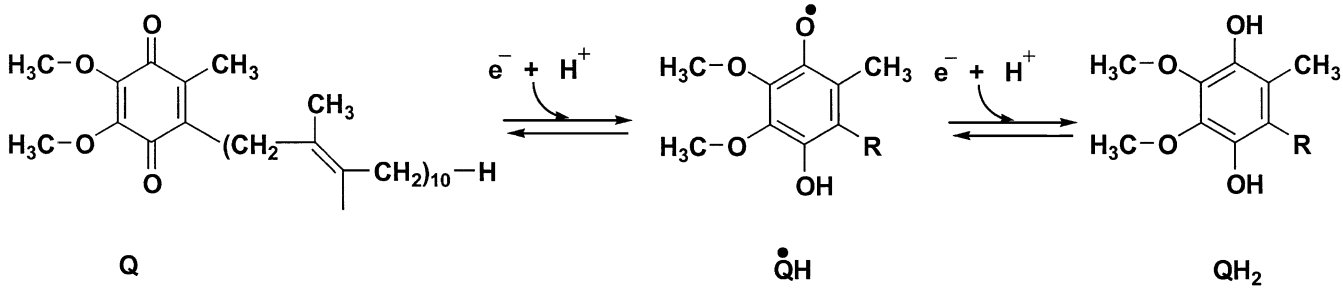


Fig. 22. Ubiquinone (Q) is reduced through a semiquinone radical intermediate (·QH^-) to ubiquinol (QH_2). R, isoprenoid substituent. Data from Miki et al. (1992) and Kubota et al. (1992).

also ascribed to flavonoids, i.e., another point of resemblance (Brown & Rice-Evans, 1998).

A key enzyme in the biosynthesis of cholesterol, 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase) is inhibited by ubiquinone. As a result, the cholesterol concentration in blood serum in the case of a hypercholesterolemia can be reduced by the oral intake of coenzyme Q (Sharma, 1979). The same is claimed for the flavonoids (Chai et al., 1981; Oganesyan et al., 1989).

A substance that, for example, is important to the stability of erythrocytes is glutathione (GSH). The oxidation of GSH to GSSG by superoxide leads to hemolysis. The GSH thiol free radical can be eliminated by reaction of GSSG with a semiquinone free radical, e.g., of coenzyme Q or a flavonoid (Iio et al., 1993; Galati et al., 1999; Kaneko & Baba, 1999). However, some flavonoids exist that inhibit GSH reductase (Elliott et al., 1992; Khushbaktova et al., 1991). Hence, the oxidised form of GSH, GSSG, in that case can no longer be reduced. The result will be an enhancement of hemolysis. Thus, the effect of different flavonoids in a mixture can antagonise each other. To the therapist, this means that it is advisable to analyse a natural product for its flavonoids before its use, or to apply a pure flavonoid. Flavonoids can also inhibit GSH S-transferase, which can compromise the transport of amino acids across membranes (Frohlich et al., 1989; Yokozawa et al., 1999).

The resemblance of the oxidation reactions of ubiquinol and flavonoids is apparent when Figs. 22 and 23 are compared.

Flavonoids offer protection from free radicals by their scavenging ability (Uma Devi et al., 1999; Re et al., 1999; Merati et al., 1992).

3.7. Linear free-energy relationships applied to the flavonoids

3.7.1. The nature of the problem

The elucidation of the many diverse physiological properties of the flavonoids is a considerable challenge to biochemists. Moreover, these properties are not equally shared by all members of the group. Hence, the experience collected by natural product chemists teaches that some relationships between structure and function based on substituent effects are to be expected. However, such an analysis is complicated in this case because the flavonoids are very reactive compounds. They can enter into almost any type of reaction known to organic chemistry, e.g., oxidation-reduction reactions, carbonyl reaction, acid-base reactions, free-radical reaction, hydrophobic interactions, tautomery, and isomerisations. The substituents may also exert their influence by electronic induction, hyperconjugation, resonance, steric hindrance, and complexation with heavy metal ions. Naturally, this multifariousness should not deter the search for structure-activity relationships, but simple explanations are not to be expected. A few reports have already appeared that confirm this view (see Section 3.7.2).

3.7.2. Linear free-energy relationships

Since this problem can be solved only with the methods of physical organic chemistry, some basic principles must be reviewed. One of the most important to this kind of analysis is the concept of the linear free-energy relationship. It was originally conceived by Hammett (1935), who analyzed the effect of substituents on the acidity of aromatic carbonic acids (Ficking et al., 1959; Jencks & Carriuola, 1960). The Hammett theory had an immediate impact on the soap industry, and became a standard concept in pharmacology and natural product chemistry. In retrospect, the idea of the linear free-energy relationship is only to be considered as an approximation, but it is often a good one (Eigen, 1964). Actually, the idea can be traced back to the work of Brønsted on acidity (Brönsted & Pedersen, 1923), but Hammett did not appear to be aware of this connection.

The basis of linear free-energy relationships is the similarity of the shapes and positions of the reaction energy profiles in a series of related compounds that undergo the same type of conversion. If the energy profiles of the reactant and the product are also approximately linear near the point of their intersection, then a linear free-energy relationship can be expected (see Figs. 1 and 11 in Eigen, 1964). The conformity of the energy profiles of a series of structurally related compounds means that their ground level energy is directly influenced by the electronic properties of the substituents, and the linearity of the potential energy curves at the intersection means that the exponential terms

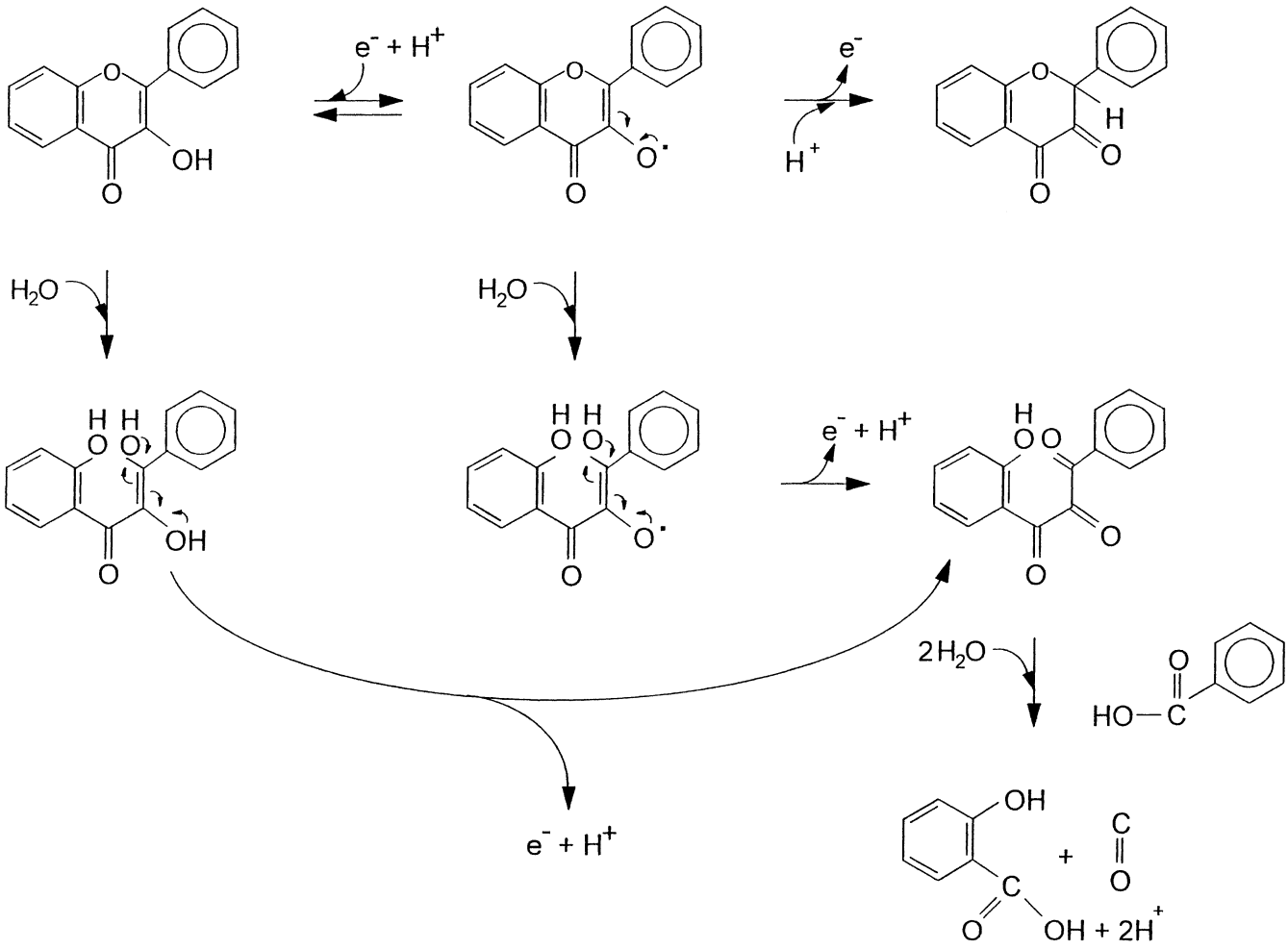


Fig. 23. Oxidation-reduction reactions of flavonols.

in the Morse curve equation (Eyring & Polanyi, 1931) are small. That will be the case if the internuclear distance between the donor and acceptor, e.g., of a proton or an electron, is near the equilibrium value.

The substituent effect may be electrostatic or inductive (between dipoles through space or along the carbon chain) (Hine, 1956). Besides, the substituents can influence the resonance energy of the compound, e.g., sterically since resonance requires planarity. Such effects may cause departure from the linear free-energy relationship. Another source of such nonlinearities is hydrogen bonding. A few authors have already tried to correlate structure with the biological activity of flavonoids. Such attempts are particularly complicated for this group of natural products due to the great variety of reactions in which its members can take part. Therefore, to the knowledge of the author, none of these cases has been clarified exhaustively. Examples of structure-activity investigations that carry the potential of representing linear free-energy relationships are described, e.g., by Duarte et al. (1993), Schwartz and Middleton (1984), Iio et al. (1980, 1983, 1985), Baumann et al. (1980, 1981), Sharma et al. (1971), Paintz and Metzner (1979), Hendrickson et al. (1994), Lee et al. (1994), Kalkbrenner et al. (1992), and Krol et al. (1994).

4. The occurrence of flavonoids

4.1. Distribution in nature

The flavonoids are qualitatively and quantitatively one of the largest groups of natural products known. Since almost all flavonoids are pigments, their colors are undoubtedly associated with some of their important biological functions. The ubiquity of the flavonoids to all geographical zones of herbal growth supports this argument. Since all colors of the spectrum, including its UV region, are represented in the spectra of the flavonoids, their electronic properties appear to include not only energy capture and transfer, but also biological selectivity. The latter is not only associated with the attraction of suitable pollinators, e.g., insects and birds, but also with the selective activation of light-sensitive genes (Kirby & Styles, 1970). A carefully studied example of the latter phenomenon is the light-sensitive growth gene of

barley. Although there is strong light absorption by the flavonoids and they are present in all plant cells containing plastids, no evidence of a participation of the flavonoids in the primary photosynthetic process is known. Evidently, plants are using light not only as a source of energy, but also for gene regulation.

Another striking electronic property of the flavonoids is their fluorescence. It remains yet to be proven whether this property is used physiologically. However, such a function is conceivable, since fluorescence can transfer small amounts of energy, which may suffice to activate pigments associated with light-sensitive genes.

The ubiquity and great diversity of the flavonoids render these pigments suitable for a taxonomical classification. Their usefulness for this purpose is enhanced by the close association of the flavonoids with vital genes, especially those involved in growth regulation. Such genes would be expected to be particularly sensitive to environmental cues and, hence, mirror both the nature of the biotope and the competitive strength of the species.

The basis of the great variability of the flavonoids is:

1) differences in the ring structure of the aglycone and in its state of oxidation/reduction;
2) differences in the extent of hydroxylation of the aglycone and in the positions of the hydroxyl groups;
3) differences in the derivatisation of the hydroxyl groups, e.g., with methyl groups, carbohydrates, or isoprenoids.

A permutation of these sources of variability reveals that theoretically, more than 2×10^6 different flavonoid species can occur. So far, more than 2×10^3 different flavonoids have been identified, and their number is growing rapidly (Bilyk & Sapers, 1985; Farkas et al., 1986; Cizmarik & Matel, 1970, 1973; DuBois & Sneden, 1995). Since flavonoid family members are closely related structurally, it is difficult to separate them (Hostettmann & Hostettmann, 1982). Besides, they are easily decomposed. Due to the relatively high molecular weight and the complicated structure of these compounds, their identification and chemical synthesis represent a challenge to the organic chemists, even if they possess modern equipment.

Since the flavonoids, depending on their content of glycosides, isoprenoids, and aliphatic ethers, can acquire almost any polarity, a range of solvents from water to ethyl ether must be used for their extraction from a complex mixture, e.g., in propolis (bees glue), honey, wax, syrup, or plant tissue. The extracts are often subfractionated on hydroxylapatite before a final separation is carried out by capillary electrophoresis (Cancalon, 1999) or HPLC (Galensa & Herrmann, 1979; Garcia-Viguera et al., 1993; Greenaway et al., 1987, 1991; Watson & Pitt, 1998; Watson & Oliveira, 1999; Ishii et al., 1996; Gawron et al., 1952).

Several publications specializing in the identification of flavonoids have appeared. Prominent examples are Mabry et al. (1970), Markham (1982), Harborne (1988a, 1988b), Bankova et al. (1982, 1987), Pangarova et al. (1980, 1986), Garcia-Viguera et al. (1993), and Bonvehi et al. (1994).

5. Identification of flavonoids

The complete analysis of the absolute structure and configuration of a flavonoid is usually a complicated task, which requires the application of advanced techniques, e.g., [^{1}H]- and [^{13}C]-NMR-spectrometry, [^{1}H-^{1}H]-correlated spectroscopy, circular dichroism, optical rotatory dispersion, mass spectrometry, and X-ray diffraction. Since only a few laboratories are equipped and staffed to make all of these expensive methods available, simpler approaches to the characterisation of flavonoids are desired. Modern chromatographic techniques like HPLC have become standard equipment in biochemistry laboratories, and often yield not only an excellent resolution, but also retention times that can be very useful in the identification of a flavonoid. A much less expensive method to acquire an impression of the nature and amounts of individual flavonoids in an extract is a combination of thin-layer chromatography and fluorescence (Jay et al., 1975; Ghisalberti, 1979; Nikolov et al., 1976; Hladon et al., 1980; Lavie, 1978; Chi et al., 1994; Glencross et al., 1972; McMurrough et al., 1985; Issaq, 1997; Karting & Hiermann, 1978).

The preparation of a sample for analysis can present a problem since flavonoid glycosides are predominantly polar structures and, hence, water-soluble, whereas the aglycones are nonpolar (Calman, 1972). The latter, therefore, must be extracted by nonpolar solvents. Methanol is often a useful compromise that permits the extraction of the majority of the flavonoids. A particularly mild and efficient extraction procedure for lipophilic flavonoids is triple-point extraction with CO_2. This procedure is rapidly gaining acceptance. If a primitive method must be applied, a sample of 50 mg of solid material may be extracted with 1 mL of methanol or amyl alcohol at room temperature in 15 min with shaking. A standard mixture of known flavonoids may be used as references. The positions of the flavonoids can be observed in UV light from a hand lamp. The characteristic colors emitted by individual flavonoids in a mixture, when exposed to UV light, aids in their identification (see Section 4.1). Jay et al. (1975) have published an extensive table of the mobilities in various solvents and the fluorescence colors of flavonoids ($\sim$175). In addition, the main medical uses of some of the prominent flavonoids are listed.

If more information that just the nature and relative amounts of the flavonoids in a sample is required, then each component must be isolated in amounts (>10 mg) sufficient for an organic chemical analysis: elementary composition (C, H, O), melting point, UV-, IR-, NMR- and mass spectra. Guides for the systematic identification of flavonoids have been published by Mabry et al. (1984) and Markham (1982).

Examples of the content of flavonoids and their catabolites in propolis and one of its important sources, poplar exudate, are given in Table 3. More details are found in a series of articles by Greenaway et al. (1987).

5.1. Magnetic resonance spectrometry of flavonoids

5.1.1. Introduction

In modern biochemistry, magnetic resonance spectroscopy has proven to be a particularly useful method, e.g., for the determination of intramolecular distances between atoms or functional groups, for the determination of the orientation of substituents about chiral centers, and to assess molecular motion. In the field of the flavonoids, NMR spectroscopy has prevailed over ESR methods, in spite of the relatively small signals and higher instrumental costs of the former technique. The reason is the universal applicability of NMR techniques to organic substances and the greater variety of accessible experimental parameters that NMR spectrometry offers. A distinct advantage of the NMR approach is that measurements can be made on the native molecule without any introduction of foreign isotopes or reporter groups that might disturb the structure, thus giving rise to artifacts. The atomic nuclei, which in flavonoids most often are used for NMR experiments, are ^{1}H and ^{13}C. Both of these isotopes are stable and occur naturally. Therefore, complications due to radioactive decay and protective measures against ionizing radiation are avoided. The natural abundancies of ^{1}H and ^{13}C are 99.98 and 1.11%, respectively, if hydrogen is used as a standard. Each of these nuclei must be measured with a specific transmitter. The one for ^{1}H (100 MHz at a field of 23.49 kG) is more expensive, but it also yields the highest sensitivity of all nuclei. The ^{13}C-sender has a frequency of 25 MHz (for a field of 23.49 kG), but the sensitivity of the measurement is only 1.6% that of ^{1}H. Since both nuclei supply useful structural information on almost any compound, a comparison of the two kinds of NMR spectra is desirable. The principle of NMR spectroscopy is to measure the energy of a radiofrequency wave required to alter the direction of the spin of a given type of nucleus. At first, the sample is placed in a strong static magnetic field, which orients the spin of all nuclei in its direction. Then, a radiofrequency wave is radiated into the sample from a direction perpendicular to the static field. The interaction between the fields, especially the static, the radiofrequent, and the one created by the rotating nucleus, results in a change in the direction of the spin of the latter. As soon as the radiofrequent wave is shut off, the nuclear spin relaxes to its previous direction, the one of the static field. Then the static field is scanned through a range sufficient to switch the nuclear spin to a new direction allowed by the laws of quantum chemistry. This process requires energy that is taken from the radiofrequent wave. Hence, a measurement of the amount of energy absorbed by the sample as a function of the strength of the static field will show a peak at the field strength characteristic of the change of the nuclear spin. This phenomenon is also called resonance. Since this condition depends on the environment, i.e., on the fields of neighbouring nuclei, information on the molecular structure is obtained. This environmental information is expressed as a shift in the field strength called the chemical shift, relative to the resonance field strength of a standard substance (Bloembergen et al., 1948; Bovey, 1969; Lee, M. W., 1994; Luz & Meiboom, 1964; McConnell et al., 1955).

5.1.2. Information available from proton relaxation rates

(1) Evidence of specific binding of ligand to the paramagnetic probe may be obtained.
(2) At least three types of ligand-probe complexes have been found and may be distinguished.
(3) Binding constants and the number of binding sites can be obtained. The values found have usually compared well with those obtained by other methods. The proton relaxation rate (PRR) approach offers the advantage of being fast.
(4) Even small conformational changes may be detected by PRR.
(5) Changes in the state of oxidation may be detected by PRR.

The limitations of the PRR studies are that a paramagnetic species must be present and that the concentrations required are rather large. However, a small volume may be used, 10–100 μL may suffice. Precautions must be taken to remove any chelating agents, which may interfere with a paramagnetic metal ion probe.

5.1.3. The theory of pulsed nuclear magnetic resonance

Although the concepts of quantum mechanics are required for a rigorous treatment of the relaxations from pulse perturbations of atomic nuclei in a magnetic field, the classical theory of mechanics suffices to explain the principles and the experimental procedures of PRR (Kowalsky & Cohn, 1964). The discussion is further restricted to nuclei of the spin angular momentum quantum number $I = 1/2$. Such nuclei are partitioned between two energy levels when they are exposed to a static magnetic field, H_o, which is applied in the Z-direction. The equilibrium distribution of nuclei aligned parallel or anti-parallel to H_o has a small excess of the former population, which creates a net macroscopic magnetic moment, M_o, in the direction of H_o. This equilibrium distribution may be disturbed by the irradiation of the nuclei with an electromagnetic wave of a frequency, ν_o, corresponding to the energy difference between the two populations, $\Delta E = h \cdot \nu_o$, where h is Planck's constant.

Classical mechanics predicts that an isolated nucleus exposed to a magnetic field of the strength H_o, is subjected to a mechanical torque $\mu \times H_o$, where μ is the magnetic

Table 3
Flavonoid composition of some typical European propolis varieties and of an important source, poplar bud exudate as found by GC-MS

Common name	Retention time (methylene units)	Exudate (percent of total ion current)	Propolis	
			Oxfordshire, UK (percent of total ion current)	Warwickshire, UK
Benzyl alcohol	11.42	0.5	0.5	0.2
Phenylethyl alcohol	12.18	0.1	0.1	<0.1
Benzoic acid	12.32	9.9	9.5	7.0
Phosphate	12.78	0.1	0.2	0.7
Glycerol	12.93	<0.1	0.2	0.6
Succinic acid	13.06	<0.1	<0.1	<0.1
4-Hydroxybenzaldehyde	13.48	<0.1	<0.1	0.4
Fumaric acid	13.49	–	–	0.1
Hydrocinnamic acid	13.94	0.2	0.1	0.4
Hydroquinone	14.02	–	<0.1	<0.1
Cinnamyl alcohol	14.06	<0.1	<0.1	0.3
Vanillin	15.00	0.1	<0.1	0.4
Cinnamic acid	15.17	0.1	0.2	2.2
Isovanillin	15.20	<0.1	<0.1	<0.1
Protocatechualdehyde	15.98	0.2	0.1	0.2
p-Hydroxybenzoic acid	16.17	<0.1	<0.1	0.1
Sesquiterpene alcohol	17.54	–	–	0.2
4-Methoxycinnamic acid	17.91	0.4	0.5	0.7
Cinnamylideneacetic acid	18.04	0.1	0.1	<0.1
Protocatechuic acid	18.26	0.1	<0.1	0.1
3,4-Dimethoxycinnamic acid	18.34	0.1	–	<0.1
Myristic acid	18.49	0.1	0.3	0.1
Fructose	18.62	–	0.3	2.1
Fructose	18.70	–	0.5	2.8
Glucose	19.02	–	<0.1	0.3
p-Coumaric acid	19.29	0.4	0.8	4.7
Glucose	19.43	–	0.3	2.9
Benzyl salicylate	19.85	0.1	0.1	0.3
3,4-Dimethoxycinnamic acid	19.86	0.9	0.8	2.1
Cinnamoyl benzoate	20.18	0.1	0.1	–
Glucose	20.43	–	0.3	5.3
Pentenyl coumarate	21.24	0.1	0.4	0.1
Caffeic acid	21.44	0.8	0.8	4.8
Pentenyl coumarate	21.77	0.2	0.5	0.2
Oleic acid	22.13	0.1	0.2	0.3
Stearic acid	22.43	0.5	0.2	0.8
Pent-4-enyl-isoferulate	22.79	0.3	0.2	0.2
C_{23} straight-chain hydrocarbon	23.00	–	0.3	0.2
Pentenyl ferulate	23.28	0.5	0.7	0.4
Pentenyl caffeate	23.45	1.7	2.1	1.3
Trihydroxychalcone	23.66	2.0	<0.1	–
Pentenyl caffeate	23.45	1.7	2.1	1.3
Pentenyl caffeate	23.98	1.2	2.2	0.9
Pinostrobin chalcone	24.53	0.5	0.4	0.3
Benzyl *p*-coumarate	24.64	1.6	2.8	0.7
Benzyl caffeate	24.71	0.5	<0.1	–
Pinocembrin	24.92	0.5	0.4	0.4
Pinocembrin chalcone	24.99	11.8	6.9	3.0
C_{25} straight-chain hydrocarbon	25.00	0.9	7.9	2.2
Alpinitin chalcone	25.28	0.2	<0.1	0.1
Phenylethyl *p*-coumarate	25.45	<0.1	0.6	0.6
3-Acetylpinobanksin chalcone	25.67	2.9	2.7	1.9
Trihydroxyflavonoid aglycone	25.77	5.3	3.9	2.1
Benzyl isoferulate	26.00	0.7	0.7	0.1
Chrysin	26.02	–	0.4	0.5
Monomethoxy-dihydroxyflavonoid aglycone	26.16	2.6	2.4	1.5
Benzyl ferulate	26.18	1.5	1.4	0.5
Behenic acid	26.36	–	<0.1	0.5
Tectochrysin	26.41	<0.1	<0.1	<0.1
C_{27} straight-chain hydrocarbon	27.00	1.5	7.0	1.2
Chrysin	27.04	5.2	4.0	1.7

Table 3 (*continued*)

Common name	Retention time (methylene units)	Exudate (percent of total ion current)	Propolis	
			Oxfordshire, UK (percent of total ion current)	Warwickshire, UK
Galangin-3-methyl ester	27.08	1.0	1.0	<0.1
Sucrose	27.36	–	0.9	6.5
Galangin	27.45	6.6	4.3	2.1
Phenylethyl caffeate	27.65	3.3	2.6	2.0
Dihydroxymethyxyflavone	27.66	0.1	0.1	0.1
Cinnamoyl p-coumarate	27.80	0.2	0.2	1.5
Lignoceric acid	28.37	–	2.9	2.1
C_{29} straight-chain hydrocarbon	29.00	0.2	1.1	1.0
Cinnamyl isoferulate	29.13	0.6	0.4	1.1
Cinnamyl caffeate	29.96	0.6	0.3	1.7
Cerotic acid	30.36	–	0.3	0.3
Kaempferol-7-methyl-ether	30.73	<0.1	<0.1	–
Kaempferol	30.94	0.2	0.1	<0.1
Kaempferol-3-methyl-ether	30.96	<0.1	–	–
C_{31} straight-chain hydrocarbon	31.00	–	0.6	0.6
Apigenin	31.45	<0.1	<0.1	–
Rhamnetin	32.24	<0.1	<0.1	–
Montanic acid	32.55	–	0.3	0.3
C_{33} straight-chain hydrocarbon	33.00	–	0.6	0.7

moment of the nucleus. Like a top spinning with its axis at an angle to the vertical, the nucleus will precess with an angular frequency of $\omega_o = \gamma H_o = (2\ \mu z/h)H_0$, where γ is the gyromagnetic ratio. The motion is conveniently described in a new coordinate system, (X′, Y′, Z′), which, in contrast to the original coordinate system (X, Y, Z), which is fixed, rotates with the angular frequency of the nucleus. Thus, the magnetic spin moment is stationary in the reference frame. Now, an additional magnetic field H_1, which rotates, like the spin moment, with the reference frame, is applied to the nucleus. This field induces a new precessing motion of the magnetic moment in the rotating frame. Its frequency is ω_1 (Fig. 24). This type of motion is called a nutation. It is stationary in the rotating frame (X′, Y′, Z′).

Although a sample contains many nuclei, e.g., $\sim 10^{20}$/mL, the ensemble behaves like a single nucleus, and a net magnetisation M_o, which is parallel to H_o, arises. After the application of the additional field H_1, M_o begins to nutate, i.e., its direction is no longer parallel to H_o. The sample is placed in a coil, the axis of which is perpendicular to H_o, and which conducts a signal induced by the rf-wave of the resonance frequency (Fig. 25). The oscillation field produced by this coil may be decomposed into components, one of which, H_1, rotates in the XY-plane with the precessing nuclei. If H_1 is switched on at zero time, then at a later time, t_1, the angle through which M_o nutates is $\gamma H_1 t_1$. Accordingly, the time required by M_o to nutate through the angle θ is $\theta/\gamma H_1$. A common technique is the application of a pulse of a duration corresponding to a 90° nutation of M_o (Fig. 25). Similarly, a pulse of the duration of a 180° nutation, i.e., twice as long as the 90° pulse, is often used.

At completion of a 90° pulse, M_o has reached the X′Y′-plane, but in the XY-plane, it is rotating at the resonance frequency. This motion, in turn, influences the magnetic flux

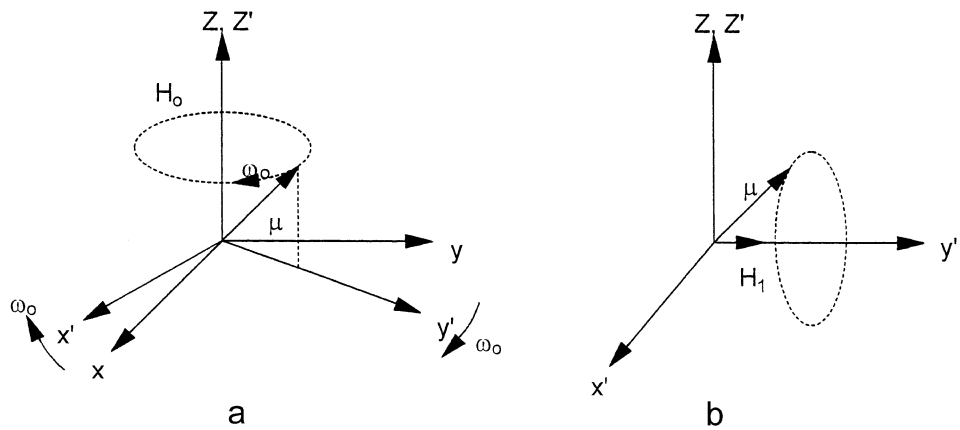


Fig. 24. Classical motion of a nuclear spin vector of the magnetic field H_o. **a**: The precession in the fixed (X, Y, Z) and in the rotating coordinate system (X′, Y′, Z′). **b**: Nutation caused by the radio frequency field H_1 viewed in the rotating frame.

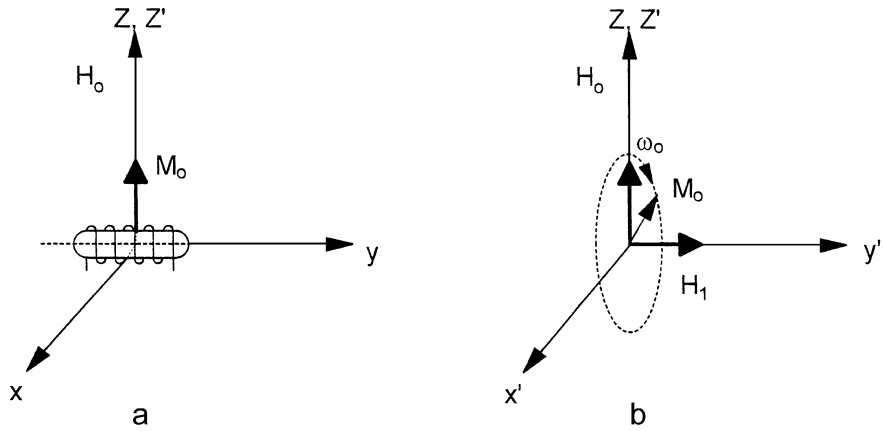


Fig. 25. **a**: The magnetization of a macroscopic sample in a tube surrounded by a solenoid and placed in a static field H_o. **b**: The nutation of M_o by the radiofrequency field H_1, as viewed in the rotating coordinate system.

of the coil, with the result that a voltage of the resonance frequency is induced in the coil. This signal, which is called the free precessional signal, is amplified and displayed on an oscilloscope. It is maximal after a 90° pulse, but 0 following a 180° pulse, since in the latter case, the magnetisation M_o is on the Z-axis (pointing in the negative direction). Hence, there is no detectable component in the X′Y′-plane. The free precessional signal from a 90° pulse decays to 0, due to 2 relaxation processes. The perturbation by the pulse disturbs the spin equilibrium. The magnetisation M_o, therefore, must subsequently return to the position in which it points in the positive direction of the Z-axis with the characteristic time T_1, the spin-lattice, or longitudinal relaxation time, according to the equation:

$$M_z(t) = M_o[1 - \exp(-t/T_1)] \quad (5.1)$$

Simultaneously, the magnetisation component in the XY-plane decays with the characteristic time T_2, the spin-spin, or transversal relaxation time, according to:

$$M_{xy}(t) = M_o\exp(-t/T_2) \quad (5.2)$$

The relaxation processes are caused by local magnetic fields, H_2, which are produced by nuclear magnetic dipoles, e.g., those of paramagnetic probes. At the site of a nucleus, the magnetic field is $(H_o + H_2)$ and its precessional frequency is $\omega(H_o + H_2)$. H_2 is small compared with H_o, and varies locally. Hence, after a 90° pulse, the precessional frequencies are spread and the spins, which were in phase at the instant of the pulse, begin to dephase. Consequently, the signal induced in the coil approaches zero as the phase randomises. Field inhomogeneities can also spread the spin phases and, thus, obscure the natural T_2 decay, which is to be measured. Such problems can be solved by the spin echo technique.

5.1.4. The measurement of relaxation times

The spin-lattice or longitudinal spin relaxation time T_1 usually is measured using a protocol, according to which two pulses of the same amplitude are applied with a variable interval (Fig. 26). A new sequence of the two consecutive pulses separated by a different interval may not be applied until the spin system has regained equilibrium, i.e., after the elapse of a period of at least 5 T_1 (corresponding to 99% equilibration). The amplitude of the signal from the second pulse may be plotted semilogarithmically against t, according to Eq. (5.1). The slope of the line yields T_1. The pulse sequence 90°-t-90° gives rise to signals like those illustrated in Fig. 27.

A more direct method of measuring T_1 uses a sequence of two pulses, of which the first is a 180° pulse and the second a 90° one. The two pulses are separated, as in the 90°-t-90° protocol, by a variable interval. The first pulse reverses the direction of the magnetisation vector. Subsequently, the latter relaxes back with the characteristic time T_1. If the 90° pulse is released before the magnetisation vector is zero the signal detected will be negative. Otherwise, a positive signal will be recorded. According to Eq. (5.1), its time course will be exponential and, as above, may be plotted semilogarithmically to yield T_1 from the slope of the line (Fig. 28).

The relaxation time T_2, the transversal or spin-spin time relaxation time may be measured if the second pulse of a sequence of two is applied in the Y′-direction. The first pulse is a 90° pulse in the direction of the Z-axis. The tipped spins dephase with individual precessional frequencies, causing apparent divergence of their spin vectors (Fig. 29, positions p and q). The second pulse of H_1, the one of 180°, which is applied along the Y′-axis, causes a nutation of the spin vectors about this axis to the opposite positions, i.e., from p and q to p′ and q′, respectively. However, now they converge due to the first pulse, which is still decaying. The reason for this is that their relative precessional frequencies remain unchanged. This procedure eliminates the part of the decay of the first pulse, which is due to field inhomogeneity. The spins have returned to a common phase after the elapse of the time 2t, where t is the interval between the two pulses. Consequently, an echo arises that is detected by the sample coil. Subsequently, the individual spin vectors dephase again

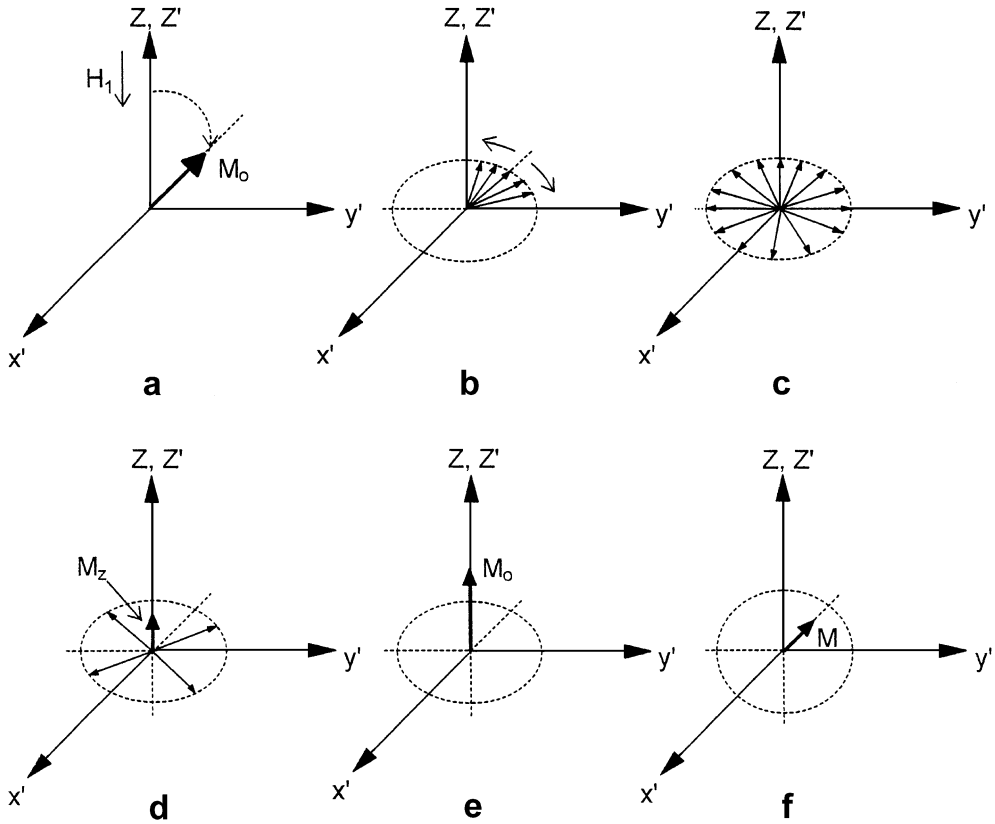


Fig. 26. Evaluation of the spin system after a 90° pulse of the field in the Z-direction viewed in the rotating coordinate system (X′, Y′, Z′). **a**: The static magnetization M_o is tipped from the Z-axis into the X′, Y′-plane by the 90° pulse. **b**: The individual spins begin to dephase. **c**: The spin phases have reached a random distribution. **d**: The growth of the magnetization vector M_Z in the direction of H_o during relaxation. **e**: Regain of the spin equilibrium existing before the pulse. **f**: A second pulse applied at the time at which the situation of the spins is the one depicted in **d**. A coherent magnetization, M, is produced in the X′,Y′-plane, which induces a pulse in the detector coil. Its amplitude, which is smaller than the one of the first 90° pulse, is proportional to $M_Z(t)$ and can be used to measure T_1.

causing the decay of the echo. Although the 180° pulse compensates for the decay due to field inhomogeneity, other artefacts from local fields can lower T_2. The latter relaxation time may be evaluated using the equation:

$$Y(t) = Y_{o\,exp}(-t/T_2) \tag{5.3}$$

where Y is the echo amplitude and t the interval between the two pulses. When T_2 is long, diffusion may significantly deter the rephasing process. However, the resulting decay is nonexponential. The effect, therefore, can be detected. Experimentally, diffusion artefacts can be reduced by the application of the pulse sequence 90°-180°-180°-180° (Carr & Purcell, 1954). The 180° pulses are applied at the times t, 3t, and 5t, and the echoes, which occur at the times 2t, 4t, and 6t, will be exponential (Fig. 29).

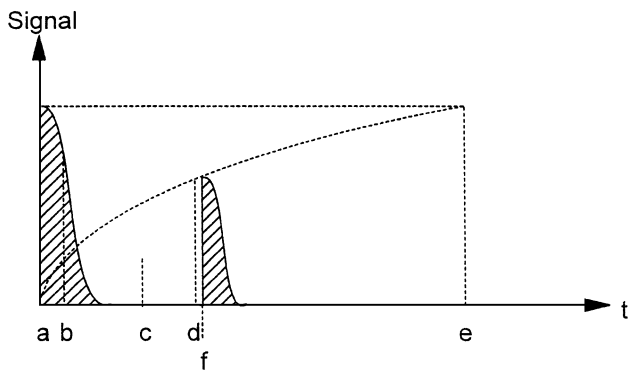


Fig. 27. Signals observed when the 90°-t-90° protocol is applied to measure T_1. The letters refer to the spin conditions illustrated in Fig. 26. The time axis represents the interval between the two 90° pulses.

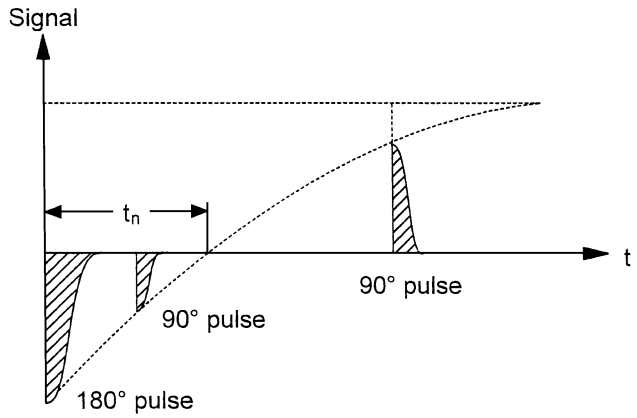


Fig. 28. Signals observed after the use of the 180°-t-90° protocol to measure T_1. The 90° signal is zero at $t = t_n = T_1 \ln 2$.

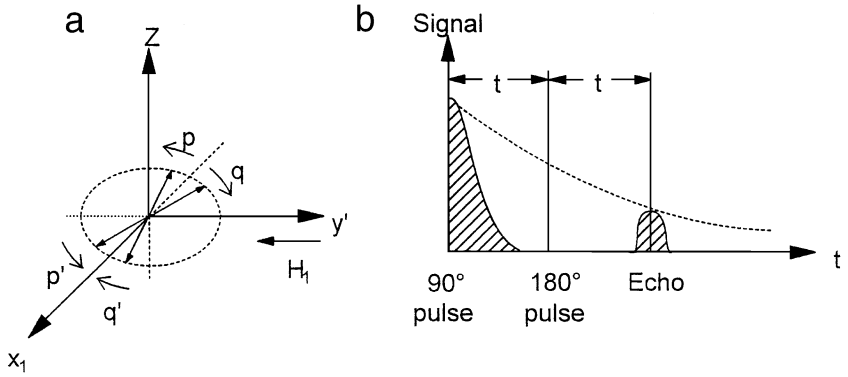


Fig. 29. **a**: The arrival of a spin echo. In the rotating frame (X′,Y′) two dephasing spins p and q are tipped by a 180° pulse from the Y′-direction to the new positions p′ and q′, respectively, from which they rephase. **b**: The signals observed in the 90°-t-180° method of T_2 measurement. The application of the 180° pulse at time t causes the dephased spins to get back in step. Subsequently, they produce an echo at time 2t.

5.1.5. Applications of proton resonance relaxation

A promising application of the proton resonance relaxation (PRR) technique is the determination of the binding parameters for the interaction of flavonoids with heavy metal ions. Such studies are of interest, e.g., to plant physiologists studying the influence of metal ions on the foliage colors and to toxicologists investigating the possibility of removing a harmful excess of heavy metal ions from the human body, using the high affinities of these ions to flavonoids. A necessary condition for the use of PRR for such purposes is that a paramagnetic species is present and that the addition of the complexing agent causes a significant change in T_1. To the author's knowledge, no such experiments have been performed so far, but to those who have access to the equipment, they are likely to offer the advantages of speed and accuracy over alternative methods, e.g., spectrophotometry and fluorometry.

In the analysis of PRR data, the evaluation of relaxation rate enhancements of complexes and their associated binding parameters has been based on graphical extrapolation (Mildvan & Cohen, 1965, 1970; O'Sullivan & Cohn, 1966). Such procedures yielded values that were in satisfactory agreement with those derived by other experimental methods. However, even for binary complexes, extrapolation can give rise to problems (Danchin, 1969; Reed et al., 1970; Bernheim et al., 1959). Therefore, extrapolation has now been replaced by an iterative procedure performed by a computer (Deranlean, 1969). This analysis revealed that satisfactory data are obtained only if the titration is carried to at least 75% saturation. Such an extent of reaction should be obtainable with binary complexes, but its attainment may be problematic, if the interaction is polyvalent.

In the case of binary complexes, e.g., between a flavonoid (F) and a transition metal ion (M), two measurable species are present, a free form and a bound one of the species that is capable of spin relaxation. The measured spin relaxation rate enhancement upon complexation is:

$$\varepsilon^* = \frac{([M]\varepsilon_0 + [FM]\varepsilon_1)}{M_T} \tag{5.4}$$

where [M] is the concentration of the free metal ion, $[M]_T$ the total metal ion concentration, and [FM] the concentration of the complex. Customarily, ε_1 is the enhancement of the complex and ε_0 is set equal to 0 for the free Mn^{2+} ion. Since the dissociation constant of the complex is defined by:

$$K_d = [F][M]/[FM] \tag{5.5}$$

where [F], [M], and [FM] are the equilibrium concentrations of the interacting species, an insertion of Eq. (5.5) into Eq. (5.4) reveals that as [F] is increased, [FM] approaches $[M]_T$ and $\varepsilon^* \rightarrow \varepsilon_1$. Therefore, linear extrapolations of $1/\varepsilon^*$ versus the reciprocal of the concentration of the PRR-silent species have been used to evaluate ε^* and K_d (Fig. 30). This procedure usually yields a satisfactory estimate of ε_1, if a suitable range of the degree of complexation has been reached, but the K_d value is prone to be erratic (Danchin, 1969; Deranlean, 1969). Hence, a nonlinear regression computer program should be applied (Reed et al., 1970). If an analysis of the free metal ion concentration by electron spin resonance is combined with PRR measurements of ε^*, ε_1 may be evaluated directly (Fig. 30) (Mildvan & Cohen, 1965). A check of the possibility of multiple interactions may be made with a Scatchard plot (Reuben & Cohn, 1970). If the sites are nonequivalent, the calculations become lengthy. Assistance may be obtained in the articles of Danchin (1969) and Cohn et al. (1969).

Complexes of higher order are treated similarly (O'Sullivan et al., 1973), but their analysis is more complicated and the errors are usually greater.

5.1.6. Concluding remarks on nuclear magnetic resonance

NMR spectroscopy has been described at some length because of its great importance to the elucidation of the structure of organic compounds in general and to the flavonoids in particular. Further details on the experimental methods and applications, especially to biological macromolecules, are given in Metcalfe (1970), Batterham and Highet (1964), Brünger et al. (1993), Clark-Lewis et al. (1964), Cohn (1970), Ferguson and Phillips (1967), and Jadetzky (1964). Recently, the scope of NMR spectroscopy

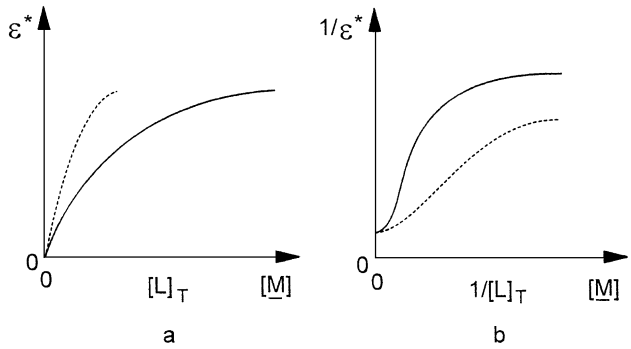


Fig. 30. Test of plots for the evaluation of ε* and K_d using the assumptions: $\varepsilon_1 = 100$, $K_d = 100$ μM (full line), and $K_d = 10$ μM (stippled line). $[L]_T$ is the total concentration of the PRR-silent species. **a**: Direct plot. **b**: Double-reciprocal plot.

has been widened by the development of two-dimensional NMR spectroscopy. Several variants of this method exist, e.g., ENDOR. The particular advantage of two-dimensional NMR is that it permits distance measurements and direct sequencing of heterologous polymers in solution. This possibility renders a direct comparison between structures in crystals analysed by X-ray crystallography and the same molecules in solution possible (Wüthrich, 1976).

5.2. Identification of flavonoids by gas chromatography-mass spectrometry

5.2.1. Scope

Whereas the elucidation of the structure of a flavonoid by NMR requires its previous isolation in high purity, this is not necessary, if equipment that combines gas chromatography with mass spectrometry (GC-MS) is available. Since the analysis of mass spectra from flavonoids in natural products, e.g., propolis, can be difficult, it is advisable to supplement the investigation with analyses using other techniques, e.g., NMR, which yields very specific information on the structural details, and HPLC, which, like GC-MS, relies on the specific, but different, retention time. An example of the analysis of flavonoids in a propolis sample is given by Garcia-Viguera et al. (1993).

5.2.2. Analysis of propolis by gas chromatography-mass spectrometry

A sample of propolis (0.5 mg) is prepared for GC by derivatisation for 30 min at 100°C in 50 μL pyridine and 100 μL bis-(trimethylsilyl) trifluoro-acetamide in a stoppered glass tube. Another sample of propolis (1 mg) is extracted with 70% ethanol to obtain the balsam. This sample is also derivatised as explained above to increase its volatility on the column. The components of the samples are separated and analyzed on an automated GC-MC apparatus (e.g., Finnigan 1020). The detected substances may be identified by a computer search of reference libraries containing GC retention times and mass spectra. The tentative identifications of the compounds are confirmed by co-chromatography of the experimental sample with samples of the pure authentic substances. The latter verify both the retention times and the patterns of the mass spectra. The peaks may be examined by single ion chromatographic reconstruction to confirm their homogeneity. The poorly resolved peaks may be resolved with a computer program that attempts to separate overlapping mass spectra (Greenaway et al., 1987; Lee, M. S. et al., 1993).

5.3. Analysis of propolis by high performance liquid chromatography

5.3.1. Scope

Since GC-MS requires expensive equipment that usually is only available in specialised laboratories and may yield ambiguous results, it is recommended, in addition, to separate and identify the flavonoids by an alternative procedure, HPLC. The instrument required for this purpose is much more widely accessible and often yields a better separation. The disadvantage of the latter method is its lack of structural information that may be discerned from GC-MS under favorable circumstances (Mauri et al., 1999).

5.3.2. The analytical procedure

A sample of propolis (0.5 mg) is extracted with methanol for 10 min in an ultrasonic bath. The extract is filtered for HPLC and injected into the apparatus (e.g., Merck-Hitachi L-6200 intelligent pump furnished with photodiode array detector Merck-Hitachi L-3000 with a Lichrochart 100 RP-18 reversed-phase column, 12.5 × 0.4 cm, particle size 5 μm). The following mobile phase is suitable: Solvent A, water-formic acid (95:5); solvent B, methanol. The substances may be eluted at a flow rate of 1 mL/min using a linear gradient starting with 30% B for 15 min, increasing to levels of 40% B at 20 min, 45% B at 30 min, 60% B at 50 min, 80% B at 52 min, and 80% B at 60 min to re-equilibrate the column. The substances may be detected by their light absorbance at 290 and 340 nm. Reference compounds, which may be commercial, synthetic substances, or isolates from propolis or honey, should be co-

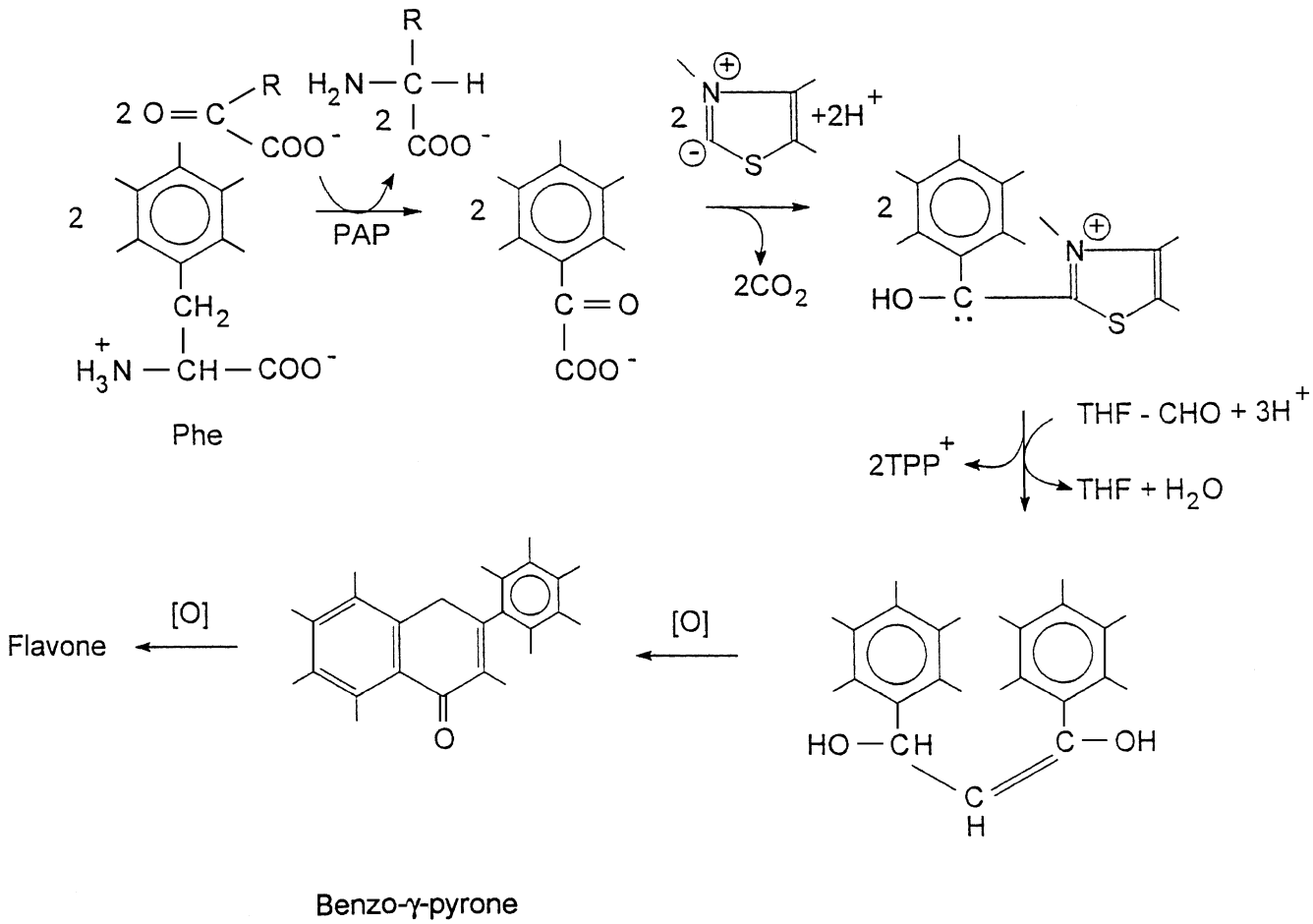


Fig. 31. Biosynthesis of a flavone. PAP, pyridoxal-P; TPP^+, thiamine pyrophosphate.

chromatographed with the experimental sample to confirm the retention times and the UV spectra.

6. The biosynthesis of flavonoids

6.1. Anabolism

All green plant cells are capable of synthesizing flavonoids. The biosynthesis invariably begins with the ubiquitous amino acid phenylalanine. It takes different, but related, courses, depending on the kind of flavonoid that is required (Czihay et al., 1976).

At first, the amino group is removed by transamination or oxidative desamination, which produces phenylpyruvate, whereas the amino group is transferred to a keto acid of the citric acid cycle or liberated as an ammonium ion. Two molecules of phenylpyruvic acid may then be oxidatively decarboxylated by thiamine pyrophosphate in the pyruvate dehydrogenase complex producing two molecules of active aldehyde, which together with a C_1-fragment (-CHO) in an oxidative step form the phenyl-γ-chromone nucleus (Fig. 31).

The flavone nucleus is subsequently multiply hydroxylated by a number of specific oxygenases to produce individual flavonoids. The flavone ring may be formed

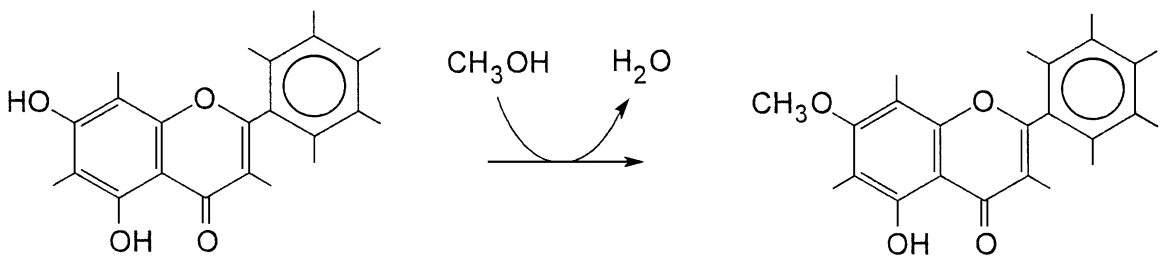


Fig. 32. Methylation of a flavone catalyzed by a methylase.

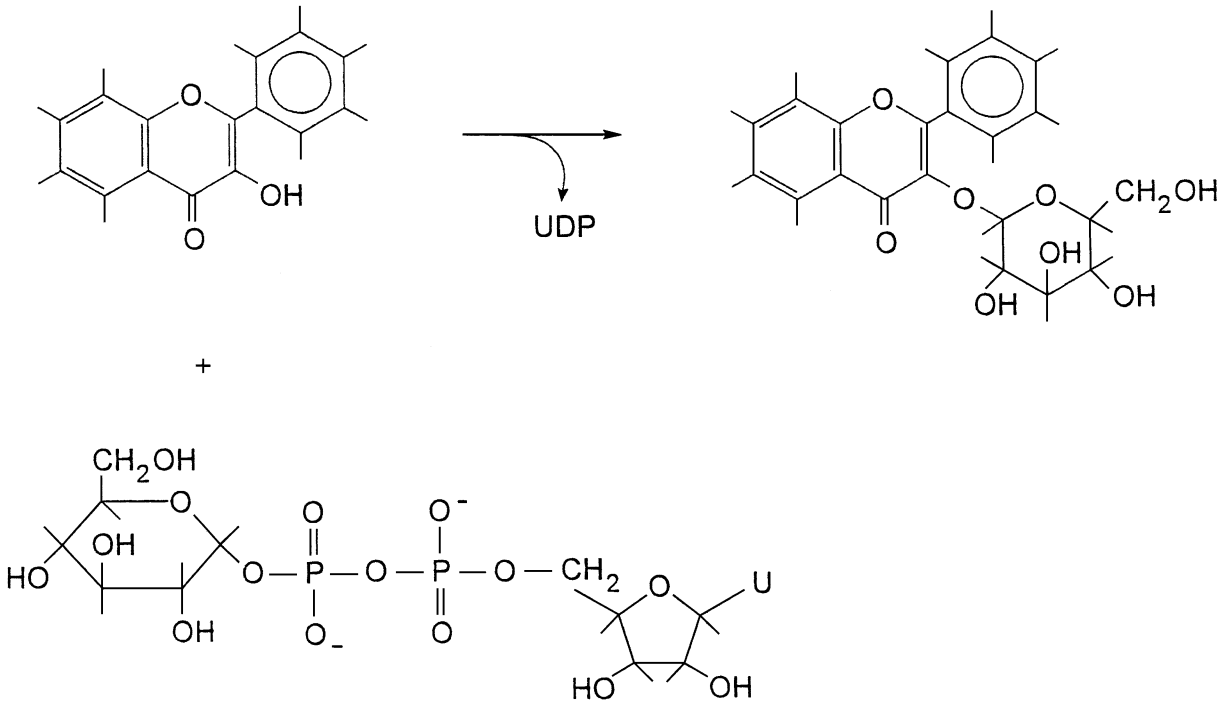


Fig. 33. Glycosylation of a flavonol by UDP-glucose catalyzed by uridyl transferase.

from the γ-chromone nucleus by reduction with tetrahydrofolate (THF). The iso-flavone series and, hence, also the isoflavanes may be formed in an analogous process, in which the stereo-specificity of the condensation step, most likely due to the assistance of an enzyme, is different (Gardiner et al., 1980; Lahann & Purucker, 1975; Link et al., 1943; Funa et al., 1999).

The methylation of the hydroxyl groups most likely occurs with methanol catalyzed by specific methylases, since similar reactions are known from animal cells (Fig. 32) (Gauthier et al., 1998). The carbohydrates to be attached to the hydroxyl groups, which preferentially occurs in the C_3-position, arrive as monosaccharides activated by UDP at the anomeric C-atom (C_1), and are consecutively linked to

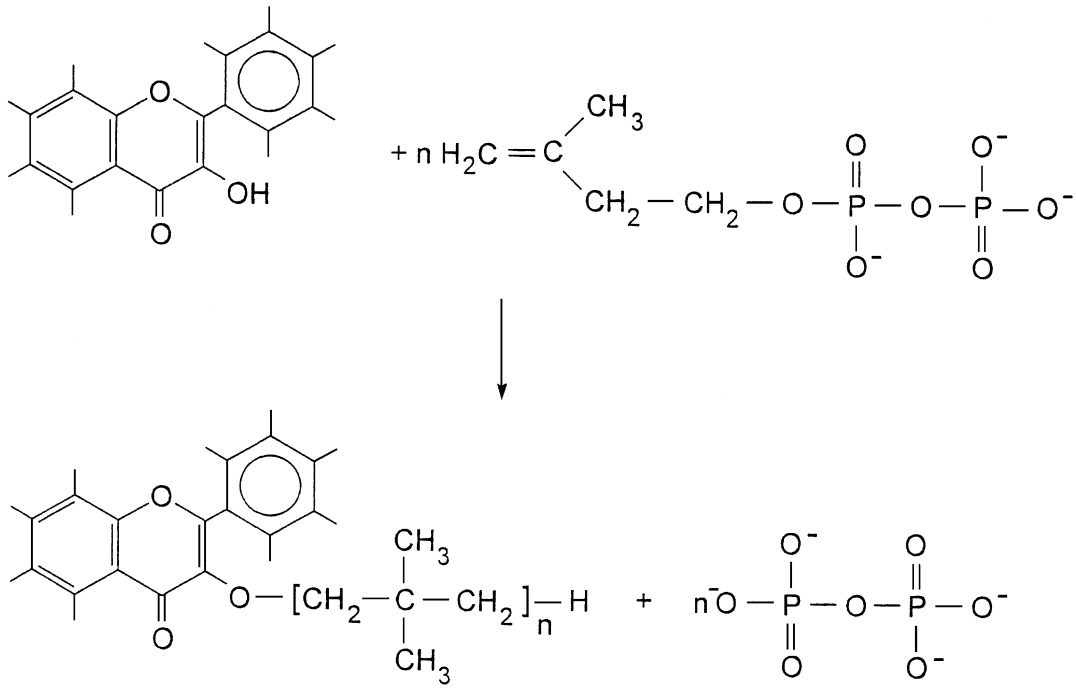


Fig. 34. Isoprenylation of flavonol by isopentenyl pyrophosphate (the biological isoprene unit). Usually, $n = 5-10$.

the aglycone (Fig. 33) (Matern et al., 1981; Heller & Forkmann, 1988).

The isoprenoid conjugates of the flavonoids are formed by the action of the biological isoprene unit, isopentenyl pyrophosphate. The attachment most often, but not invariably, occurs at position C_3 of the γ-chromone (Fig. 34).

6.2. The genetics of flavonoids

The genetics of the flavonoids was an early subject of much scientific interest for the reason already mentioned in Section 6.2, the application of the flavonoids to the taxonomical classification of plants. The reason is that the genes for the enzymes that mediate the biosynthesis of the flavonoids are easily expressed, and that the products are vivid, easily recognisable, colored pigments, and diversified enough to be readily distinguishable. Furthermore, the flavonoids also offer taxonomical criteria that are based upon a more direct expression of the genetic structure than the classical morphological structures, e.g., the shape of the leaves, the curvature of the leaf rim, the number of seeds in a fruit, etc. Since the genes contain all of the information

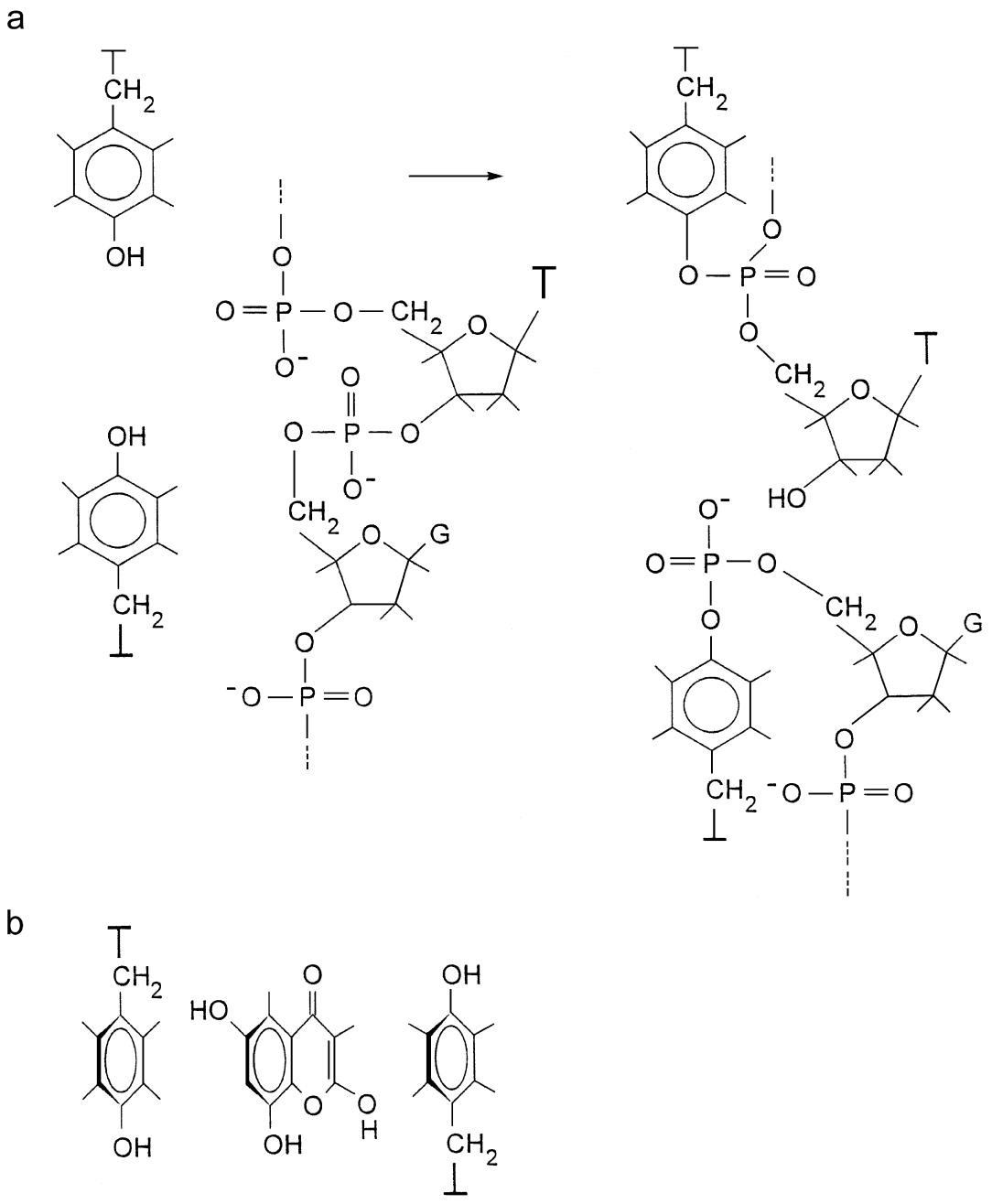


Fig. 35. **a**: Two phenolic side chains of tyrosine in the active site of topoisomerase II normally form ester bonds with the phosphates at the chain end of DNA. T, thymine. **b**: Apposition of the two tyrosine side chains with the flavonoid (quercetin).

about an individual specimen, the genetic approach to taxonomy appears to be the most fundamental basis available. Moreover, flavonoids are not only produced in response to many external stimuli, but also by metabolic feedback reactions capable of inducing other genetic activities. The result may be gene activation, mutation, or repair of DNA (Zahringer et al., 1978; Cea et al., 1983; Peters et al., 1986; Disseling, 1996; Fekermann et al., 1998; Ching & Baguley, 1987; Chin, 1999).

The biochemical basis of the activity of the flavonoids on genes recently has been studied from a nutritional view point (Singleton, 1981). Normal human nutrition has always contained much vegetable material. It has been estimated that an average healthy individual consumes 1–2 g of flavonoids daily. Considering the chemical reactivity of the flavonoids, this is an amount that gives rise to some concern. Evidently, humans have fared rather well on such a diet since their appearance on earth, but even a slight toxicity, at such an extensive exposure, might give rise to some disease or malfunction (Ritov et al., 1995). It was recognised that some individuals actually are sensitive to flavonoids because antibodies to these compounds were found in human blood. It was also discovered that about 3–5% of the population is allergic to flavonoids. Such a fraction is hardly alarming, considering the great number of commonly occurring allergens. Red wine, which has a high content of flavonoids, has been recommended by prominent nutritionists for its favorable influence on cardiovascular health. This effect was ascribed to the antioxidative effect of the flavonoids. Pathologists support this observation by noting that the vascular walls of alcoholics at dissection are found to be in remarkably good condition, i.e., smooth and free of atherosclerosis (Donovan et al., 1999; da Luz et al., 1999; Lairon & Amiot, 1999; Cishek et al., 1997; Miyagi et al., 1997). The proven mutagenic activity of some flavonoids, a prominent example is quercetin, gave reason for concern because some mutations ultimately lead to cancer. However, the mutagenic activity of the flavonoids in the Ames test, in which a histidine-requiring mutant supplemented with mammalian mitochondria is mutated back to the wild-type, was not higher than that of similar compounds, which also are indigenous to our nutrition (Ames et al., 1975; MacGregor & Jurd, 1978; Maruta et al., 1979; Cea et al., 1983; Sahu et al., 1981). Besides, no connection between flavonoids and human cancer has been found. Some flavonoids possess an antimutagenic effect (Choi et al., 1994; Agullo et al., 1996, 1997; Anderson et al., 1997, 1998).

However, one biochemical mechanism of the action of flavonoids on DNA was found, since quercetin inhibits topoisomerases II (Duthie & Dobson, 1999) and IV (Bernard et al., 1997). This enzyme cleaves the DNA chain in the superhelix form to create additional turns or to unroll some such superhelical turns. Then, it normally joins DNA ends again, but this final step is competitively inhibited by quercetin. The result is a single-strand breaks, which may suffice to cause double-strand breaks that result in the loss of genetic information. A further consequence of single-strand breaks is the initiation of a repair mechanism. If successful, the latter delivers an intact gene, but on a rare occasion, it is faulty, and in that case, a mutation may occur. The two DNA ends are transiently linked to two tyrosine side chains positioned opposite to each other in the active site of the topoisomerase. Quercetin structurally resembles the phenol ring of tyrosine. Hence, this flavonoid may intercalate between the two phenol rings, e.g., forming a charge transfer complex or by hydrophobic interactions. Thus, it may sterically hinder the points to which the DNA chain ends must attach to become rejoined (Fig. 35) (Howard et al., 1994; Leteurtre et al., 1994; Freudenreich & Kreuzer, 1994).

Flavonoid-sensitive genes are associated with modulation, longitudinal growth, stress response, and petal coloration. Only few details about the mechanisms are known, but some information will be discussed in Section 19. The stress response mediated by the flavonoids may well involve the inhibition of DNA topoisomerase II described in Section 7.

7. The role of the flavonoids in plant physiology

The flavonoids are essential constituents of the cells of all higher plants. They resemble in their regulatory properties most of the lipid-soluble vitamins, but serve in addition, due to their color and odor, as communicators with the environment (Middleton & Teramura, 1993; Harborne et al., 1976; Brouillard & Cheminat, 1988; Harborne, 1986, 1988a, 1988b). They are recognised by pollinators, e.g., insects, birds, and animals, which contribute to the dispersion of seeds. The growth regulation of plants by flavonoids has attracted considerable scientific interest because it is important to plant breeding, agricultural economics, and world health (Moyano et al., 1996). Besides, the mechanism is sufficiently similar to growth-regulating processes in animals to invite the suspicion that flavonoids might also influence the growth metabolism of animal cells, including those of humans (Groteweld et al., 1994; Jiang et al., 1999; Ceriani et al., 1999; Ghosal & Jaiswal, 1980; Fragner, 1964; Albrecht et al., 1999). Investigations of such aspects are impeded by the difficulty of composing a long-term diet that is sufficient for sustenance, but absolutely free of flavonoids.

The effect of flavonoids on plant growth, which is known, is at least partly indirect and associated with the action of the auxins. The prominent representative of this group, indolyl acetic acid (IAA), is formed from tryptophan by pyridoxal phosphate (Pyr-P)-mediated transamination or desamination followed by decarboxylation (Fig. 36).

This process occurs in the cytoplasm, but the growth hormone may leave the cell to disperse in the plant via the vascular system. Two routes of exit from the cell of origin are conceivable: either directly through a cell membrane channel, which is permeable to aromatic compounds, or,

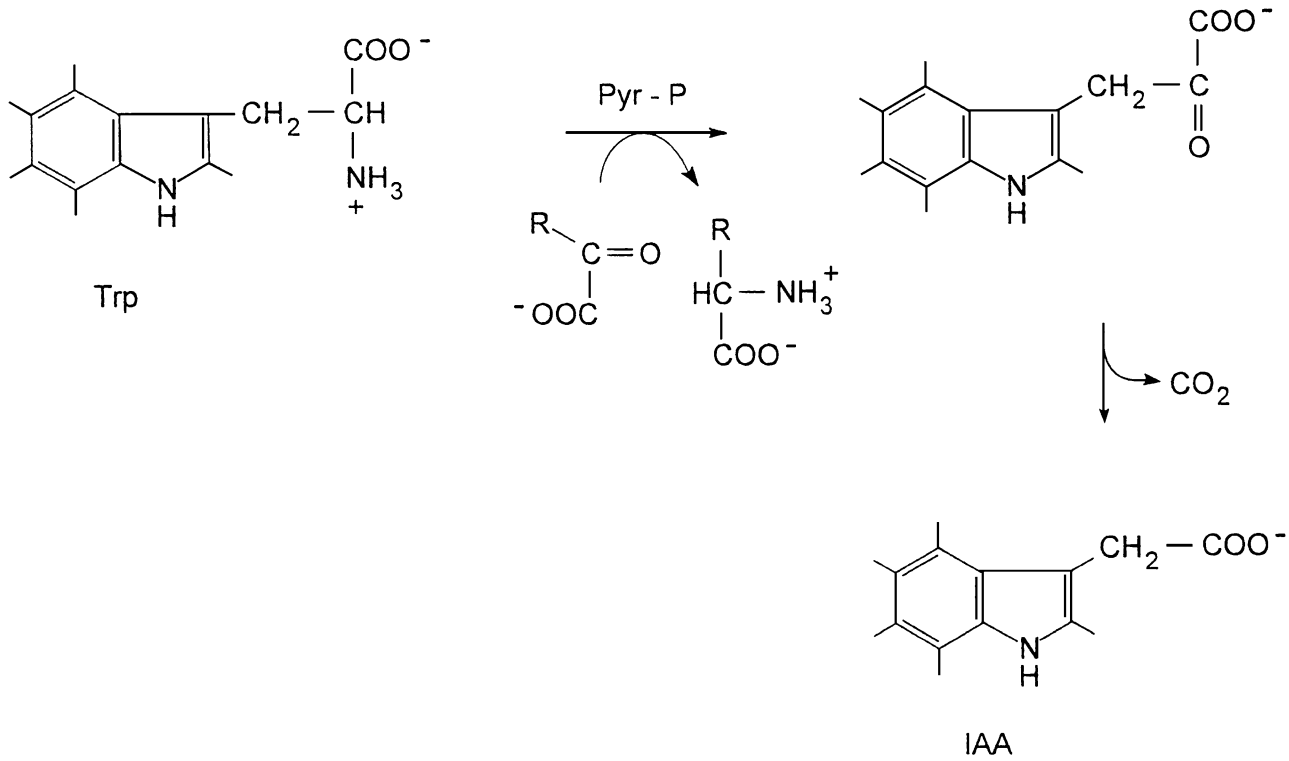


Fig. 36. The metabolic conversion of tryptophan to IAA.

more likely, by inclusion in vesicles formed by the trans-compartment of the Golgi apparatus or by the endoplasmic reticulum, followed by exocytosis.

Other cells can take up the IAA by a receptor-mediated endocytosis or through a cell membrane channel specific for aromatic compounds. It is known that the human blood-brain barrier contains such a transporter for an IAA-like hormone serotonin (5-hydroxytryptamine). It requires ATP for the active cotransport of serotonin and glucose into the brain (Crone, 1965, 1986; Shoshan et al., 1980; Kimmich & Randles, 1978). Granula containing serotonin and histamine in mast cells are exocytosed into the blood by a mechanism that is Ca^{2+}-dependent (Wilson et al., 1991; Fewtrell & Gomperts, 1977a, 1977b; Van Canegham, 1972), but inhibited by flavonoids because it is cyclic AMP (cAMP)-dependent (kinase action), and flavonoids inhibit the hydrolysis of cAMP by phosphodiesterase (PDE) (Pene et al., 1988; Bradley & Cazort, 1970; Conti & Setnikar, 1975; Herbst, 1970; Setnikar et al., 1960; Saponara & Bosisio, 1998).

Since a major target of the flavonoids is the synthesis of eicosanoids, especially PGs, which they prevent by steric hindrance of the binding of the substrate arachidonic acid, these signal substances probably are also involved in transport processes across the cell membrane. By analogy to the inhibition by flavonoids of the exit of IAA molecules from a plant cell, the implication is that eicosanoids are also required for that transport process (Jacobs & Rubery, 1988; Schubert et al., 1999). It is yet unknown whether PGs or related compounds open specific plant cell membrane channels or whether they participate in the exocytosis of granula. However, specific cell membrane receptors for PGs are known in animal cells (Kurachi et al., 1989; van Canegham, 1972), and eicosanoids can influence some of the protein phosphokinase signal chains. The latter effect is likely to be genetic, since some eicosanoids can activate the expression of enzyme genes (Medina et al., 1994; Fine et al., 1992; Lorenzo et al., 1996).

The eicosanoids themselves are formed after the induction of COX by the hormones and cytokines epidermal growth factor, basic fibroblast growth factor, platelet-derived growth factor, interleukin (IL)-1β, tumor growth factor-β, and tumor necrosis factor (TNF)-α (Vane, 1971; Vane et al., 1994). In this perspective, the flavonoids are most properly classified as auxillary auxins in plant physiology (Jacobs & Rubery, 1988; Stenlid, 1976). They increase the concentration of IAA by the prevention of leakage of this substance from the cell. Thus, the gene expression resulting in longitudinal growth of the plant cell, which is induced by IAA, is enhanced. Factors that contribute to the increase in the concentration of free flavonoids after infection include increased flux from phenylalanine, inhibition of glycosylation, and glycosidase action (Partridge & Keen, 1976) (Fig. 37).

Another effect of flavonoids on plant physiology that is known is the inhibition by quercetin of energy transfer during photophosphorylation (Mukohata et al., 1978; Cantley & Hammes, 1976). However, the mechanism of this inhibition remains to be elucidated. In this connection, it is

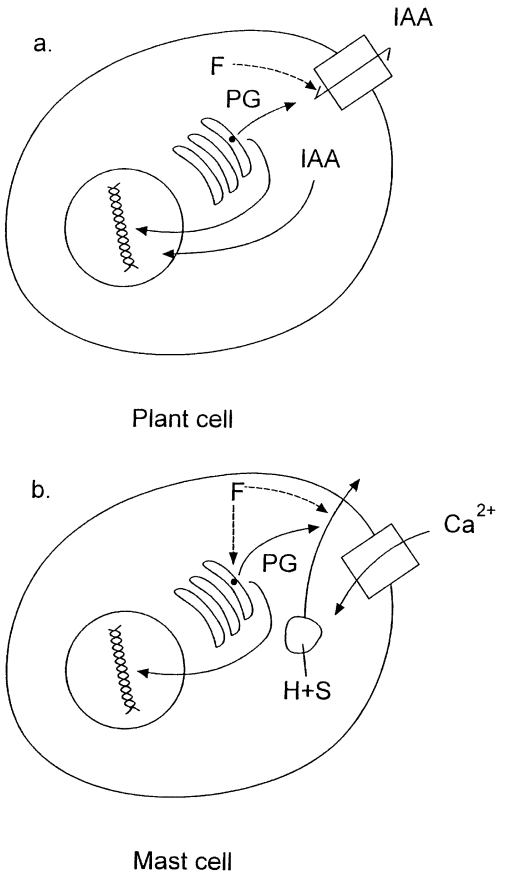


Fig. 37. **a**: Model of the growth-promoting action of IAA in the plant cell and of the indirect effect of flavonoids (F) on this process. F prevent the exit of IAA by inhibition of the key enzyme in the biosynthesis of PGs. The latter mediate the transport of IAA across the cell membrane. **b**: The mast cell produces serotonin (S) and histamine (H) that are stored in granula, the exocytosis of which is indirectly inhibited by F (Picot et al., 1994). The latter inhibit the biosynthesis of PGs by blocking the binding site for the substrate arachidonic acid on the key enzyme PG COX, also called PG H_2-synthase or PG-endoperoxide-synthase (Kulmacz et al., 1994). PGs facilitate the exocytosis of the granula containing S and H. Exocytosis is accompanied by the uptake of Ca^{2+} ions.

interesting that Cantley has found binding sites for quercetin on the chloroplast coupling factor I. Since ubiquinone and flavonoids have structural features in common, a competitive inhibition or a sequestration of intermediate free radicals may take place.

Flavonoids play an important role in the nitrogen metabolism of nitrogen-fixating plants, because they induce the nodulation of their roots. These nodules contain dinitrogen-fixating bacteria, e.g., of the strain Rhizobium, which live in symbiosis with leguminous plants. The plant prevents the inhibition of the conversion of dinitrogen to ammonia by keeping the dioxygen level low, and the bacteria express the three proteins that are required for nitrogen fixation: nitrogenase reductase, nitrogenase, and the coenzyme FeMo-co. The latter is extremely sensitive to inhibition by oxygen (Mortenson & Thorneley, 1979). The target of this inhibition is assumed to be an Fe-Mo-S cluster that participates in the transfer of six electrons from a very strong reductant, e.g., ferredoxin. This process requires the hydrolysis of 12 molecules of ATP. In contrast, dinitrogen reduction is a much less energy-requiring process. The main effect of the flavonoids is probably the induction of genes expressing proteins required by the nodule cells, but they are also very suited for participation in the elimination of dioxygen, since they are antioxidative.

7.1. Flavonoids as signals of symbiosis

Cooperation requires communication and organisation. In the case of symbiosis between potentially nitrogen-fixating bacteria and leguminous plants, flavonoids play several roles as signal substances. Apparently, leguminous plants in need for biologically useful nitrogenous substances, e.g., amino acids or ammonia, release exudates containing several flavonoids from the roots into the surrounding soil, where they enter bacterial cells containing nodulation-inducing genes, as well as genes for nitrogen fixation. Srivastava et al. (1999) detected six flavonoids by HPLC, e.g., naringenin, daifzein, hesperitin, naringin, 7-hydroxy-coumarin, and luteolin, in such an exudate. The individual flavonoids were capable of inducing the expression of the bacterial nodABC genes, but a combination of naringenin and daidzein yielded the strongest biological effect. After the initial contact between the flavonoids from the nitrogen-starving plant and the bacterial cell, the latter is guided towards the leguminous plant by chemotaxis. Experiments with mutants showed that this phenomenon is different from the chemotaxis arising from nutritive substances, e.g., sugar or amino acids (Pandya et al., 1999). When the bacterial cells arrive at the plant root, they release lipochitooligosaccharides, which function as nodulation factors (Prome, 1999). The result is root hair deformation, cortical cell division, and admittance of the bacterial cells to the space between cortical and endodermal cell layers, so-called lateral root cracks (Gough et al., 1997). A transcriptional analysis of the effect of nod gene-inducing flavonoids showed that 19 nod boxes controlled nodulation, whereas 16 conserved NifA-sigma54 regulatory sequences coordinate the expression of the nitrogen-fixation genes (Perret et al., 1999). The driving force in the evolution of the symbiotic relationship between nitrogen-fixating bacteria and leguminous plants appears to be the ability of the strain to transfer the nodulation ability laterally in the form of a plasmid carrying the essential genes (Broughton & Perret, 1999).

8. The pharmacology of flavonoids in animals

So far, the science of pharmacology has concentrated its efforts mainly on potent plant toxins that accidentally may be ingested, if not given or taken with the intention to kill, and on drugs that are being considered for a medical application. In contrast, natural products, which are regu-

larly ingested in high amounts as components of a normal human diet, but which are only slightly toxic, have almost been ignored (Hughes & Wilson, 1977; Gábor, 1981). The flavonoids belong to the latter category. The justification of this policy is the high cost of a full-scale pharmacological investigation and the moral obligation to develop a defence against accidental or criminal, potentially fatal acute intoxication. However, since the long-term effects of the ingestion of compounds of low acute toxicity may impair health, e.g., by accumulation in major organs, especially the liver, or by initiation of immune disorders, an awareness of the need for attention to this class of substances is rising. The flavonoids recently have been included in such investigations (Chipault et al., 1952; Di Carlo et al., 1999; Wada et al., 1999). Another concern is that dietary components such as flavonoids can influence the metabolism of drugs (Conney et al., 1981). Important reasons for the low toxicity of the flavonoids are the low solubility of the aglycone in water and the rapid catabolism of the pyrone nucleus in the liver. The low solubility of the flavonoids in water often presents a problem for medical applications of these substances. Hence, the development of semi-synthetic, water-soluble flavonoids, e.g., for the treatment of hypertension and microbleeding, has been an important advance. Examples of such flavonoids are the hydroxyethylrutosides (see Section 17.1) and inositol-2-phosphate-quercetin (Calias et al., 1996).

8.1. Pharmacodynamics

Nutritional flavonoids are absorbed from the gastrointestinal tract (Crespy et al., 1999; Pforte et al., 1999), whereas medical flavonoids are administered directly to the diseased tissue, if it is accessible, e.g., in the skin or the throat, or along a route leading immediately to the target, e.g., the nasal or the vascular systems (Metzner et al., 1979; Heilmann & Merfort, 1998; Masquelier et al., 1979; Spilkova & Hubik, 1986, 1988; Spilkova & Dusek, 1996; Vinson, 1998; Zloch & Sidlova, 1977; Zloch, 1977; Hollman & Katan, 1997, 1998; Hollman et al., 1996; Hollman, 1997; Rice-Evans & Miller, 1996; Maxwell & Thorpe, 1996; Di Carlo et al., 1999; Balentine et al., 1997; Gabor, 1988; Graham et al., 1978; Gugler et al., 1975; Booth et al., 1956; Cheng et al., 1969; de Eds, 1968; Griffiths & Barrow, 1972; Griffiths & Smith, 1972a, 1972b; Griffiths, 1975; Herrmann, 1976; Honohan et al., 1976; Murray et al., 1954; Petrakis et al., 1959; Piller, 1977; Simpson et al., 1969; Struckmann, 1999). After liberation of the glycosides from the aglycone by bacterial enzymes in the intestine, about 15% of the flavonoid aglycones are absorbed with bile micelles into the epithelial cells and passed on to the lymph (Day et al., 1998; Spencer et al., 1999). An important factor determining the efficiency of the absorption of flavonoid glycosides from the intestine is the sugar moiety. Hollman and colleagues (Hollman & Katan, 1999; Hollman et al., 1999a) showed that quercetin glycosides from onions were absorbed better (52%) than the pure aglycone (24%).

Some flavonoids inhibit the non-Na^+-dependent facilitated diffusion of monosaccharides in intestinal epithelial cells (Kimmich & Randles, 1978). Consequently, the parallel concentrative Na^+-dependent transport ATPase for monosaccharides gains efficiency (Sharma et al., 1981). The remainder of the flavonoids are excreted with the faeces and some in the urine (Choudhury et al., 1999). The lymph carrying the flavonoids enters the portal blood near the liver, and the majority ($\sim$80%) probably is absorbed in the first pass (Casley-Smith & Casley-Smith, 1986). Part of the flavonoids probably are attached to serum albumin (Podhajcer et al., 1980). Another part is found in conjugates that have retained their antioxidative properties (Manach et al., 1998). The hepatocytes transfer the flavonoids to the Golgi apparatus and possibly also to the peroxisomes, in which they are oxidatively degraded (Griffiths & Smith, 1972a, 1972b). Some oxidative degradation of flavonoid aglycones also takes place in the intestine because some bacterial enzymes can open the C-ring of the flavonoid skeleton (Winter et al., 1989).

The products are secreted by organic acid transporters into the blood and subsequently excreted through the kidneys (Graefe et al., 1999; Bourne & Rice-Evans, 1999). The half-life of a typical flavonoid in the body has been measured to be 1–2 hr, but data of sufficient accuracy for a compartmental analysis have not been published. Since very little information on the rates of transportation of flavonoids and their decomposition products across membranes are known, the proposition of a complete, realistic dynamic model is premature (Honcha et al., 1995; Ueno et al., 1983; Griffiths & Barrow, 1972; Tesi & Forssmann, 1971; Lenne-Gouverneur et al., 1999). However, the last step, the active secretion of organic acids in the kidney into the urine, has been studied in the isolated rat kidney (Möller, 1969). That excretion curve can be simulated with two exponential terms, i.e., the process can be explained by a two-compartment model (Fig. 38). The assignment of these compartments to the morphological structures has not been possible yet.

Since flavonoids are not accumulated in the liver and their decomposition products (caffeic and cinnamic acids, as

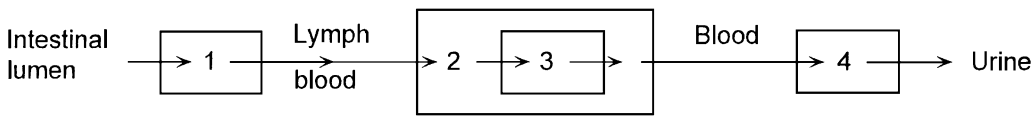


Fig. 38. Minimal model of the pharmacodynamics of flavonoids in higher animals. Passage of flavonoids from 1, intestinal epithelial cell; over blood and lymphs to 2, hepatocyte; 3, Golgi apparatus or peroxisome; again over blood to 4, renal tubulus cell.

well as their derivatives) are completely excreted with the urine at a rate similar to that of caffeine, the study of further details of the process has not been deemed urgent.

8.2. Acute toxicity of flavonoids

By injection of large amounts of flavonoid aglycones in solution into the blood of rats, it has been possible to determine the LD_{50} value for this animal. It is ~ 2 g/kg of body weight (Casley-Smith & Casley-Smith, 1986). Due to the low solubility of flavonoid aglycones in water, to the short residence time of flavonoids in the intestine, and to the low coefficient of absorption, it is not possible for humans to suffer acute toxic effects from the consumption of flavonoids, with the exception of a rare occurrence of allergy (Petersen, 1977; Petersen & Afzelius, 1977; Wozniak & Braun, 1972; Hausen & Wollenweber, 1988; Hausen et al., 1987a, 1987b). Flavonoid concentrations in the blood after the infusion of soluble flavonoids, e.g., pure hydroxyethylrutosides, directly into the blood for the purpose of the control of blood pressure or the fortification of leaky blood vessels have maximally reached levels 2–3 orders of magnitude below the only recorded LD_{50} value (for the rat). The margin of safety for the therapeutic use of flavonoids in humans, therefore, is very large and probably not surpassed by any other drug in current use (Grotz & Günther, 1971). However, here a note of caution is necessary against the use of unpurified flavonoid extracts from plant materials for intravenous injections. Such an application would be irresponsible, since accompanying substances might give rise to an anaphylactic shock or other acute immunological crisis. Such incidences have occurred already. Hence, only single, pure flavonoids should be injected into the blood circulation.

Besides, the effects of several flavonoids may not be additive. Moreover, highly toxic flavonoids have been found in tropical areas, e.g., Africa. They colored the local propolis variants strongly black. Hence, very dark propolis types should be avoided, unless they have been tested. Such samples are very rarely found outside of the place of their origin.

8.3. Long-term effects of flavonoids

Flavonoids have been consumed by humans since the arrival of human life on earth; i.e., for about 4 million years. The daily consumption of flavonoids by humans has probably remained almost constant over this period, since the nature of the vegetable components of the diet, according to the archaeologists and to the anthropologists, appears to have remained almost the same. Consequently, the heavy exposure to flavonoids during the entire life of a human cannot have grave consequences to health. However, since flavonoids in plants are known to induce gene expression and flavonoids in cultures of human cells have given rise to mutations, the daily exposure to dietary flavonoids might cause some concern, although their half-life is only on the order of a few hours. For the reasons mentioned, the risks incurred by the regular consumption of flavonoids is slight and undoubtedly much less than those that we willingly accept, e.g., by smoking or driving. Besides, a considerable, almost constant exposure to flavonoids is unavoidable because they cannot be removed from their main source, the vegetables, beverages, and fruits, which we absolutely need to maintain our health. Hence, we have to live with the flavonoids, and there is no substantial reason to expect that they present any significant toxicological risk, except under extreme circumstances, e.g., by intravenous injection of large amounts. When that is done in the course of a toxicological experiment to assess the safe limits, one observes in rats after 3 weeks of a constant, extremely high flavonoid concentration, morphological changes in the membrane structure of hepatocytes, which lead to cell necrosis and eventually to the death of the animal. Hence, although the flavonoids that are normally absorbed by the human body are probably the safest drugs ever known, any substance, even oxygen and water, without which life cannot be sustained, in high concentrations or after special activation can become toxic (Nagao et al., 1981; Ambrose et al., 1951, 1952). It should be noted that a significant mutagenic effect has been detected by some flavonoids (Ames et al., 1975; Cea et al., 1983; Sahu et al., 1981; Seino et al., 1979; Beretz et al., 1978; Brown et al., 1977; Grigg, 1978; Hardigree & Epler, 1978; Sugimura et al., 1977), as well as by many other components of normal food products.

8.4. The catabolism of flavonoids

Flavonoids, like other aromatic compounds, at first are prepared for ring opening by hydroxylation at suitable vacant positions. Such potential hydroxylation sites are C_3, C_5, C_8, C_1', and C_5' in the flavones and C_2, C_5–C_8, and C_1'–C_5' in the iso-flavone subclass. Like many therapeutic drugs, the flavonoids induce the expression of the genes of isoenzymes of oxygenases specific for the particular flavonoid aglycone. The reaction that is mediated by cytochrome P_{450} (Nesnow & Bergman, 1981; Seidel & Endell, 1978; Brown et al., 1976; Dock et al., 1978; Meehan & Straub, 1979; Boulton et al., 1999; Dai et al., 1997), THF, NADPH, flavin adenine dinucleotide, Cu^{2+}, and Fe^{2+} introduces one of the atoms of dioxygen into the flavonoid, whereas the other oxygen atom forms water (Fig. 39).

Side reactions of activated oxygen can produce superoxide and H_2O_2, which both cause pronounced toxic effects, in this case, primarily to the liver. The cistron encoding the oxygenases also contains the genes of several other enzymes that participate in the catabolism of the flavonoid (Canivenc-Lavier et al., 1996). These other genes are coexpressed with that of the oxygenase, thus producing a dehydratase and an epoxide hydratase. The dehydratase, from vicinal hydroxyl groups in the aromatic ring, can form

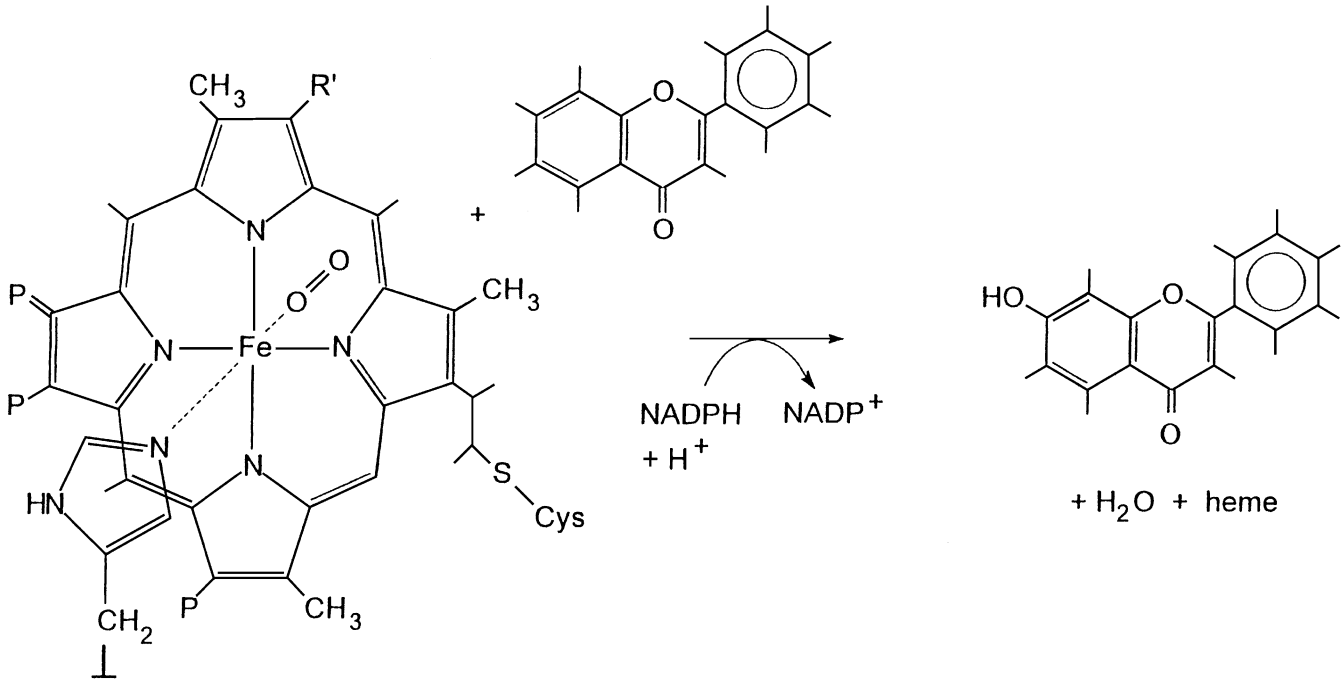


Fig. 39. Activation of dioxygen by cytochrome P450 in a specific oxygenase isoenzyme in preparation for the hydroxylation of the flavonoid and liberation of water.

an epoxide. Such compounds can be toxic because they can add amino groups, especially those on guanine, or sulfhydryl groups in the active sites of numerous important enzymes (Fig. 40).

These enzymes, which accompany the oxygenases, are a mixed blessing because they create epoxides that can cause a mutation and subsequently lead to cancer. However, one of these enzymes is an epoxide hydrolase that eliminates

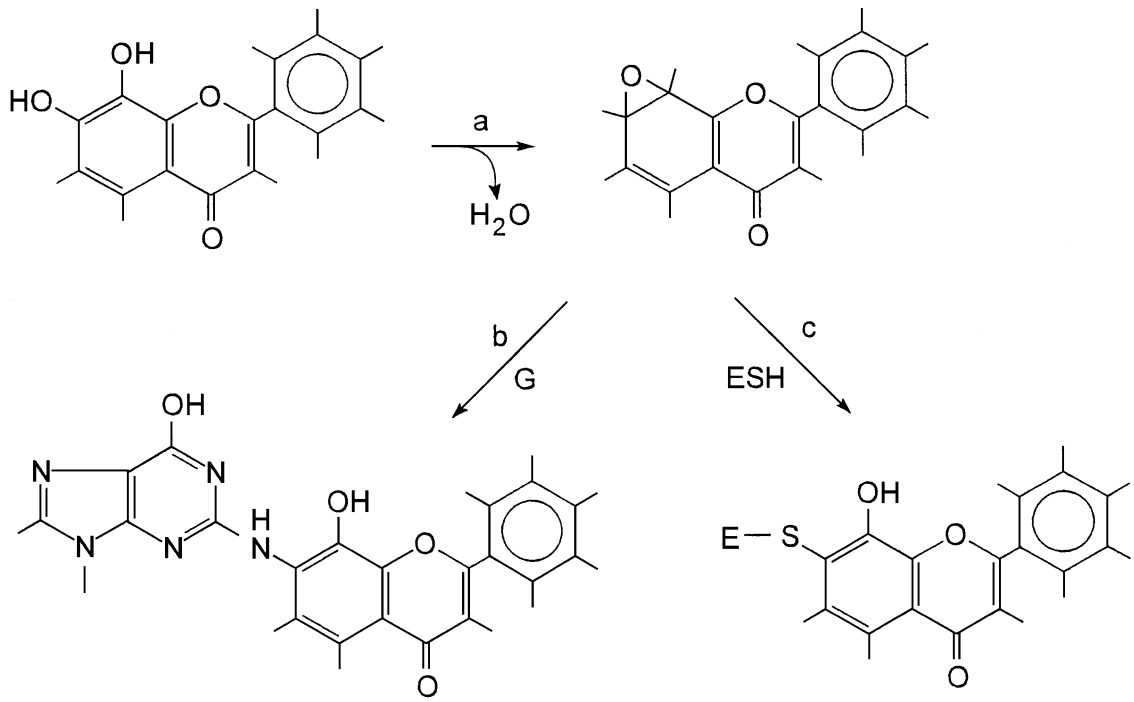


Fig. 40. **a**: Formation of an epoxide from a flavonoid by removal of a molecule of water. **b**: Addition of an amino group of guanine in DNA to the flavonoid epoxide, thus creating a mutation. **c**: Addition of a sulfhydryl group in the active site of an enzyme (ESH) to the exposite. This reaction inhibits the enzyme irreversibly.

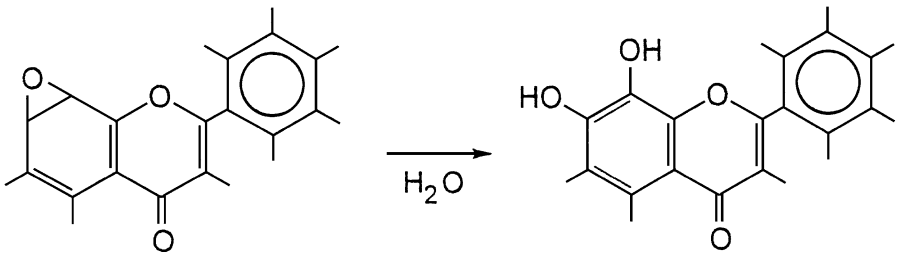


Fig. 41. Action of an epoxide hydrolase.

epoxides by addition of a water molecule. Thus, a number of reactions compete. Whether the outcome is beneficial or not to the individual depends on the genetic structure, the quality of the control and repair systems, and environmental factors, as well as on other yet unknown and uncontrollable factors (Olifson et al., 1978; Huang et al., 1981, 1982, 1983; Hammons et al., 1999). In the case of aflatoxins, which can promote cancer by forming epoxides that can add amino groups from nucleotide bases, notably on guanine, thus creating mutations, flavonoids can act as protective agents (Schwartz & Rate, 1979; Souza et al., 1999; Lake & Parke, 1972) (Fig. 41).

The prerequisite for the opening of the aromatic ring structure is the existence of vicinal hydroxyl groups. They provide a site for an oxidative attack that opens the ring. Unfortunately, the necessary preparatory steps are fraught with the danger of mutation and carcinogenesis, but the alternative is an intolerable accumulation of aromatic compounds in the liver (Fig. 42). Hence, this organ would be destroyed, which would be fatal, and we have no other choice than to accept the minor risk, the possibility of a mutation, since the latter is curable.

Whereas the A-ring must be opened by the mechanism described above, the B-ring is easily and reversibly opened by a simple oxidation/reduction reaction.

9. The immunology of the flavonoids

Small organic compounds such as the flavonoid aglycones are only antigenic if they are bound to macromolecules in the blood, i.e., to plasma proteins. Although immune reactions rarely are problems by the consumption or therapeutic application of flavonoids, they do occur

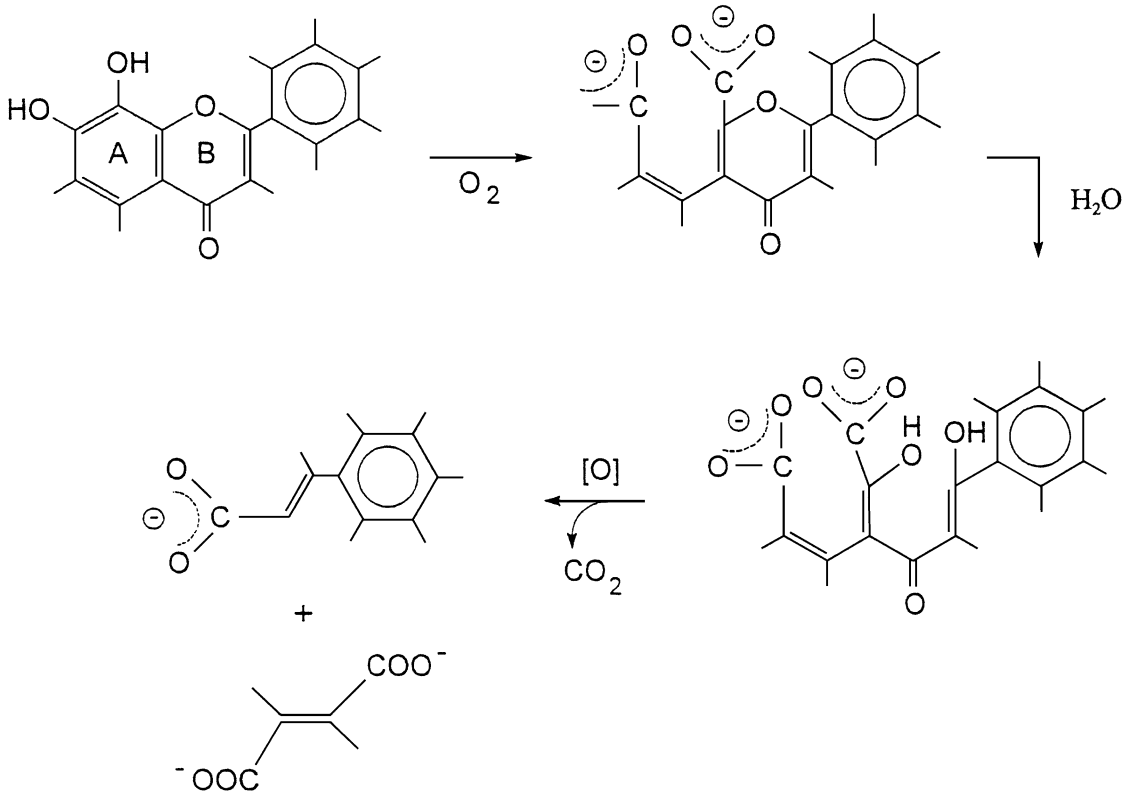


Fig. 42. Oxidative ring opening of a flavonoid producing coumaric, cinnamic, fumaric, and caffeic acids, as well as their derivatives. All of these end products are rapidly excreted through the kidney; $t_{1/2}$, ~1 hr.

occasionally. At least some flavonoids, therefore, are capable of binding to one or more of the plasma proteins, probably primarily to serum albumin and lipoproteins, since flavonoids are hydrophobic and are transported in bile acid complexes, as well as in chylomicrons.

9.1. The flavonoids as antigens

Flavonoids are only weakly antigenic, but antibodies against flavonoids have been found in human blood. As already mentioned in Section 9, allergic reactions occur in about 3–5% of the population after the intake of considerable amounts of flavonoid-rich products (Hausen et al., 1987a, 1987b, 1992; Hausen & Wollenweber, 1988; Hegui et al., 1990; Schuler & Frosch, 1988; Ginanneschi et al., 1989; Janes & Bumba, 1974; Hashimoto et al., 1988). However, almost any substance to which we are exposed can give rise to allergy in sensitive persons.

9.2. Flavonoids as immune modulators

Several reports have been published on the specific activation of cytotoxic and natural killer T-lymphocytes (NK-T-Ly, T_8) by flavonoids (Wiltrout et al., 1988; Berkarda et al., 1983; Schwartz & Middleton, 1984; Hume et al., 1979; Berton et al., 1980; Trnovsky et al., 1993; Fewtrell & Gomperts, 1977a, 1977b; Schwartz et al., 1982; Hughes, 1999; Middleton, 1998). However, no simple mechanisms are known that can explain this phenomenon (but it is believed to be due to the inhibition of COX, since PGs can suppress T-lymphocytes). In view of the importance of these T_8-lymphocytes (8 stands for the presence of the plasma membrane protein CD8) in the second line of the immune defence against invading foreign cells, e.g., metastases, bacterial cells, or virus-infected cells of the body, it appears appropriate to consider possible indirect mechanisms. Although T-lymphocytes circulate in the peripheral blood, they rarely recognise antigens directly because they hardly possess antibodies mounted on the surface to detect such intruders. Instead, they receive messages of such occurrences from macrophages and other cells. The first stop of such an antigen is normally a macrophage in the spleen or a lymph node, but may also be another antigen-presenting cell (APC), e.g., a B-lymphocyte. On the surface, these APCs are densely covered with a specific antibody. The cell that recognises the antigen, dimerises the antibody-antigen complex, endocytises it, and cleaves the antigen into small fragments. If the antigen is a protein, it is cut up into peptides that are $\sim$10 amino acids long. This decomposition takes place in a lysosome, a former endosome, which sorted the membrane receptor for the antibody from the remainder of its content and returned the receptor in a receptosome to the cell surface for reuse. The lysosome fuses with the Golgi transcompartment to deliver the antigenic peptides to newly synthesised major histocompatibility complex (MHC) Class I molecules. Each MHC molecule possesses a cleft of a length just suitable for the binding of the antigenic peptide. The loaded MHC molecule then travels to the cell surface, where it exposes the antigenic peptide and part of itself to the environment. Other APCs possess different, but functionally similar, MHC molecules. Passing T-lymphocytes recognise the antigenic molecule in conjunction with the MHC protein using its specific antigen receptor in the plasma membrane, and respond by producing and secreting cytokines. The latter comprise IL-1 to IL-16, interferon (IFN)-α, in addition to colony stimulating factor (CSF) and chemotactic substances. The ILs arouse B-lymphocytes and other T-lymphocytes in the vicinity to proliferate, and some of the former to differentiate to plasmacytes. The latter produce antibodies, but only of a kind that specifically binds the antigen recognised at the epitope that is exhibited by the MHC protein. In the course of this intensive cell communication via the ILs, also the cytotoxic T-lymphocytes, as well as the so-called NK-T-Lys, are activated (Fig. 43). The activity of the NK-T-Ly is known to be enhanced in human peripheral blood by flavone acetic acid (Urba et al., 1988; Wiltrout et al., 1988; Miranda et al., 1999). However, it should be noted that flavone acetic acid is a synthetic compound devoid of hydroxyl groups. Hence, its biological effect may differ from that of natural flavonoids.

Flavonoids bound to proteins probably enter macrophages by this mechanism (Mullink & von Blomberg, 1980; Bolton & Casley-Smith, 1975; Piller, 1978). They are known to interfere with both protein phosphokinases and transport ATPases, i.e., enzymes involved in the regulation of cell homeostasis (Suolinna et al., 1974; Spector et al., 1980a). Any such perturbation would stress the macrophage sufficiently to induce the production and secretion of cytokines. These alert the immune apparatus, thus fortifying a timely defence against infectants. Showell et al. (1981) have reported the inhibitory effect of quercetin and other compounds on lysosomal secretion, arachidonic acid metabolism, and Ca^{2+} fluxes in rabbit neutrophils. Flavonoids inhibit the activity of IL-5, which largely is chemotactic (Park et al., 1999).

Several immune cells produce various forms of IFNs: T-lymphocytes form IFN-α, whereas macrophages and granulocytes synthesise IFN-β. Evidence has been presented that shows that many flavonoids stimulate the production of IFNs. In this way, a different part of the immune system is activated (Cutting et al., 1953; Hornung et al., 1988).

The IFNs are acting in several different ways, some of which are not fully clarified yet. However, the following principles of their action on viruses have been established:

(1) IFNs induce the expression of nucleases that cleave viral genomes.
(2) IFNs inhibit the translation of viral proteins by altering the pattern of phosphorylation of the elongation initiation factors (eIFs).

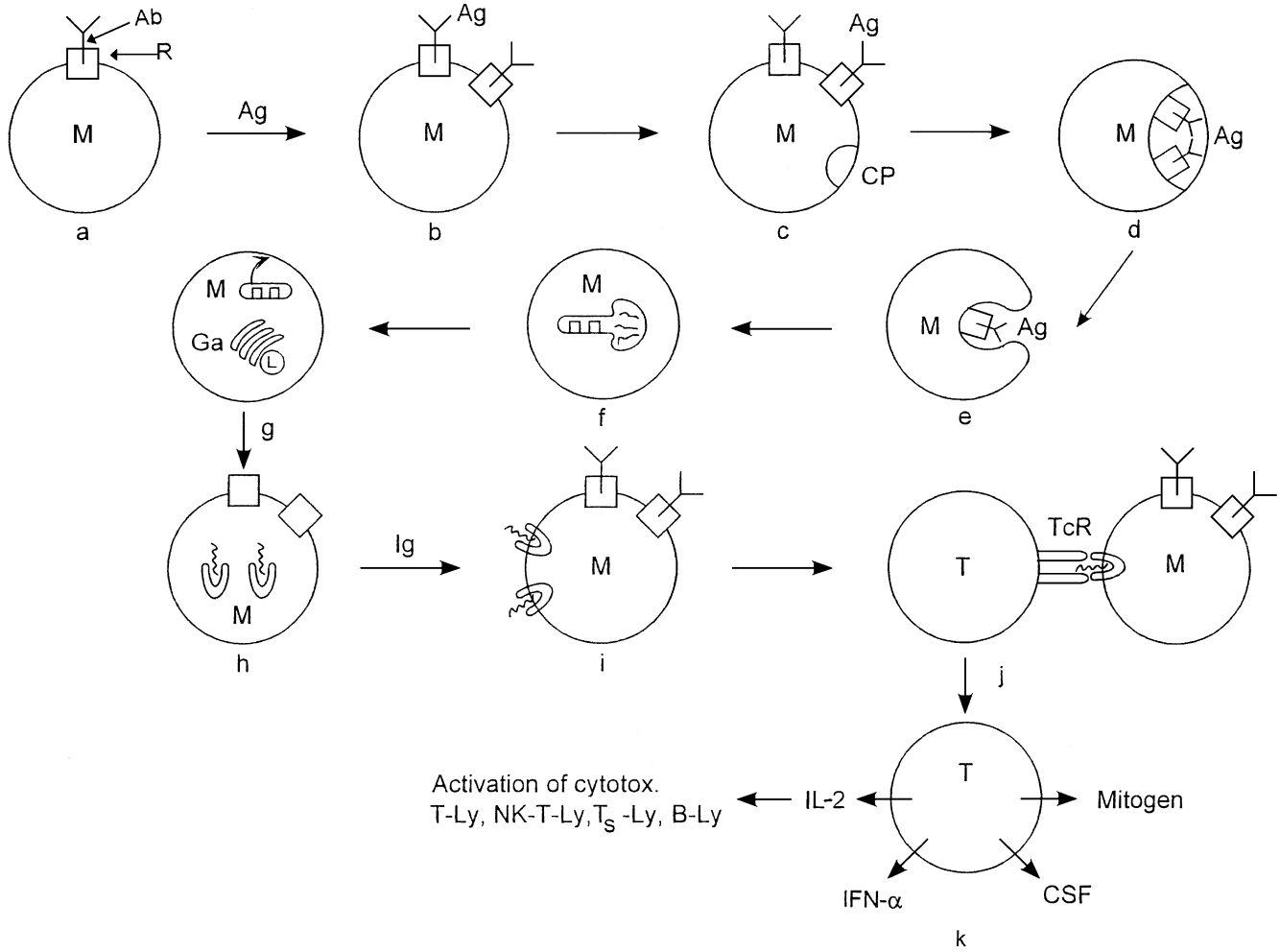


Fig. 43. Activation of cellular immunity. **a**: Macrophage (M) carries a receptor (R) for the terminal part (F_c) of the antibody. The antibody carries at the distal end two identical antigen (Ag)-recognizing sites. **b**: An Ag has been recognized by the antibody (Ab). A second Ab molecule joins the complex. **c**: The receptor-Ag-Ab complex diffuses along the plasma membrane to the coated pit (CP). **d**: The protein clathrin catches the R-Ab-Ag complex. **e**: The loaded coated pit is invaginated. **f**: In the resulting endosome, the F_c receptors, which are soluble, are sorted out and transferred to a separate compartment, while enzymes and a proton pump imported from the Golgi apparatus (GA) turn the endosome into a lysosome, in which the insoluble complex of antigenic protein and antibody is cleaved to peptides. **g**: The receptosome returns the FcR unharmed to the cell membrane for further duty. The lysosome (L) fuses with the GA to surrender the antigenic peptides to the MHC molecules, which reside here for their final glycosylation and trimming in preparation for their posting on the cell surface. **h**: Each MHC protein binds an antigenic peptide, leaving about one-half of it extruding from the cell surface. **i**: The MHC proteins, each presenting one antigenic peptide, have reached their positions in the cell membrane and the FcR have been reloaded with specific Ab. **j**: A T-lymphocyte (T), by its T-cell-antigen-receptor (TcR), simultaneously engages both the Ag and the MHC to detect whether any or both are foreign. **k**: The specific recognition has taken place, with the result that protein phosphokinase signal chains have activated genes for the expression of the cytokines, IL-2, IFN-α, CSF, mitogens, etc. The former activates several cell types, including cytotoxic (cytotox.) T-Ly, NK-T-Ly, T-suppressor (T8)-Ly, and B-Ly. In addition, the latter are induced to differentiate to plasmocytes that produce specific antibodies against the recognized epitope.

(3) IFNs cause the fortification of the plasma membrane of a neighbouring cell, which renders it more resistant to the penetration of attacking virus particles.

Although flavonoids are known to modulate the activity of protein phosphatases that are involved in both gene expression and the regulation of protein translation, their main effect appears to be to alert macrophages (Berton et al., 1980; Amoras et al., 1992a, 1992b). Flavonoids are capable of inhibiting the slow anaphylactic reaction by a yet unknown mechanism (Hope et al., 1983).

10. Scavenging of free radicals by flavonoids

One of the more prominent properties of the flavonoids is their excellent radical scavenging ability. It is also a valuable aspect for therapeutic and prophylactic applications of flavonoids, e.g., after infection, inflammation, burns, or radiation injury (Fritz-Niggli & Frohlich, 1980; Fritz-Niggli & Rao, 1978; Fritz-Niggli, 1968; Panthong et al., 1989; Hladon et al., 1980; Gabor, 1972a, 1972b; Schmidt et al., 1980; Wozniak & Braun, 1972; Casley-Smith & Bolton, 1973; Casley-Smith et al., 1974; Casley-Smith & Piller,

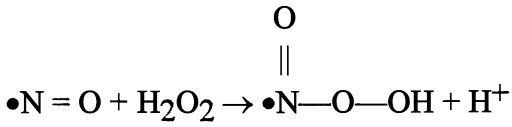


Fig. 44. The formation of nitrous peroxy acid.

1974; Van Cauwenberge & Franchimont, 1968; Crismon et al., 1951; Dieckmann, 1973; Felix, 1972; Danö et al., 1979; Calzada et al., 1999). As mentioned in Section 2, the radical scavenging ability is intimately connected with the oxidation/reduction potential and the activation energy for electron transfer of the substance (Marinov & Evtodienko, 1994; Salvayre et al., 1981; Pratt & Watts, 1964; Okonenko, 1986; Hodnick et al., 1986, 1998; Spilkova & Hubik, 1992; Fremont et al., 1998). This connection has been investigated and discussed in relation to the flavonoids by Halpern (1961) and Marcus (1963). The particular appeal of the flavonoids for radical scavenging in biological systems arises from their very low toxicity and low cost.

Free radicals are formed by activation of dioxygen in the initial response of macrophages to the recognition of an antigen. This rapid oxidative burst, of which neutrophils are also capable, is the first line of the immune defence. The process, which usually kills the invading foreign cells, e.g., a bacterial, metastatic, or virus-infected intruder, involves haemoenzymes of the oxygenase type and flavin nucleotide-dependent oxygenases (Kujumgiev et al., 1999; Limasset et al., 1999). A single electron is transferred from one of several available substrates, e.g., an amino acid to dioxygen-forming superoxide. This aggressive free radical oxidises double bonds in unsaturated FAs located in the cell membrane of the target cell, thus forming radicals and initiating a chain reaction in which free radicals are rapidly destroyed by electrophilic substrates and new ones are created. In addition to unsaturated compounds, sulfhydryl groups and aromatic substances also participate in this chain reaction. The immediate result is, among others, the rupture of double bonds, which by peroxidation have become very sensitive to oxidation, cleavage of disulfide linkages, oxidation of sulfhydryl groups, and dimerisation of thymine. Soon the rupture of the cell wall, which can no longer resist the osmotic pressure differential; the inactivation of vital enzymes, especially the anabolic ones; and the deficiencies in the genetic activity follow. One of these mechanisms alone suffices to kill the target cell, and a combination of them leaves hardly any doubt about the outcome.

An additional toxin liberated during the oxidative burst of macrophages is H_2O_2, which is the product of the eradication of the activated oxygen species by SOD. H_2O_2 reacts with nitrogen oxide, ·NO, a short-lived, physiological free radical, which serves several important functions in the cell, e.g., as a second messenger, as a neurotransmitter, and as an immune modulator (Ignarro et al., 1987; Furchgott & Zawadzki, 1980). The product of H_2O_2 and NO is nitrous peroxy acid, an extremely powerful oxidant (Fig. 44).

Free radicals also accelerate eicosanoid formation, which intrinsically depends on the presence of such agents, especially on the tyrosine radical (Stubbe, 1994). In turn, some eicosanoids, e.g., PGs, induce the expression of the genes for enzymes, such as elastase and collagenase, that are needed to dispose of damaged tissue and that initiate the repair processes.

Some flavonoids stimulate macrophages; stop further production of eicosanoids, some of which release pain-inducing peptides, e.g., substance P and bradykinin; and destroy excess oxidants. Thus, they support the resumption of the normal state in inflamed tissue. Irradiation of biological tissue with X-rays, nuclear particles, especially α- and β-particles, or γ-rays causes the cleavage of water to ·OH and hydride radicals. The latter instantly forms hydrogen gas, which is presumed not to be very deleterious, but the hydroxyl radicals are. They have a sufficiently long half-life to be the primary damaging agent. Widespread chain reactions are started, which destroy membranes, enzymes, and genes, and which lead to major organ damage. Such patients need avid radical scavengers of low toxicity. Aliphatic alcohols may be considered as antidotes since they are effective, but their toxicity is considerable. The application of flavonoids appears to be a very attractive alternative, but to the knowledge of the author, no scientific reports on such an application have appeared so far (Fig. 45).

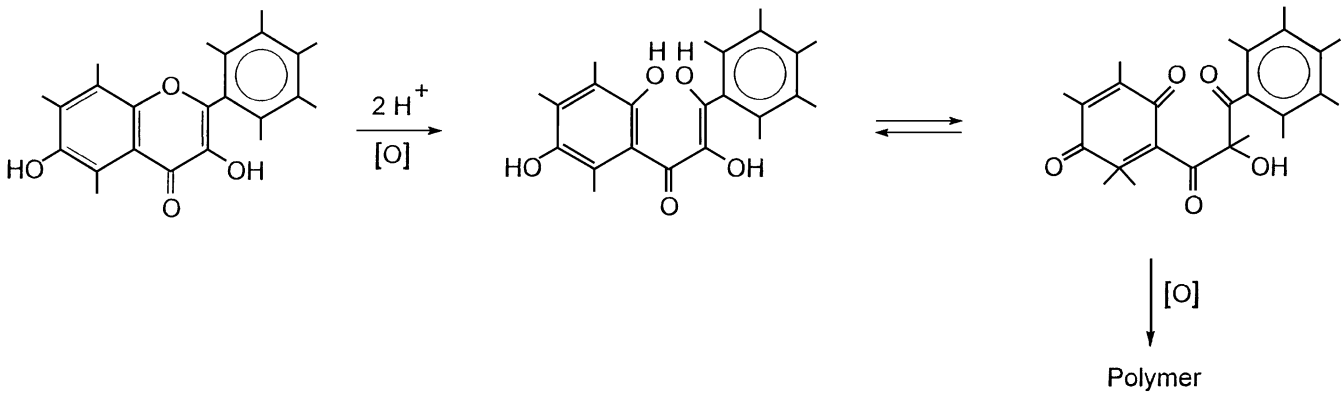


Fig. 45. Products of the oxidation of flavonoids.

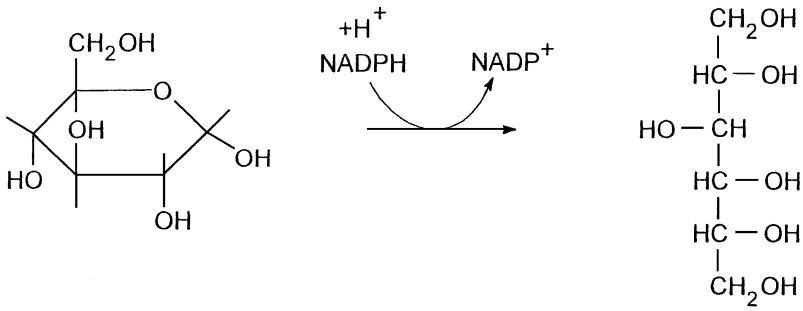


Fig. 46. Reduction of D-glucose to sorbitol by NADPH through aldose reductase catalysis.

Flavonoids are easily oxidised irreversibly to a p-hydroquinone, which in a reversible reaction, is further oxidised to a p-quinone. The latter easily polymerises to an insoluble substance, which is of no further use to the organism (Fig. 45). Hence, it must be decomposed. The oxidation of flavonoids is catalyzed by heavy metal ions and by light. These ubiquitous catalysts are likely to take part in many normal physiological reactions of plants.

11. The electron transfer catalysis by flavonoids

Flavonoids readily participate in biological oxidation-reduction processes and thus, are effective catalysts of electron transfer reactions. This implies firstly that their physiological standard potentials are located near that of important biochemical oxidation/reduction couples and secondly, that their activation energies for the uptake or donation of electrons are low. Since flavonoids are inactivated by oxidation, they much more easily lose than gain an electron. In this connection, it is appropriate to remember that the protection of biological reductants, especially ascorbic acid, is considered to be one of the most important functions of the flavonoids (Korkina & Afanas'ev, 1997; Jablonski & Anderson, 1984; Fujita et al., 1988; Robak & Gryglewski, 1988; Lonchampt et al., 1989; Laughton et al., 1989; Afanas'ev et al., 1989; Kostyuk et al., 1988; Miura & Nakatoni, 1983; Chen et al., 1990; Miyahara et al., 1993; Das & Ramanathan, 1992; Ratty & Das, 1988; Kukreja et al., 1988; Bohm et al., 1998). Ascorbic acid is irreversibly destroyed by oxidation, but flavonoids are oxidised in preference, i.e., they are sacrificing themselves, thus saving the indispensable vitamin C. Consequently, flavonoids are continuously consumed at a high rate to scavenge the omnipresent active oxygen species. Hence, the high daily consumption of 1–2 g of flavonoids in the form of vegetables, fruits, and beverages is justified.

Aldose reductase is an enzyme that, in spite of its low turnover number in certain pathological states, e.g., diabetes mellitus, can become important (see Section 17.3). It is inhibited by flavonoids (Keller & Leuenberger, 1980; Varma et al., 1962, 1975, 1977; Heyman & Kinoshito, 1976; Hers, 1960; Dons & Doughty, 1976) (Fig. 46).

Another oxidoreductase that is inhibited by flavonoids is the HMG-CoA reductase. This key enzyme in cholesterol biosynthesis is subject to allosteric feedback inhibition by cholesterol. Besides, its activity is modulated by phosphorylation catalyzed by a protein phosphokinase. Flavonoids can replace cholesterol as the allosteric inhibitor that, in the case of inborn errors, restores endogenous steroid regulation. In addition, flavonoids modulate the activity of some protein kinases (End et al., 1987; Gamet-Payrastre et al., 1999). Hence, flavonoids can spare many patients from the high risk of vascular diseases (see Section 17.9). Since the HMG-CoA reductase is NADPH-dependent, the binding site of the flavonoids is most likely the nucleotide fold (Fig. 47).

Also, several folic acid-mediated reactions are flavonoid sensitive, e.g., the restoration of THF from dihydrofolate after one of the numerous oxygenase reactions (Figs. 6 and 48). Since both the folic acid derivative and the reductant,

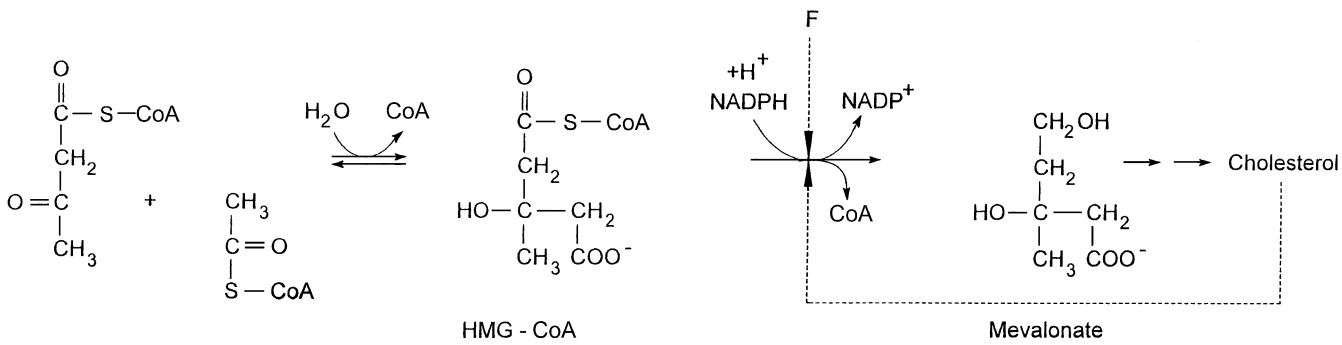


Fig. 47. Inhibition of HMG-CoA reductase by cholesterol (allosteric feedback regulation) or by flavonoids (F).

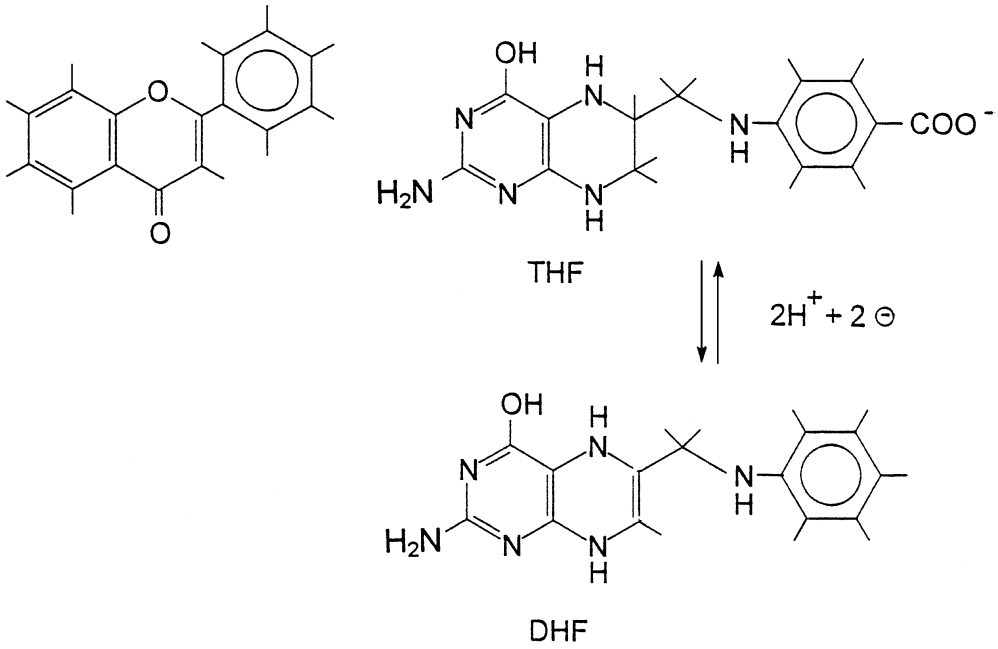


Fig. 48. Structural resemblance between folic acid and flavonoids. DHF, dihydrofolate.

NADPH, resemble flavonoids, it seems possible that they could be displaced by flavonoids, but it is too premature to make conjectures about detailed mechanisms.

The oxygenases represent a large group of oxidoreductases that, for several reasons, are inhibited by flavonoids (Park et al., 1998):

(1) They all operate by free radical mechanisms that are stopped by the radical scavenging action of the flavonoids.
(2) They all use THF as the electron transfer catalyst, but its participation may be prevented by the flavonoids, as described above.
(3) They also need pyridine and flavin nucleotides in their electron chain, but these prosthetic groups may be put out of action by flavonoids, as previously mentioned.
(4) All oxygenases contain Fe^{2+} and Cu^{2+} as essential components of their catalytic mechanism, but flavonoids have a strong affinity for heavy metal ions. As a consequence, the oxidation/reduction potentials of these ions are displaced and their locations, as well as their ligand architecture in the enzyme, are changed.

Examples of such oxygenases are cytochrome oxidase (Horn et al., 1970), xanthine oxidase (XO), proline hydroxylase, PG COX, NO synthase, and lipoxygenase. The PG COX is the key enzyme in the biosynthesis of the eicosanoids. The latter are tissue hormones that play a major role in inflammation, pain sensation, and tissue repair (Kuehl & Egan, 1980).

The PG COX is a large, complex enzyme with two active sites, one for the cyclisation of arachidonic acid and another for the subsequent peroxidation.

12. The flavonoids as enzyme inhibitors

Numerous enzymes, some of which were mentioned in Section 11, are inhibited by flavonoids. They include hydrolases, oxidoreductases, DNA synthetases, RNA polymerases, phosphatases, protein phosphokinases, oxygenase, and amino acid oxidases. This list is probably not complete since frequently new reports appear on additional examples of enzyme inhibitions by these substances. In some cases, the type of inhibition is competitive, but more often it is allosteric. Examples of allosteric activation of enzymes are also known. The stunning variety of the types of enzymes, the activities of which are influenced by flavonoids, spans across almost all enzyme classes. Yet, the flavonoids do not precipitate widespread chaos in metabolism, but restrict their influence to small branches. This considerable degree of tolerance to these chemically quite reactive substances, which structurally bear distinct resemblance to many compounds of human biochemistry, may be explained in part by their poor solubility in water, which keeps their concentration low; to their short half-life; and to the compartmentalisation of the organs and their cells, which segregates incompatibles.

12.1. Hydrolases

Conspicuous among the hydrolases that are inhibited by flavonoids is hyaluronidase because of its importance to the integrity of the loose connective tissue (Hasato et al., 1979; Li et al., 1997). The barrier that the glycones in the connective tissue present to the spread of infectants, e.g., bacterial cells, metastases, and viruses, is deteriorating during inflammation due to the action of this enzyme. The

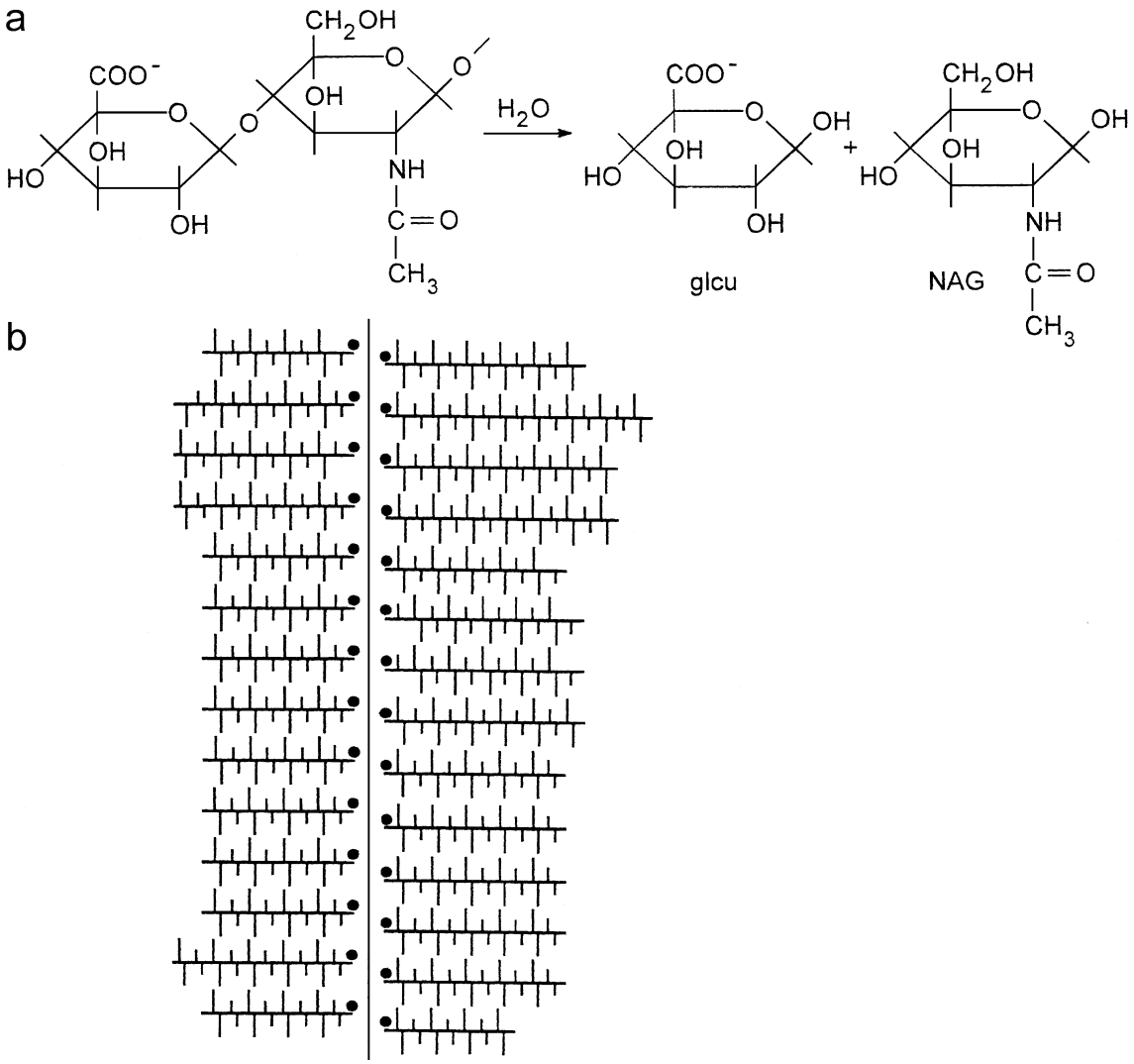


Fig. 49. The hydrolysis of hyaluronic acid by hyaluronidase. In this case, the structural basis of the inhibition by flavonoids is neither obvious nor known. **a**: Segment of the hyaluronic acid chain. **b**: Structure of a proteoglycan. The central vertical chain is hyaluronic acid to which globular proteins (black) adhere. Some of the latter carry long peptide chains to which keratan sulfate and chondroitin sulfate are attached as side chains. glcu, glucoronate; NAG, *N*-acetylglucosamine.

outstanding and recognised protective value of flavonoids is, therefore, to a large extent simply due to the inhibition of such glycanases (Tesi & Forssmann, 1971; Ramaswamy et al., 1972; Bonvehi et al., 1994; Metzner & Schneidewind, 1978; Pepeljnjak et al., 1985; Shub et al., 1978; Lee et al., 1997; Cecchini et al., 1997; Huang et al., 1997) (Fig. 49). Other effects of the flavonoids are ascribed to their influence on proteases (Mantle et al., 1999; Lee et al., 1998).

An important subclass of the hydrolases that is inhibited by flavonoids is the phospholipases (PLs) (Kyo et al., 1998; Kawaguchi et al., 1997). These enzymes cleave phosphodiester linkages in biological membranes. Ring (1976) has

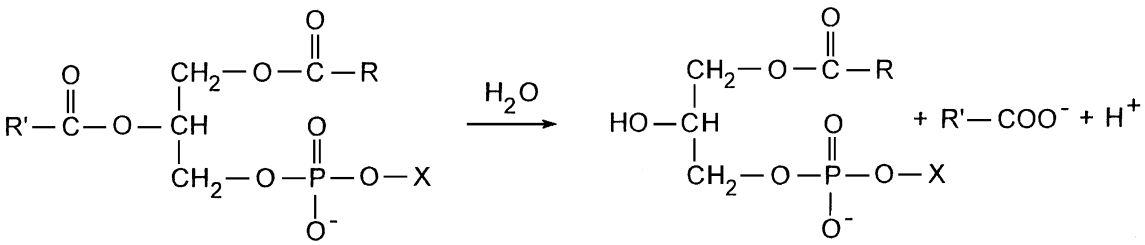


Fig. 50. The process catalyzed by PLA_2. $R'\text{-}COO^-$ is an arachidonate and the second product is a lysophosphatide. The latter constitutes a weak point in the membrane that often causes its rupture. X may be, e.g., choline, serine, ethanolamine, or inositol (phosphate).

studied the influence of flavonoids on the permeability of biological membranes. Since many of the products of PL action have signal functions as second messengers in metabolism, the regulation of the PL activities has widespread consequences. One example is the inhibition of PLA_2 by flavonoids. This enzyme liberates arachidonic acid, which is not only the originator of all eicosanoids, but also has a capability of its own to regulate the permeability of specific plasma membrane channels (Fig. 50).

A particularly interesting flavonoid-sensitive hydrolase is the cAMP PDE. Its substrate, cAMP, is the first discovered and best known second messenger. It activates a special class of protein P kinases, which initiate several signalling pathways that regulate many different components of metabolism. cAMP also moves from its place of origin, the cytoplasm, through the nuclear pores to the chromosomes, where it activates genes by binding to repressors, cAMP-dependent response element binding protein; which subsequently undergo conformational changes, with the result that they lose their passivating grip on the DNA.

The cAMP-P-diesterase is not only inhibited by flavonoids (Fig. 51) (Ruckstuhl & Landry, 1981; Petkov et al., 1981; Ferrell et al., 1977, 1980; Beecher et al., 1999; van het Hof et al., 1999), but also by caffeine and theophylline. The latter substance is the stimulating component of tea. The relationship between flavonoids and caffeine is reflected in the nature of the decomposition products of the former: caffeic acid and its derivatives. The inhibition type is noncompetitive (Arts et al., 1999).

Among the hydrolases that are inhibited by flavonoids, the phosphatases are a large and important group (Iio et al., 1980; Kavutcu & Melzig, 1999). It includes acid and alkaline phosphatases, as well as pyrophosphatases. These enzymes are Zn^{2+}-containing metalloenzymes that drive many anabolic processes by removal of primary products. The molecular basis of the inhibition is still unknown, but may well involve the ligandation of the flavonoids to the metal atom.

The protein phosphatases present a special case. These enzymes, which regulate signal chains and cell cycle proteins, can become activated or inhibited by flavonoids, depending on the system (Ait-Si-Ali et al., 1998). The most important subclasses of these enzymes are those specific for the phosphates of tyrosine and serine/threonine.

12.2. Oxidoreductases

Most biological electron transfer processes require coenzymes of the nucleotide type, although the catalytic function is located in an aromatic moiety, which is usually linked to the nucleotide by a phosphodiester bond. Since flavonoids structurally resemble both nucleotides (Wattenberg et al., 1968; Iio et al., 1983, 1985; Chang et al., 1994; Yamauchi et al., 1992; Le Bail et al., 1998) and the aromatic oxidation/reduction catalyst, they, in some cases, compete with the nucleotide for its binding site on the enzyme, whereas in other cases, they interfere directly with the transition state, e.g., by intercepting a free radical intermediate. The latter activity is known to be one of the favorite occupations of flavonoids (Arora et al., 1998). Since pyridine nucleotides, flavin nucleotides, and pteridines all operate by free radical mechanisms, a working hypothesis for the inhibition of these oxidoreductases, which comprise the great majority of this class, can easily be constructed (Chang et al., 1994; Hoffman et al., 1981).

The PG COX has been crystallised in complex with arachidonate. X-ray diffraction studies showed the binding groove of the substrate and the localisation of the functional groups that are involved in inhibition reactions. Aspirin transfers its acetyl group to the N-terminal serine side chain, thus perturbing the transition state (Vane et al.,

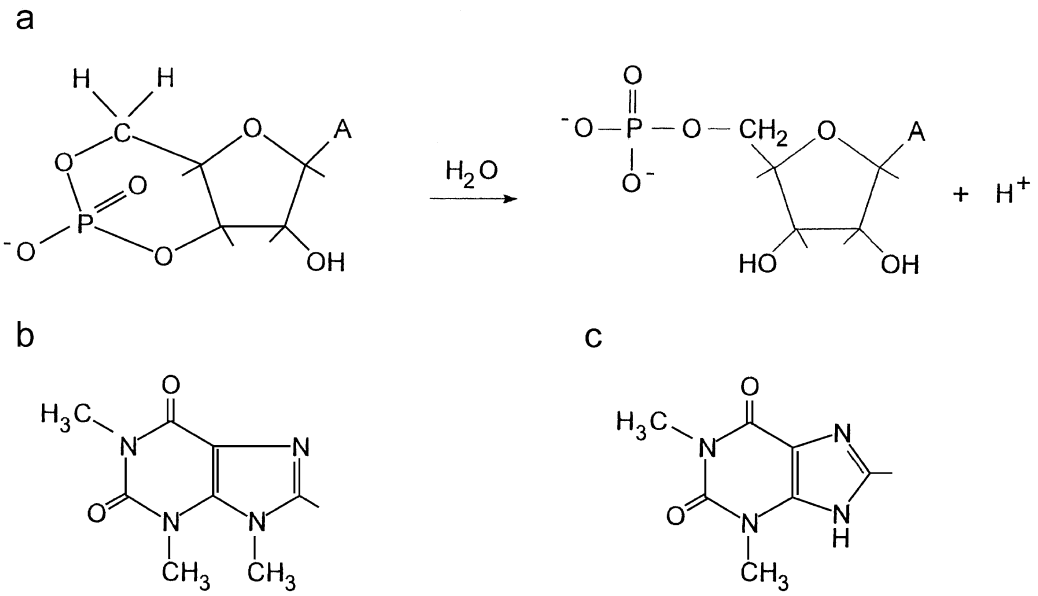


Fig. 51. **a**: The reaction catalyzed by cAMP-P-diesterase. A, adenine. **b**: caffeine. **c**: theophylline.

1994; Shimokawa & Smith, 1992; Picot et al., 1994; Yoshimoto et al., 1983; Roth & Siok, 1978), whereas flavonoids probably bind noncovalently at a site where they sterically hinder the correct binding of the substrate arachidonate. Many antioxidants inhibit the PG COX by competing with arachidonic acid for its binding groove. However, some COX inhibitors can induce the expression of the genes of metalloproteinases. These enzymes decompose the tissue and are necessary for the removal of cell debris. Simultaneously, they can facilitate the liberation of metastatic cells to the circulation (Ito et al., 1995). Another oxidoreductase of clinical significance is aldose reductase. This enzyme reduces aldoses, such as glucose and galactose, to hexitols, which are osmotically very active since their breakdown is slow (van Heyningen, 1959). In diabetic patients, this reaction can lead to problems, especially in the eye and the intestine because such patients have a reduced blood and lymph flow due to lipid deposition on the vascular wall. Consequently, the water drawn osmotically from the tissues into the intestinal lumen and the eye increases the local hydrostatic pressure. This leads to failure of the absorption of nutrients from the intestinal lumen into dessicated epithelial cells and to disturbances of vision. Fortunately, the enzyme can be inhibited by many flavonoids (Kader & Sharpless,

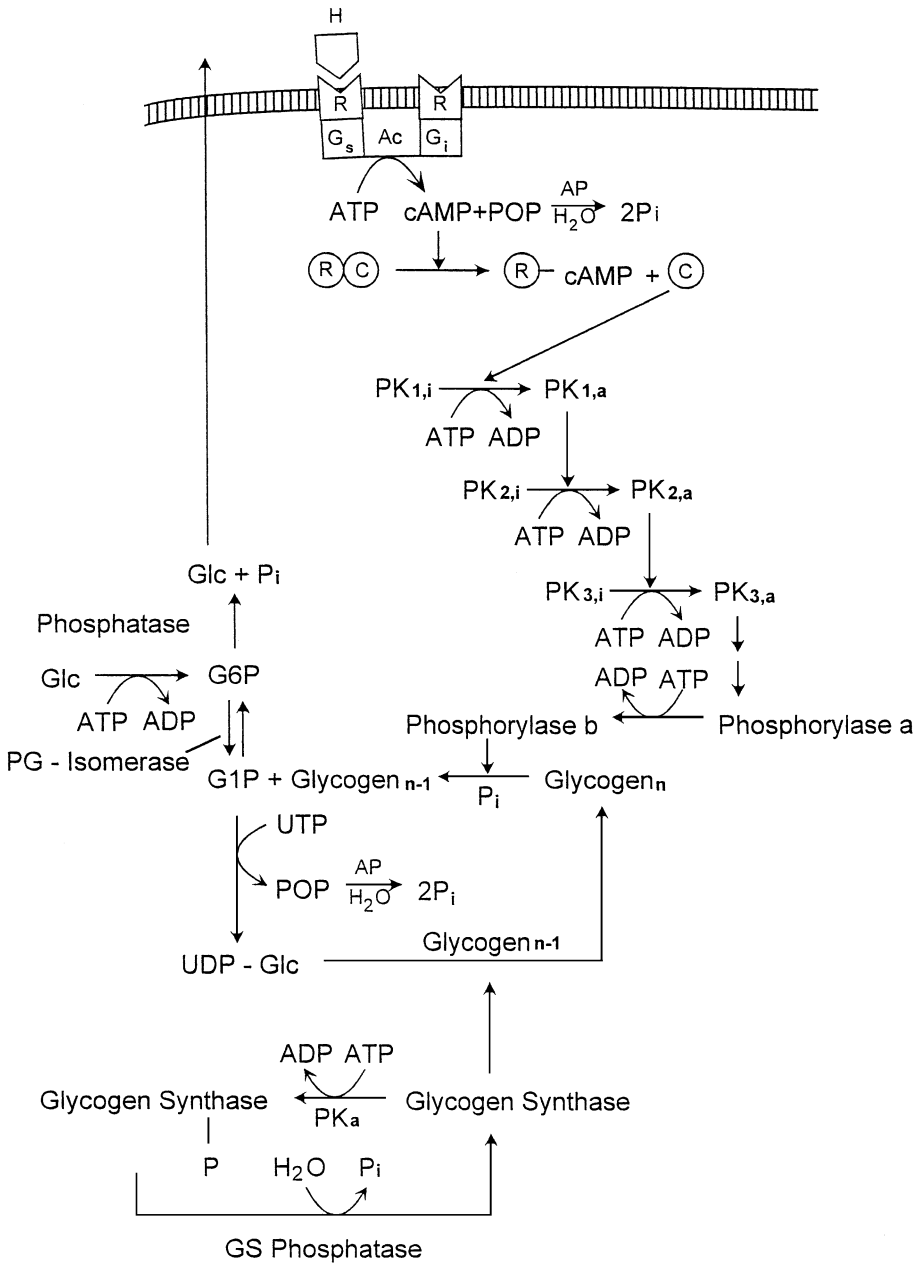


Fig. 52. Simplified scheme of glycogen metabolism. H, hormone (e.g., epinephrine or glycogen); AP, alkaline phosphatase; G1P, glucose-1-P; Glc, glucose; G_i, inhibiting G-protein; G_s, stimulating G-protein; GS, glycogen synthase; PK_a, active form of PK; PK_i, inactive form of PK; POP, pyrophosphate; R and C, regulating and catalytic subunits of cAMP-dependent protein P kinases (PK).

1978; Parmar & Ghosh, 1979; Turner & Hryszko, 1980; Varma et al., 1975, 1977; You et al., 1999).

Two isoenzymes of COX exist (COX 1 and 2). One releases the primary inflammatory response and should be inhibited, whereas the other supports repair mechanisms and should be spared. Acetylsalicylate (aspirin) and salicylate inhibit both of these isoenzymes, thus causing serious side effects, such as bleedings, suppression of lymphocyte activation (Barasoain et al., 1980) and neurological disorders when they are taken in high doses, but modern alternatives, e.g., some flavonoids, allow a differentiation, i.e., they inhibit only the isoenzyme that forms harmful products.

12.3. Kinases

The special significance of the kinases in relation to their inhibition by flavonoids is their involvement in metabolic regulation. They transmit environmental and local cues along signal chains to effector systems, e.g., gene activation or energy release from glycogen stores. All kinases use ATP as one of the substrates, i.e., they possess the conserved nucleotide fold that, in all likelihood, also accommodates flavonoids. Particularly far reaching is the regulation of the protein phosphokinases by the flavonoids. Characteristically, the attack on the P-kinase signal chains is two-pronged because, apart from the direct inhibition of these enzymes by flavonoids, the latter substances also influence, positively or negatively, the protein phosphatases that reverse the action of the protein P-kinases (Fig. 52). Thus, in principle, the flavonoids are capable of regulating the signal chains both ways, depending on the circumstances (Cochet et al., 1982; Kyriakidis et al., 1986; Picq et al., 1989; Ferriola et al., 1989; Hagiwara et al., 1988; Jinsart et al., 1992; Apps & Glover, 1978).

The hormone binding to its receptor (R) activates via G_s a P-kinase cascade that activates glycogen breakdown and inhibits glycogen synthesis. The R-site, which is associated with G_i, has a lower affinity for the hormone than the one linked to G_S and inhibits adenylate cyclase (AC).

12.4. Isomerases

The best known example of an isomerase that is inhibited by flavonoids is the quercetin inhibition of the DNA topoisomerase II. This case already has been described in Section 7.

12.5. Transferases

No reports on the effects of flavonoids on the action of the transferases are known to the author.

12.6. Ligases and lyases

The ligases use ATP as a substrate and form covalent linkages with it. Due to the structural resemblance, they are potentially inhabitable by flavonoids. However, so far, no such examples have been reported. The remaining class in the international enzyme classification is the lyases. To the knowledge of the author, only a few examples of the inhibition of these enzymes (e.g., carbonic anhydrase) by flavonoids have been found (Conney et al., 1981).

13. The hormone action of flavonoids

Flavonoids can act as hormones in both plants and animals (Sonnenbichler et al., 1980; Sonnenbichler & Pohl, 1980; Baker, 1998; Baker et al., 1998). The roles of flavonoids as plant hormones already have been discussed (see Section 6). The discovery that flavonoids also have hormonal effects in animal systems was a surprise (Mitcher et al., 1982). The origin was the observation that sheep that had eaten fermented clover became sexually aroused. An analysis showed that the active substance was silybin. The structure of this complex flavonoid was elucidated by Sonnenbichler and Pohl (1980). They found that the hydroxyl groups of the aglycone were positioned in space just like those of an estrogen (Fig. 53). Since estrogens also have anabolic effects (Sokolova et al., 1978; Sharma et al., 1971), one might suspect that flavonoids might be able to act as growth hormones in animals also. However, so far only few indications of such a function have been found.

Flavonoids can also display an estrogenic and pregnancy inhibitory function (Jain et al., 1993; Singh et al., 1990). This effect was already known by lay practitioners in the Middle Ages, but it now has been confirmed using modern methods. After ovariectomy, endothelial dysfunction resulting from the lack of estrogen can be improved by the supplementation with either genistein or 17-β-estradiol (Squadrito et al., 2000). The authors concluded that the effects of the two substances were overlapping. However, the discussion of the phyto-estrogenic effect of flavonoids in the literature has been rather controversial, especially regarding possible replacement therapy with genistein and estrogen to improve endothelial dysfunction (Squadrito et al., 2000).

14. The mutagenic potential of flavonoids

Many organic substances, regardless of whether they are natural products or prepared synthetically, are capable of inducing mutation in the Ames test (Ames et al., 1975; MacGregor & Jurd, 1978; MacGregor, 1979, 1986a, 1986b; Brown et al., 1977; Brown, 1980; Bjeldanes & Chang, 1977; Hatcher & Bryan, 1985; Ellinger et al., 1984; Bartholomew & Ryan, 1980), which indicates that they are potentially mutagenic in humans. Since the experimental conditions of this test are far removed from the physiological environment in humans, which includes efficient

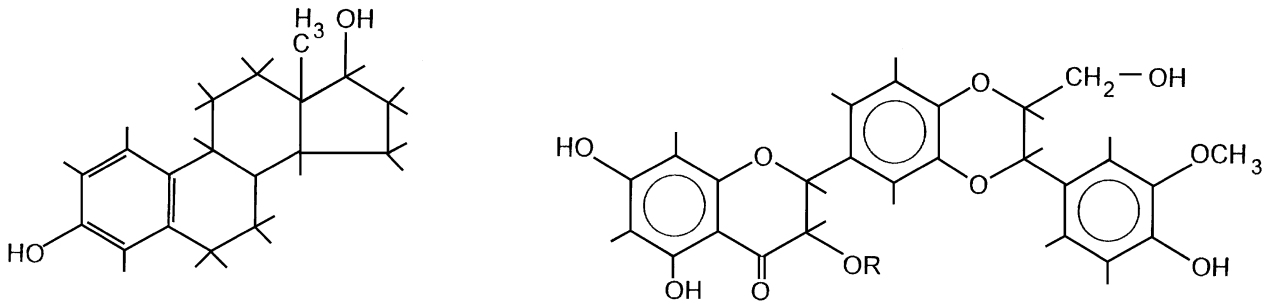


Fig. 53. Comparison of the structures of estradiol (left) and silybin (right).

filters, the suspicion raised by a positive reaction must be further tested in animals. Such checks in a more realistic model did not confirm the mutagenicity of the flavonoids being tested. However, since the tests are expensive, only a limited number of animals were used. Hence, the possibility exists that on rare occasion, flavonoids do cause mutations in animals. Of all mutations, the great majority are neutral, i.e., have no further consequences, whereas a small fraction have a positive or a negative effect. In the worst case, a mutation is one step towards carcinogenesis, but most mutations are discovered by repair enzymes and are eliminated in time. The fraction of the mutations that which are rectified by physiological mechanisms is largely unknown. It undoubtedly depends on the constitution and current state of health of the individual (Maruta et al., 1979; Sugimura & Nagao, 1979; Takahashi et al., 1979; Sahu et al., 1981; Bartholomew & Ryan, 1980; Carver et al., 1983; van der Hoeven et al., 1984; Pamukcu et al., 1980; Erturk et al., 1984; Williams, 1986; Saito et al., 1980; Hosaka & Hirono, 1981; Hirono et al., 1981; Takahashi et al., 1983; Aeschbacher et al., 1982).

The frequency of mutations induced by flavonoids in the Ames test roughly corresponds to that of structurally related compounds, e.g., naphthalene derivatives, and it is low. Therefore, the risk of pathological consequences of mutation incurred by the consumption of flavonoids must be considered to be low (Habs et al., 1984).

15. The influence of the flavonoids on the sensory system

The human sensory system continuously registers and reports the state of the internal and the external environment. Examples are the fragrance of flowers, the taste of a spiced meal, the aroma of freshly brewed coffee, and the bouquet of a wine. All of these sensations, which each in its own way improves the quality of life, are, to a considerable extent, liberated by flavonoids that have been entrained with water vapours and other volatile substances. In other words, not only the colors from the petals of the flowers, but also the gastronomy, including the drinking of wine, depend to a considerable extent on the presence of flavonoids. Therefore, a ban on flavonoids from foods, which seems to be both unnecessary and impossible, would destroy an important part of our culture (Brown, 1980; Bruillard & Delaponte, 1977; Bruillard, 1982).

15.1. The olfactory system

The mechanism of arousal of various parts of our sensory system has been studied recently in considerable detail. Therefore, it is now possible to make an educated guess about how flavonoids are perceived and how they can enhance the sensation of other flavours. Olfactory agonists, i.e., substances that arouse attention because of their smell, are recognised by specific receptors on the surface of the olfactory neurones in the nose. This interaction is analogous to the one between hormones and their plasma membrane receptors. Like many hormone receptors, the olfactory cells transduce the signal from the receptor to the cytoplasm with the help of G-proteins. The latter are proteins that bind guanine nucleotides, especially GTP and GDP. G-Proteins are also GTPases (for a review, see Bourne, 1994). They consist of three subunits, α, β, and γ, of which, the α-subunit contains the active site, whereas the $\beta\gamma$-heterodimer upon GTP hydrolysis becomes detached and binds to an ion channel in the plasma membrane. The result is the opening of the channel, the influx of ions, and the propagation of an action potential. The latter is transmitted to the brain to report about the sensation (Fig. 54).

The flavonoids are prominent olfactory agonists, but since their aglycones are lipophilic, they can also penetrate lipid membranes and interfere with cytoplasmic metabolism and regulation. Many specific membrane channels, including those for ions, are known to be regulated by phosphorylation and dephosphorylation. Examples are the Na^+/K^+-transport ATPase (Horisberger et al., 1991) and the Cl^- channel, which is incapacitated by the inborn error cystic fibrosis. Since flavonoids interact with both protein P kinases and protein phosphatases, they are likely to be involved in olfactory sensation in several ways.

The taste sensation is known to be akin to the olfactory. However, the taste buds on the tongue are a more primitive version because only few modalities are recognised, i.e., the receptors are less specific. We can distinguish between sweet, sour, bitter, and salty tastes only. The taste receptors are furnished with G-proteins that function as those of the

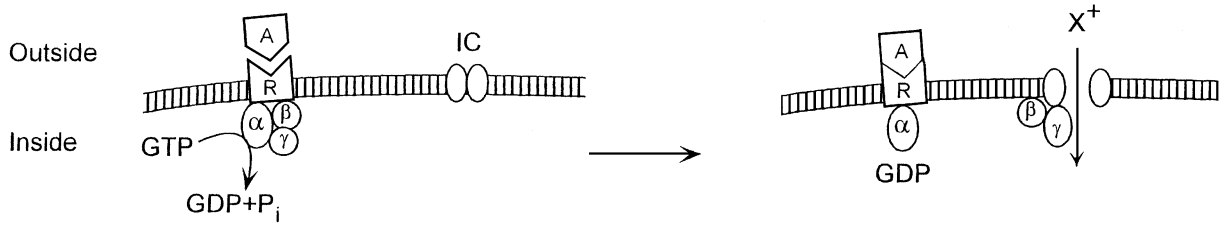


Fig. 54. Two models illustrating mechanisms of olfactory sensation. A cAMP-dependent protein kinase modulates the function of the ion channel by phosphorylation. A, olfactory agonist; α, β and, γ, subunits of the G-protein; IC, ion channel; R, olfactory receptor.

olfactory neurones, and the signal chain appears to be the same as the one described above. Flavonoids enhance the neural activity by inhibiting the cAMP PDE, thus raising the cAMP concentration (Ruckstuhl & Landry, 1981; Graziani & Chayoth, 1977, 1979; Graziani et al., 1977).

15.2. The neurostimulatory effect of flavonoids

Not only the peripheral nervous system, but also the CNS, is influenced by the flavonoids (Griebel et al., 1999). Flavonoids are agonists at adenosine receptors in the brain (Paladini et al., 1999; Blardi et al., 1999; Hasrat et al., 1997). They are also ligands for the benzodiazepine receptor (Medina et al., 1997). The result is a stimulation akin to the one experienced after the consumption of coffee, tea, or tobacco. Since these stimulants all contain appreciable concentrations of flavonoids, the latter are probably major contributors to the desired effect. The caffeic acid esters, which can arise in the catabolism of flavonoids, are considered the most important allergy provokers in propolis (Greenaway et al., 1987).

Flavonoids have an instant pain-relieving effect on skin wounds after insect or snake bites, burns, or cuts. This mechanism may easily be explained by the inhibition of PG formation explained in Section 9. When venom is injected, e.g., by bees or snakes, additional factors play a role. Such venoms contain several toxins and enzymes, e.g., PLA_2. The latter enzyme is inhibited by many flavonoids. The details remain to be clarified, but since flavonoids are lipophilic substances and the activity of this enzyme requires the interaction with a lipid membrane, a reasonable assumption is that the flavonoid prevents contact of the enzyme with a special membrane structure, which normally releases an activating conformational change in the enzyme (see Fig. 50).

The local anaesthetic effect of flavonoids resembles that of acetylsalicylate (see Section 12.2). For example, it is used by bee keepers, who have an easy access to propolis, and by dental surgeons, especially in Eastern Europe (Henning, 1974; Hladon et al., 1980).

15.3. The analgesic effect of flavonoids

Analgesia means the relief of pain. Higher animals possess various endogenous mechanisms by which they can achieve such an effect in situations of grave crisis. An important pathway of this kind is the enhanced biosynthesis of proopiomelanocortin, from which the endogenous opiates enkephalin, endorphin, and dynorphin are released by proteases. The justification of the classification of these substances as opioids is not only that they induce a state of anaesthesia, but also that they fold into the same spatial configuration as members of the morphium group of natural compounds. Besides, both the endogenous opiates and those of plant origin engage the same neuronal receptors. Special neurones are particularly richly furnished with this type of receptor. They are located near the synapses of the presynaptic neuron and are coupled to secretory mechanisms for granula of transmitter substances. The latter are displaced from the synapses. Hence, the propagation of action potentials is inhibited. The phenomenological effects of flavonoids and endogenous opiates have so much in common that a direct connection is suspected (Seibert et al., 1994; Rylski et al., 1979; Ahmadiani et al., 1998). Both classes of substances raise the concentration of cAMP, flavonoids (e.g., quercetin and kaempferol) by inhibiting cAMP PDE (Beretz et al., 1978), opiates by activating G_S (Simmen et al., 1989, 1998). The flavonoids may bind directly to the opiate receptors or like opiates, they may inhibit neuronal transmitter receptors from activating pain neurones (Fig. 55). The latter neurones are the targets for opiate treatment and misuse.

16. Complexes of flavonoids with heavy metal ions

Heavy metal ions are avid ligand binders (Asen et al., 1977; Hiermann & Kartnig, 1978; Middleton & Drzewiecki, 1982; Schwartz et al., 1982; Schwartz & Middleton, 1984; Miura et al., 1994). This ability is also evident in many biological systems. Examples are hemoproteins, cobalamine (i.e., vitamin B_{12}), and numerous metalloenzymes (Parellada et al., 1998). Among the heavy metal atoms that are essential to biochemical systems are Fe^{2+}, Cu^{2+}, Zn^{2+}, Co^{+1}, Mn^{2+}, and Ni^{2+}. However, other heavy metal ions are strong inhibitors of sulfhydryl groups in the active sites of anabolic and protective enzymes, e.g., γ-levulinic acid synthetase, porphobilinogen synthetase, ferrochelatase, all kinases and dehydrogenases, as well as GSH reductase. Examples of such harmful heavy metal ions are Hg^{2+}, Zn^{2+},

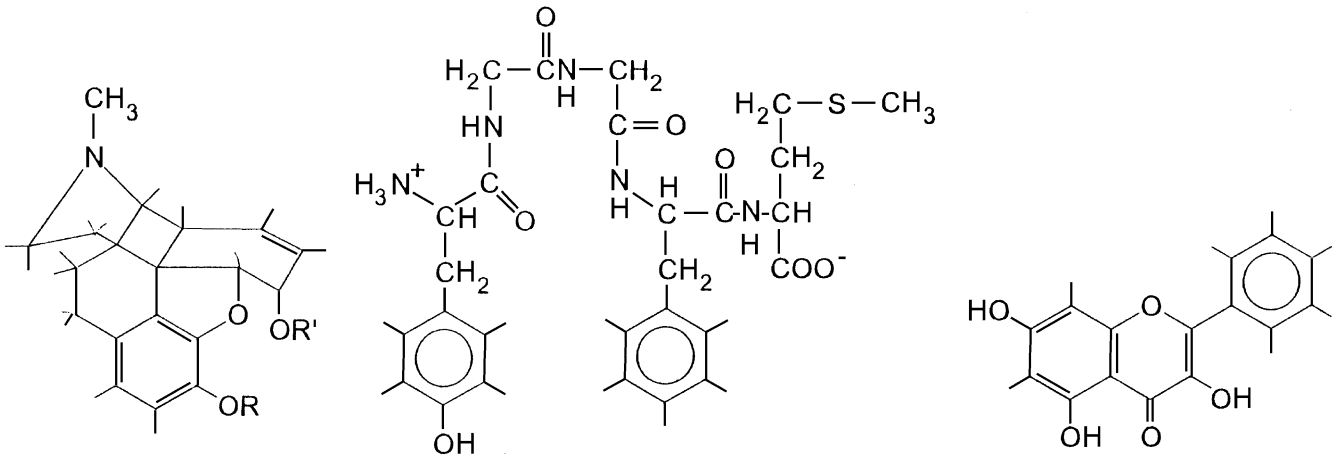


Fig. 55. Comparison of the structures of morphine (left), met-enkephalin (center), and a flavonoid (right). F, phenylalanine; G, glycine; M, methionine; Y, tyrosine.

Sn^{2+}, and the ions mentioned above when present in excess. The free elements corresponding to these ions are less toxic by a factor of 10^2-10^3. The reason is that these metals, in the form of the elements, are insoluble and chemically rather inert. However, active oxygen species and other strong oxidants in the organism are capable of oxidizing the elements to ions at a slow rate. Moreover, heavy metals in elemental form have a marked tendency to accumulate in adipose tissues and in the liver. From these locations, they are difficult to mobilise due to their low reactivity and to their inaccessibility to extraction by solvents. The latter may be even more toxic to the organs than the heavy metals (Bakir et al., 1973; Schwartz et al., 1982; Lamb et al., 1995; Wrzesinski et al., 1995).

Numerous cases of chronic intoxication with heavy metals are known. Examples are ingestion with mercury salts, leakage from deteriorating dental amalgam plumbs, and occupational hazards, e.g., painters intoxicated with lead and cinnober. Thousands of patients have been and are affected by severe heavy metal poisoning, but so far, the best curative agents that have been approved for clinical use have been synthetic polysulfhydryl compounds. They were used in the widely publicised case in Iraq in 1967, in which beized wheat sowing seeds were stolen and baked into bread that was eaten by thousands of villagers (Bakir et al., 1973). Many died as the result of acute ethyl-mercury poisoning, and still more suffered for many years from chronic intoxication, particularly of the liver.

The problem with the use of polymeric sulfhydryl compounds for detoxification is their low solubility, especially in water. Hence, it must be given orally, and is absorbed in the intestine only to a minor extent. The part that reaches the circulation is probably carried by bile micelles. Only very low concentrations of polysulfhydryl compounds are likely to reach the main target, the liver, and due to the tight association of the heavy metals with lipid stores, only a small fraction of the toxin can be mobilised and excreted with the bile.

The advantage of the flavonoids as therapeutic agents is that they should be able to mobilise heavy metals effectively, especially when water-soluble derivatives such as hydroxyethylrutosides are injected into the blood. Once they have re-entered the blood, they could be removed by dialysis combined with affinity chromatography on a matrix containing sulfhydryl groups. At present, a combination therapy using polysulfhydryl polymers together with flavonoids seems to be a promising treatment (Fragner, 1964; Strohecker & Henning, 1963; Thompson et al., 1976; Cetta et al., 1978). In contrast, the rise in the blood level of 1,3-dimercaptopropane-1-sulphonic acid seems to call for caution due to its resemblance to British anti-Lewisite, which has toxic effects. A likely problem is the removal of essential trace metals from enzymes.

17. Medical, technical, gastronomic, and other applications of flavonoids

The use of bee products, such as honey, propolis (kit wax), wax, gelé royal, and pollen, in the treatment of human diseases is very old. It is probably almost as old as humanity itself, i.e., about two million years (Eversted et al., 1997). All of these bee products contain appreciable concentrations of flavonoids, especially propolis, which is a rich source of flavonoids. Honey was probably the first of the bee product that was eaten because its taste is sweet and its high glucose concentration quickly raises the blood sugar level with an invigorating effect. Next, some of our ancestors had discovered that inflammation in the skin and throat healed more rapidly when they were treated with honey or propolis. Evidence of the early use of honey and propolis is the discovery by archaeologists of resins containing flavonoids at ancient burial sites and fire places, as well as in caves that seem to have been inhabited.

When we approach historical times, the evidence of the use of honey and propolis abounds. Jars containing residues

of high flavonoid content have been found in Egypt on the tombs of the Pharaohs. Besides, propolis, due to its sterilizing and tanning effect, in some cases has been used for balsaming of mummies. Due to oxidation, these mummies acquired a black surface colour, which at first was ascribed to tar or asphalt.

Whereas the Bible only alludes to propolis, the Koran explicitly mentions this substance (Muhammed, Ca. 500). It specifically recommends the believers to keep a supply of propolis in the house for use in the case of illness. The name propolis also originates from the Near East, "pro" in Latin means "in front of" and "polis" in Greek means "city". This name alludes to the practice by bees of smearing the landing platform in front of the entrance to the hive with propolis glue, which the young working bees have enzymatically modified by chewing the resinous material that the experienced collecting bees have gathered in trees and shrubs. Their saliva contains an amylase that liberates the flavonoid aglycones. Soldier bees on guard at the entrance oversee that all arriving bees sterilise their feet in the propolis glue before they enter the hive. The bees also covered the walls of the latter with a layer of propolis glue in order to render the hive almost sterile (Metzner et al., 1979; Bankova et al., 1983; Hladon et al., 1980; Kujumgiev et al., 1999) and to exclude draught and humidity. All invading foreign insects and rodents, e.g., mice, snails, or spiders, are instantly killed with stings and mummified with propolis, if the carcasses are too heavy to be removed from the hives.

Propolis has had numerous non-medical applications, a few of which will be mentioned here to indicate the wide appreciation of this substance in our culture (Lejeune et al., 1984).

Today, propolis is mostly used by lay medical practitioners, but in Eastern Europe, it is widely used by authorised medical doctors. It is used, for example, in cremes and ointments for use against skin lesions, as repositories against vaginal infections, and extracts of propolis, especially in ethanol, are given per os to treat a variety of diseases, some of which are discussed in the following sections. Flavonoids can also be used as sweeteners (Brown et al., 1979).

In the following sections, some of the authorised uses of flavonoids by the medical profession in Western civilisation will be described.

17.1. Hypertension and microbleeding

Hypertension is a widespread disease, and in part can be hereditary in nature. The condition can become aggravated, for example, by psychological stress, tobacco smoking, and alcohol consumption. These characteristics indicate the participation of the part of the CNS that controls the state of contraction of vascular smooth muscles. An understanding of this complex syndrome requires a brief review of the regulation of the water equilibria in the body (called water homeostasis).

In response to a nervous signal from the formato reticularis in the brain stem, prorenin is produced and secreted into the blood. Biosynthesis primarily takes place in the renal cortex, but, surprisingly, to a certain extent also in the maxillary gland. Prorenin is activated, i.e., converted to renin, by proteolytic liberation of a peptide. Renin, which like pepsin belongs to the acidic type of proteases, activates angiotensinogen (also called hypertensinogen), a prohormone formed by the liver, to angiotensin (hypertensin). The latter is shortened by a few amino acids by the angiotensin-converting enzyme to angiotensin II, which binds to plasma membrane receptors on a variety of cells, especially renal tubular cells and vascular endothelial cells (Cushman & Ondetti, 1991; Bettini et al., 1978a, 1978b, 1978c, 1978d; Kimura et al., 1997). On the cytoplasmic side, the receptor is in contact with G-proteins that activate AC. The latter produces cAMP, which activates a protein phosphokinase that opens a water channel by phosphorylation (Fig. 56).

Flavonoids can influence this mechanism in a variety of ways (Bettini et al., 1978a, 1978b, 1978c, 1978d; Gerdin & Svensjö, 1983; Casley-Smith, 1976; Ambrose & de Eds, 1949; Arturson, 1972; Bohr et al., 1949; Harper, 1966; Klemm, 1966, 1967; Lockett & Jarman, 1958; Paris & Moury, 1964; Paris & Vairel, 1949; Schiller, 1951; Svensjö et al., 1977; van Cauwenberge & Franchimont, 1967; Hodgson et al., 1999; Itoigawa et al., 1999; van Acker et al., 1997). The most obvious is the inhibition of the PDE that destroys cAMP by opening its ring (see Section 11). The result of this inhibition is an increase in the flow of water from blood into the tubular cell, from which it can escape into the urine. The removal of water lowers the blood pressure. This effect has long been claimed by lay medical practitioners, but can now be confirmed by evidence from recent biochemical research. The flavonoid class that most frequently is used to lower blood pressure is a group of water-soluble, semisynthetic compounds called hydroxyethyl-rutosides (Voelter & Doughty, 1978; Rose, 1970; Tschopp et al., 1970).

The transport of water through lipid membranes is also regulated by another mechanism, which is controlled by the nonapeptide vasopressin (adiuretin or the antidiuretic hormone). Vasopressin is produced in the hypothalamus at the base of the brain in the nucleus paraventricularis. It is formed as a prohormone linked to a larger protein neurophysin, which carries it along the axon of a long neuron, terminating in the posterior part of the hypophysis, the neurohypophysis, a gland located in the middle of the head behind the nose. In the hypophysis, vasopressin is liberated by proteolytic cleavage and stored until use. The hormone is secreted into the blood upon receipt of a neurotransmitter that binds to a surface receptor. The blood carries the vasopressin to the kidneys, where it binds to a specific receptor in the plasma membrane of tubular cells. This receptor is a transmembrane protein, which, on the cytoplasmic side, is noncovalently linked to a G-protein by a conformational change in the vasopressin-receptor complex.

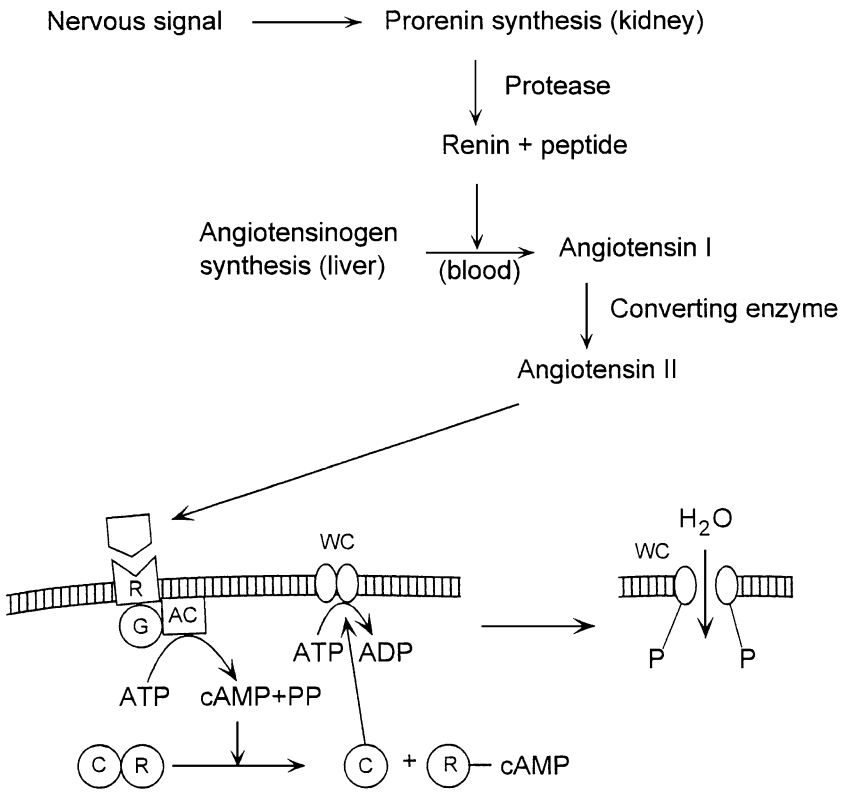


Fig. 56. Sketch of the mechanism of regulation of water transport in a renal tubular cell. C and R, catalytic and regulatory subunits of PKA; G, G-protein; R, angiotensinogen receptor; WC, water channel.

The G-protein hydrolyses GTP to GDP and phosphate, thus releasing free energy, which is used to change the conformation, not only of the G-protein, but also of the associated AC molecule. The latter becomes activated and converts ATP to cAMP. As in the case of angiotensin, a cAMP-dependent protein phosphokinase is subsequently activated and either directly phosphorylates a water channel or starts a cascade of P-kinases, which ultimately opens the water channel by phosphorylation. In this case, water, together with Na^+ ions, flows from the lumen of the renal tubule into the lining of the cells and onwards to the blood. Hence, this hormone decreases diuresis and raises blood pressure. If for any reason vasopressin is secreted in insufficient amounts, blood pressure can rise beyond the normal limit, unless other mechanisms suffice to compensate for the disorder.

Vasopressin also has a different role pertaining to water homeostasis, because this hormone also binds to the adrenocorticotrophic hormone (ACTH) receptor of the adrenal cortex. ACTH or corticotropin is synthesised in the adenohypophysis, the anterior lobe of this organ, and is secreted into the blood in response to the binding of the tripeptide corticoliberin. ACTH initiates steroidogenesis in the adrenal cortex by binding to a specific plasma membrane receptor (Lüddens & Havsteen, 1986), but this effect is simulated by vasopressin. The biosynthesis of three classes of steroid hormones takes place, each in a separate layer of the adrenal cortex. The outer layer produces glucocorticoids; the middle layer, mineralocorticoids; and the inner layer forms sexual corticoids. As the organ ages, the roles of the layers change, i.e., each cell, depending on its age, produces mainly one of the three classes of corticoids. The most prominent of the mineralocorticoids is aldosterone (Fig. 57). This hormone acts in renal tubular cells where it induces the secretion of Na^+ ions into the urine. Since each Na^+ ion is accompanied by three water molecules, this hormone is also important to water homeostasis. The formation of all of the steroid hormones involves hydroxylation by oxygenases, which are all stimulated by flavonoids, i.e., in this way, the flavonoids can contribute to the observed lowering of blood pressure.

Hypertension imposes a stress on the walls of the blood vessels, which the walls may not be able to withstand, especially if they are weakened by other diseases, e.g., atherosclerosis or diabetes mellitus (Tschopp et al., 1970; Pollock & Heath, 1975; Lean et al., 1999). The result is microbleeding or, still worse, aneurism, i.e., the formation of a rupture-prone expansion of a blood vessel. Such a rupture often leads to the death of the patient within a few minutes. The microbleedings, which may be difficult to detect, do not represent an immediate threat, but they must be stopped before the loss of circulating blood becomes dangerous, or the surrounding tissue is extensively damaged by components of the blood, e.g., proteases and phagocytes. Severe mental stress is an aggravating factor because it raises the blood pressure and increases the lesion, in which PLA_2 commences

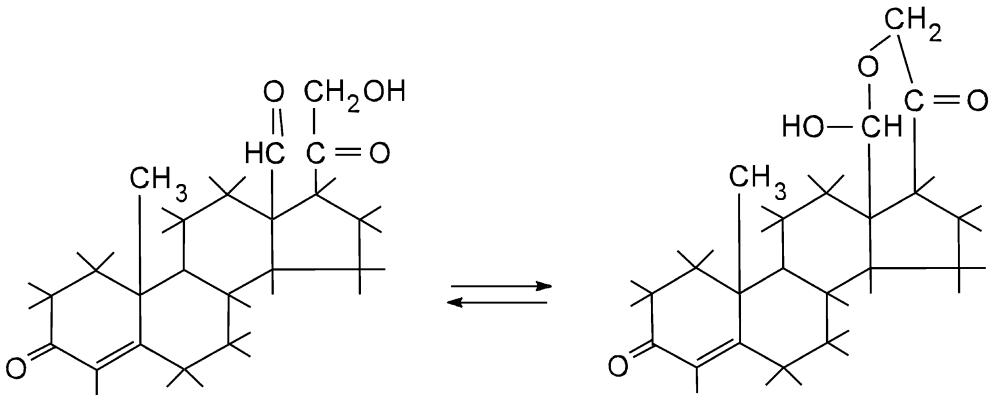


Fig. 57. Alternative structures of aldosterone. The decisive functional group is the aldehyde group.

the liberation of arachidonic acid. The latter compound opens K^+ channels, thus perturbing the local electrolyte homeostasis (Barbour et al., 1989; Kanai et al., 1995; Kim & Clapham, 1989). In addition, arachidonic acid is converted to PGs, etc., which genetically induce the biosynthesis of proteases, such as elastase and collagenases. The latter aid the repair process by hydrolyzing fibrous proteins, but simultaneously further weakens blood vessel walls.

One example of the effect of stress on the vascular system was the fate of a former chief executive in the United States, who, in the course of the Watergate scandal, suffered microbleedings. The microbleedings nearly caused his death because this disease can be very difficult to recognise. Finally, the diagnosis was made and a treatment commenced that saved his life. Flavonoids are very useful drugs for this purpose because they efficiently inhibit the key enzyme in PG synthesis, the PG COX (see Section 9; Kuehl & Egan, 1980; Sörensen & Hansen, 1970; Hammersen, 1972; Klemm, 1967; McEwan & McArdle, 1971; Niebes, 1972; Rish & Rodriguez, 1972). Besides, these compounds are so innocuous that they can be used for prophylaxis in cases in which microbleedings are suspected, but not yet demonstrable (Arturson, 1972).

Therefore, water-soluble, semisynthetic flavonoids are used widely in cases of hypertension, water disequilibrium, and microbleedings (Radouco-Thomas et al., 1964; Ten Cate et al., 1973; Sempinska et al., 1977). These substances are hydroxyethyl rutosides (Fig. 58). They are given intravenously.

17.2. Inflammation

Another prime example of the therapeutic application of flavonoids is inflammation (Van Cauwenberge & Franchimont, 1968; Brandao et al., 1997). This process is the integrated response of many defence systems of the body to the invasion of a foreign body of any kind, e.g., a wooden splinter, bacterial cells, or viruses (Hufford & Lasswell, 1978). Inflammation involves, among others, the action of the complement system, blood coagulation, humoral and cellular immunity, cytokines, tissue hormones, angiogenesis, and repair processes. Flavonoids kill many bacterial strains, but not all (Metzner et al., 1979; Mabry & Ulubelen, 1980; Schmidt et al., 1980; Cizmarik & Trupl, 1976). Some of these topics are still the subjects of intense investigation, and the scientific literature in this field could fill an entire library (Cao et al., 1997; Cao & Cao, 1999; Holden, 1999). The great interest in inflammation is due to the involvement of its components in many serious diseases, including cancer, Morbus Alzheimer's, and acquired immunodefi-

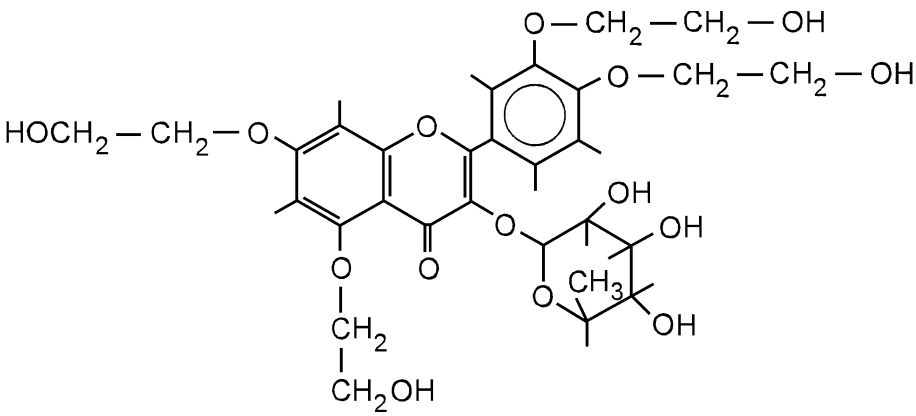


Fig. 58. The structure of a hydroxyethyl rutoside (quercetyl rhamnose).

ciency syndrome (AIDS) (Chen et al., 1999; Földi & Zoltan, 1970). In spite of the advanced level of complexity of this subject, we shall have to examine some of these biochemical processes in order to understand the curative effects of the flavonoids. An important mechanism in inflammation is the recruitment of macrophages to participate in the battle against invasion by microorganisms or their toxins. These inflammatory agents are recognised by specific antibodies mounted on the surface of the macrophages in a receptor (FcR). The toxin-antibody complexes are endocytised, and raise the alarm in the cell, with the result that the latter emits IL-1. This substance induces the expression of the COX-gene, which encodes the PG COX that produces the eicosanoids, i.e., signal substances for the pain pathway, chemotaxis, and smooth muscle contraction (Masferrer et al., 1994).

The treatment of a sore throat and fever with an ethanol extract of propolis is a classical example of a quick cure brought about by flavonoids. Usually, the fever disappears overnight, as does the erythema and the pain in the throat. As a rule, the patient then feels much better and can resume work.

Both the pain and the fever are the result of chemical signals from the point of invasion to the brain. These compounds, which release the alarm, are eicosanoids (see Section 9) formed from arachidonic acid by a series of enzymes, some of which, especially PG COX, are inhibited by flavonoids. The PGs are carried in the blood to the brain. Since they are lipids, they apparently can cross the blood-brain barrier. Some neurons in the midbrain carry specific receptors for PGs. As a result, an interneuronal communication takes place, by which granula containing substance P (P stands for pain) and bradykinin are released. The latter peptides diffuse to neurons responsible for the sensation of pain, whereas the PGs themselves bind to receptors on neurons responsible for the maintenance of normal body temperature. This thermostat is displaced, with the result being that the temperature of the body rises causing fever.

The swelling of inflamed tissue is due to osmosis. That is the phenomenon by which water-soluble substances incapable of penetrating a semi-permeable membrane are present in different concentrations on the two sides of the membrane. If the pores of the membrane are wide enough to allow water to pass, this substance will move from the dilute to the concentrated side of the membrane to establish thermodynamic and hydrostatic equilibrium. Normally, the plasma membranes of the cells are water-resistant, but in the case of inflammation, mechanical tissue damage, or hydrolysis catalyzed by virally induced enzymes, they are not (Földi & Zoltan, 1970; Földi et al., 1970; Földi-Börksök & Földi, 1975; Gabor, 1972a, 1972b). In the damaged cell membrane, among other enzymes, PLA_2 also becomes activated and releases arachidonic acid from the plasma membrane. As previously mentioned, this substance is converted into various eicosanoids (see Section 9), which with the help of yet incompletely identified signal pathways are able to induce the expression of the genes encoding elastase, collagenase, and other proteases (Medina et al., 1994; Negishi et al., 1995). These enzymes cleave structural proteins in the cell into peptides, which are too large to pass through the plasma membrane, but soluble enough to draw water through the loosened cell membrane by osmosis. Therefore, the cell blows up like a balloon. Hence, the swelling of the tissue. In the case of massive bacterial invasion into the blood, i.e., sepsis, the osmotic swelling can make an extremity, e.g., a leg, grow to grotesque proportions. This is especially true in Africa where it is often seen in elephantiasis. All of these phenomena hinge upon the formation of large amounts of PGs. They can easily be stopped by inhibition of the key enzyme by flavonoids (Casley-Smith & Casley-Smith, 1986).

An important weapon in the arsenal of the body defence is the immune system. It has two branches: the humoral and the cellular. Both of these branches are stimulated by flavonoids. The simplest of the immunities is the humoral. It consists of antibodies of all binding specificities. The antibodies, which are dissolved in the blood and, hence, reach almost all parts of the body, can be divided into the immunoglobulin (Ig) classes IgG, IgM, IgA, IgD, and IgE. IgM is only present in the initial phase of the immune response. After that, the production is switched to IgG, which quantitatively dominates. IgA acts in mucous membranes, particularly in the alimentary canal. IgE is directed against allergens, and will be discussed in Section 17.7. Antibodies against ~ 1 million different antigens are present in the blood. That covers all antigenic structures (called epitopes) that can occur. Each antibody can only bind one epitope, and is normally only present in a small concentration, but once an antigen has been recognised, the production of Igs of the same specificity is enormously expanded to cope with the challenge. Antibodies arrest antigens by binding and precipitation. In addition, they cooperate with the complement system, a cascade of proteolytic enzymes and membrane-penetrating proteins, to kill foreign cells, e.g., virus-infected cells and metastases. The antibodies mark an epitope on the surface of the attacking cell by binding (Figs. 59 and 60) and serve as guides and stabilizing

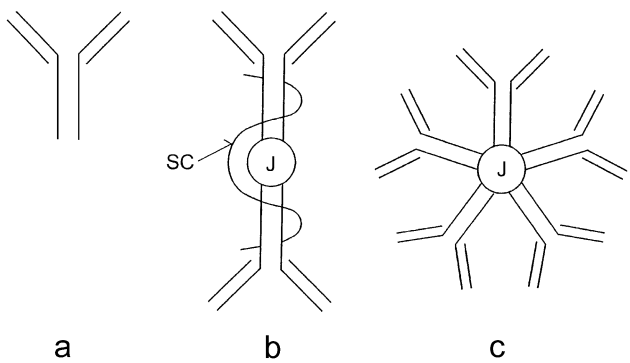


Fig. 59. **a**: IgG, IgD, and IgE. **b**: IgA. **c**: IgM. J, joining protein; SC, secretory component (a glycoprotein).

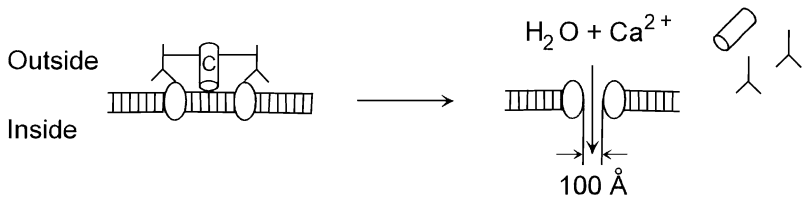


Fig. 60. Sketch of the attack of antibodies and the complement system on a cell displaying foreign antigens on the surface, e.g., viral proteins. Two IgG molecules (or one IgM) mark the target for the complement proteins, which at the tag aggregate to a hollow cylinder that nonenzymatically punches a hole of ~100 Å diameter in the target cell. Subsequently, the cell is exploded by osmolysis or killed by the influx of Ca^{2+} ions.

rigs for the complement proteins that punch a ca. 100 Å wide hole in the membrane of the target cell. Then water pours into this cell by osmosis and blows it up until it bursts. However, an even more important factor is the inflow of Ca^{2+} ions, which kills the cell by activation of protein kinase C (PKC) and Ca^{2+}-dependent proteases.

Flavonoids stimulate the production of antibodies in a yet poorly known fashion (Goodwin & Webb, 1980). However, it is likely that they do so by alerting cytokine production, since this is assisted by protein P-kinase cascades, which are known to be under the influence of flavonoids. Besides, flavonoids prevent the synthesis of PGs that suppress T-cells. The cellular immunity is more complex than the humoral. The main cells involved are macrophages, lymphocytes, and granulocytes. They communicate with the aid of cytokines. The latter have the properties of growth hormones, mitogens, and chemotactic attractors or repellents. They comprise among others at least 15 different ILs (IL-1 to IL-15), several IFNs, and various colony-stimulating factors. Most macrophages reside in the spleen and lymph nodes. They represent the first line of defence against invading antigens. The latter are carried by the circulating blood to the macrophages, where they are specifically recognised by antibodies mounted on the outer surface in receptors, which bind the Ig by the end piece, called the Fc-fragment, distal to the antibody-binding sites. Antibodies loaded with antigen rapidly diffuse along the cell surface to dimerise, and the dimer slides into a shallow cavity, called a coated pit, where it is trapped by an adhesive protein (clathrin) and endocytised. The endosome imports hydrolases and a proton pump from the Golgi apparatus, separates the receptors from the antibody-antigen complex, returns the receptors to the cell surface in a receptosome for reuse, starts the proton pump that acidifies the lysosome, and hydrolyses the antigen to fragments. This proton pump is inhibited by flavonoids (Showell et al., 1981).

Antigenic peptides are released into the cytoplasm, where they are picked up by nascent MHC Class I complexes as these emerge from the Golgi apparatus. An antigenic peptide of about 1 dozen amino acids is placed in a groove of the MHC complex, such that about one-half of it is protruding, and the loaded MHC complex moves to the plasma membrane, in which it is so lodged that the protruding peptide and part of the MHC complex are recognisable from the outside (for a review, see Kärre, 1995) (see Fig. 43).

The lymphocytes are divided into two classes, the T- and the B-lymphocytes. All of the lymphocytes originate from a stem cell in the bone marrow, but the T-cells are matured in the thymus (hence, the symbol T) and the B-cells mature in the *Bursa fabricii* equivalent (a chicken organ) in the bone marrow (hence the symbol B). The two classes of lymphocytes play quite different roles in cellular immunity. Whereas most T-lymphocytes circulate in the blood to detect antigens presented by APC (mainly B-lymphocytes, granulocytes, and macrophages), the B-lymphocytes reside in the spleen and the lymph nodes.

The T-lymphocytes are subdivided in T_4- and T_8-lymphocytes. The subindices refer to the presence of a characteristic plasma membrane protein, CD4 and CD8, respectively. The CD4 protein is an ion channel that is also the port of entry of human immunodeficiency virus (HIV) particles. Since the role of T_4-lymphocytes is to help B-lymphocytes to differentiate into plasma cells that produce antibodies, they are called T-helper or T_H-lymphocytes. Since they are the target of HIV, their transformation inhibits antibody synthesis. Hence, HIV patients are prevented from combating any antigens, including viruses with antibodies designed for the purpose.

All T-lymphocytes recognise antigenic peptides together with the MHC complex using its specific antigen receptor (see Fig. 43). This double recognition ensures that the presenting cell is endogenous and that the peptide is foreign, since all T-lymphocytes directed against self-antigens have been killed or inactivated (put into the state of anergy) during the fetal period.

The T8-lymphocytes are called suppressor T-cells (T_S), because they inhibit the helper function of the T4-lymphocytes. Important subclasses of T8-cells are the cytotoxic T-lymphocytes and natural killer T-cells (NK-T-Ly), which both belong to the first line of the immune defence. Both of these cell types kill foreign cells that have been tagged with antibodies. The cytotoxic T-cells activate the complement system, the components of which are synthesised by the liver and secreted into the blood. The enzymatic complement factors (the first stages in the complement cascade) are secreted by the liver as inactive precursors. Complement factor 1 can be activated by release of a peptide catalyzed by a variety of agents, including serine proteinases and properdin. Subsequently, it activates the complement factor 2, which, in turn, activates complement factor 3 and so

on, down to the factors in the late stages of the cascade, in which nonenzymatic proteins aggregate to form the hollow cylinder that stabs the target cell to death (Fig. 61).

NK-T-lymphocytes use not only a different pore-forming protein called porin, but also a variety of serine proteinases. Both cytotoxic and NK-T-lymphocytes are stimulated by flavonoids, but the mechanism is yet unknown. However, it is likely that their activation by cytokines is dependent upon protein phosphokinases, and the latter are known to be influenced by flavonoids.

The immune cells communicate with chemical signals called cytokines, most of which are proteins, but some of them may be eicosanoids and hence, directly subject to control by flavonoids. Most of these signals are positive, i.e., stimulatory, but some are negative, e.g., those from T_S- to T_H-cells. The immune alert starts at the macrophage that has caught an antigen on an antibody on its surface. Its Fc-receptor is noncovalently associated with a second-messenger-producing enzyme, phosphatidylinositol lipase (PIL), which liberates inositol phosphates and diacylglycerol (DAG) from the plasma membrane (Fig. 62). Several inositol phosphates activate specific protein phosphokinases, and DAG stimulates PKC. The activity of PKC is inhibited by flavonoids, most likely at the ATP-binding site. The result of the influence of flavonoids on casein PK and on protein phosphatase may be the initiation of several enzyme cascades, one of which induces the expression of the gene for IL-1. This substance is secreted into the blood and diffuses to T-lymphocytes, which bind these molecules to specific plasma membrane receptors. The latter are furnished with second messenger-producing enzymes that start P-kinase cascades to the cell nucleus, with the result being that the gene encoding IL-2 is expressed. IL-2, which is a mitogen, i.e., a substance-inducing cell division (mitosis), is secreted, diffuses to B-cells, and initiates their proliferation. IL-2 also has this effect on T-cells. IL-1 can also be formed by cells other than macrophages, e.g., by vascular endothelial cells and hepatocytes. A section of the complex network of the ILs is seen in Fig. 63.

The IFNs are important cytokines, the synthesis of which is stimulated by flavonoids (Sen & Lengyel, 1992). The protection offered by IFNs against viral attack is particularly important when the virus reaches the organ directly, e.g., the nose by an influenza virus infection, and not via the blood steam. The reason is that virus-induced IFN synthesis occurs quickly, whereas antibody formation requires considerable time (hours by repeated infection, weeks by the first incubation). Besides, the virus protection afforded by the IFNs is nonspecific, whereas antibody protection is highly specific. Hence, the virus can escape the antibody defense, but not the IFN attack by mutation.

IFN synthesis is induced by the presence of double-stranded RNA in a cell. Such RNA forms are present as intermediates in the replication of many viruses. The biosynthesis of IFN can also be provoked by injection of synthetic double-stranded RNA as prophylaxis. The action of IFN is at least two-pronged: direct inhibition of the translation of viral mRNA (Fig. 64) and the induction of an endonuclease, which specifically cleaves viral mRNA. The latter mechanism involves the induction of an oligo-2′,5′-adenylate synthase that is activated by double-stranded RNA. This enzyme catalyses the synthesis of oligo-2′,5′-adenylate, which acti-

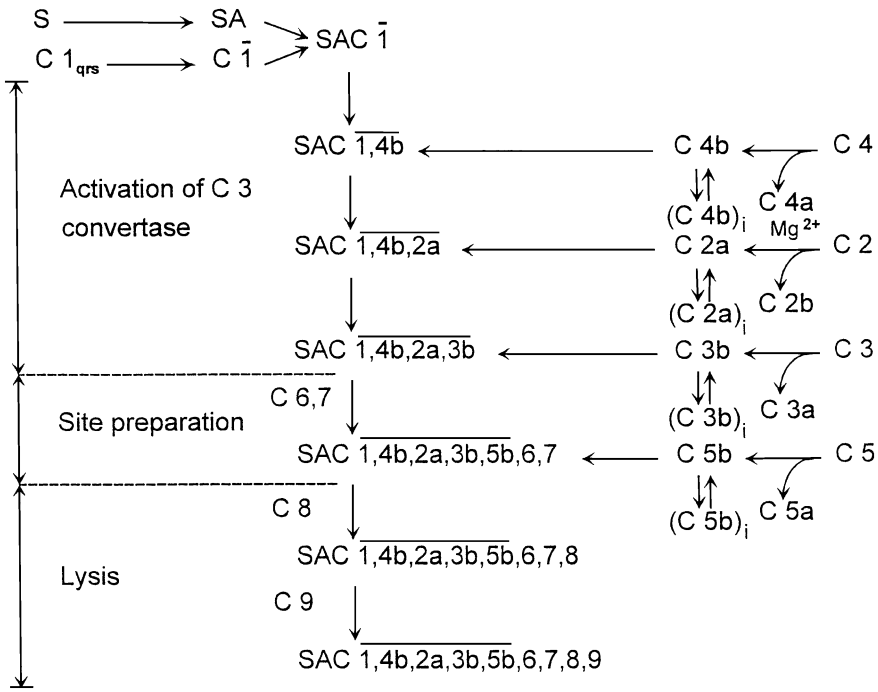


Fig. 61. Cell lysis by the classical complement cascade. A, antibody (two IgG or one IgM); C, complement factor; C1-4, activation of the lytic mechanism; C5-9, lytic agents; i, inactive; P, peptide; q, r, and s, chains of C1; S, site; SAC1, 4b, 2a, C_1-convertase; SAC1, 4b, 2a, 3b, peptidase.

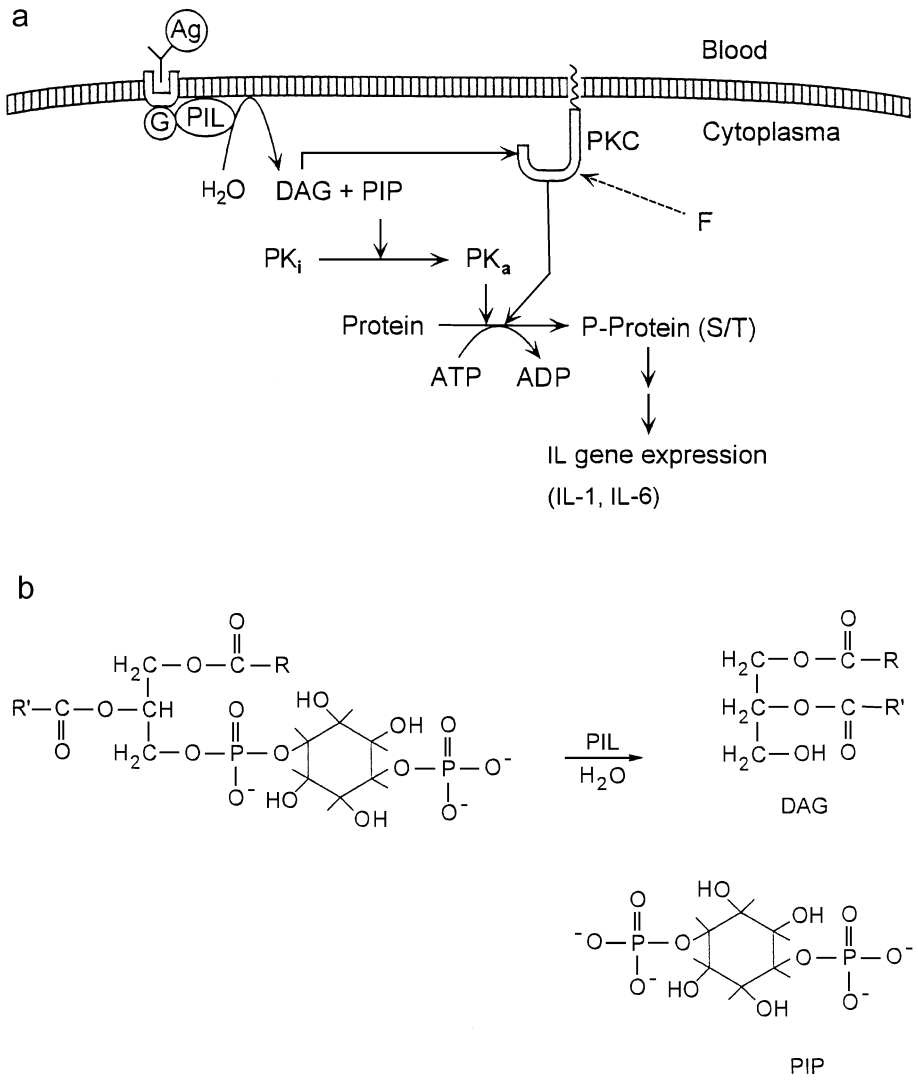


Fig. 62. **a**: Activation of a macrophage by the binding of an antigen (Ag) to an antibody mounted in a Fc-receptor on the outside of the plasma membrane. The receptor propagates an activation conformational change via the α-subunit of a G-protein (G) to an associated, phosphatidylinositol phosphate-dependent phospholipase (PIL) which liberates DAG, and inositol phosphate (PIP) from the plasma membrane. DAG activates from the plasma membrane phosphokinase C (PKC), which is anchored nearby with a myristate residue in the plasma membrane. PIP activates serine (S)- and threonine (T)-specific protein phosphokinases. Many of the substrates are protein kinases in enzyme cascades that terminate in the activating complexes at the cytokine genes in the cell nucleus. Especially the genes for IL-1 and -6 are subsequently expressed. Adapted from Gemsa and Resch (1991). **b**: Hydrolysis of phosphatidylinositol phosphate in the plasma membrane catalyzed by a specific phospholipase (PIL). F, flavonoid that inhibits PKC.

vates an endogenous endonuclease that degrades viral mRNA, thus preventing the synthesis of viral proteins (Fig. 65). The discovery of the IFNs initially gave rise to high hopes of their general usefulness in the therapy of viral diseases and cancer (for review, see Johnson et al., 1994; Pellegrini & Schindler, 1993). IFNs do have an antiviral effect, but have only proved to yield significant improvement in some special types of cancer, e.g., hair cell leukaemia and Wilm's tumor. The latter is a relatively benign children's kidney tumor, which usually can be treated successfully by classical means (surgery). Three types of IFNs are known: α-IFN, which is produced by T-lymphocytes; β-IFN, which is synthesised by fibroblasts; and γ-IFN, which is made by macrophages. Whereas γ-IFN is monomeric, the α and β varieties are homologous dimers (see Tables 4–6).

The biosynthesis of IFNs is induced by the entry of virus particles into macrophages, T-lymphocytes, and fibroblasts, but the mechanism is unknown. A likely possibility is that IFNs, together with other cytokines, are synthesised in immune cells in response to the general alert raised in macrophages when they suffer environmental stress, e.g., by a viral antigen (see above). The genes of related proteins, e.g., hormones or enzymes, are usually located on the same cistron and are expressed concurrently. Although no evidence of such an arrangement is known to the author, it is likely to apply to the cytokines, too. Another attractive hypothesis of the expression of IFN genes is that the viral genes directly or indirectly induce IFN gene expression because the uptake of double-stranded synthetic nucleic acids into a cell

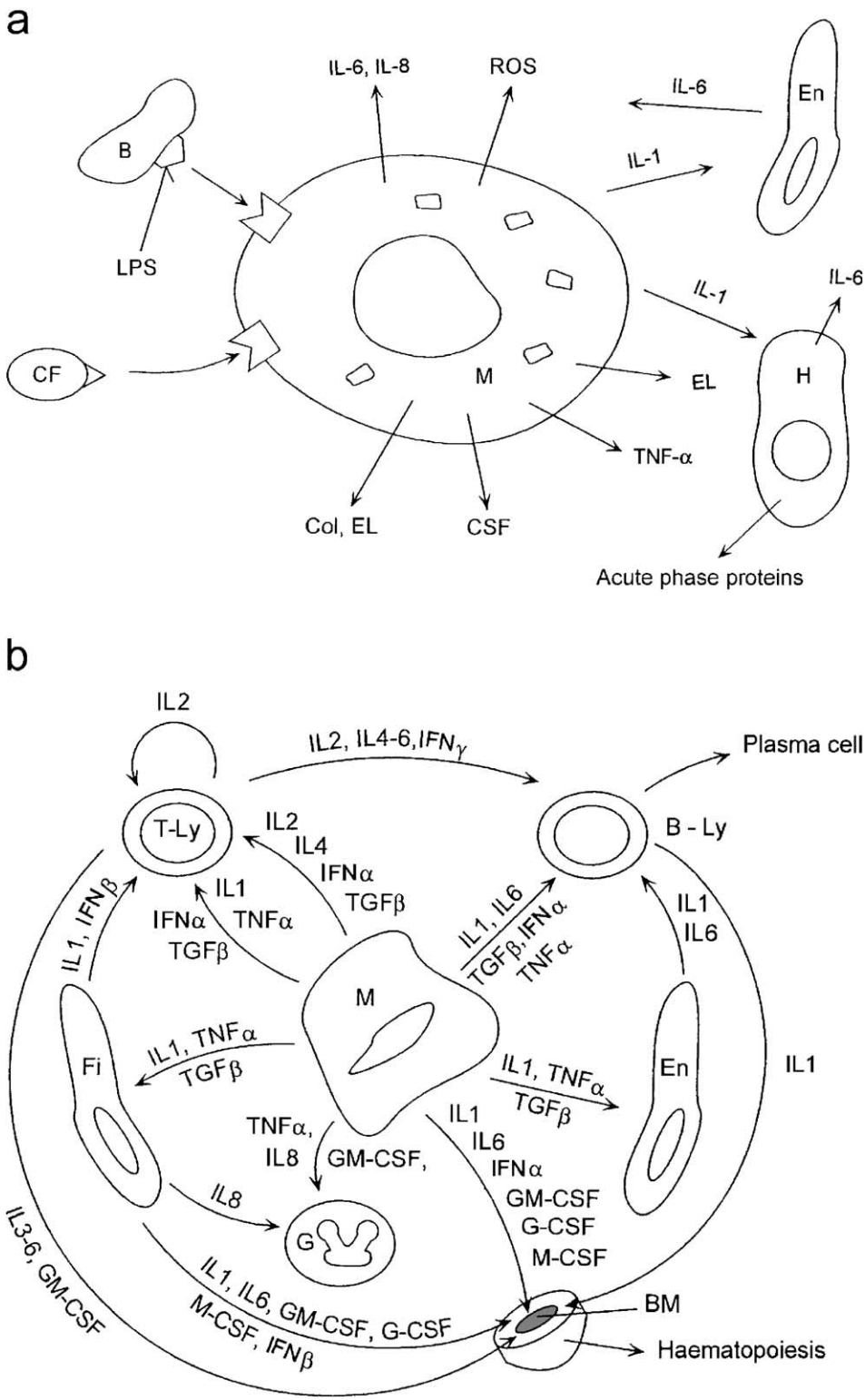


Fig. 63. Network of inflammatory cytokines regulating the synthesis of acute phase proteins. **a**: Dead bacterial cell exposing the endotoxin lipopolysaccharide (LPS). **b**: Major effects of cytokines. Flavonoid stimulates the cytokine production. Adapted from Flad and Gemsa (1991). BM, bone marrow; CF, complement factors 3a-9; Col, collagenase; EC, ecosanoid; EL, elastase; En, endothelial cell; Fi, fibroblast; G, granulocyte; H, hepatocyte; M, macrophage or monocyte; ROS, reactive oxygen species ($\cdot O_2^-$, H_2O_2, $\cdot OH$); TGF, tumor growth factor.

suffices to induce the IFN effect. An early event in the IFN-induced transmembrane signalling is the activation of PKC. This results in an increase in the concentration of the second messenger DAG (Yap et al., 1986). Flavonoids stimulate IFN synthesis in a yet unknown fashion (Mitrocotsa et al., 1999).

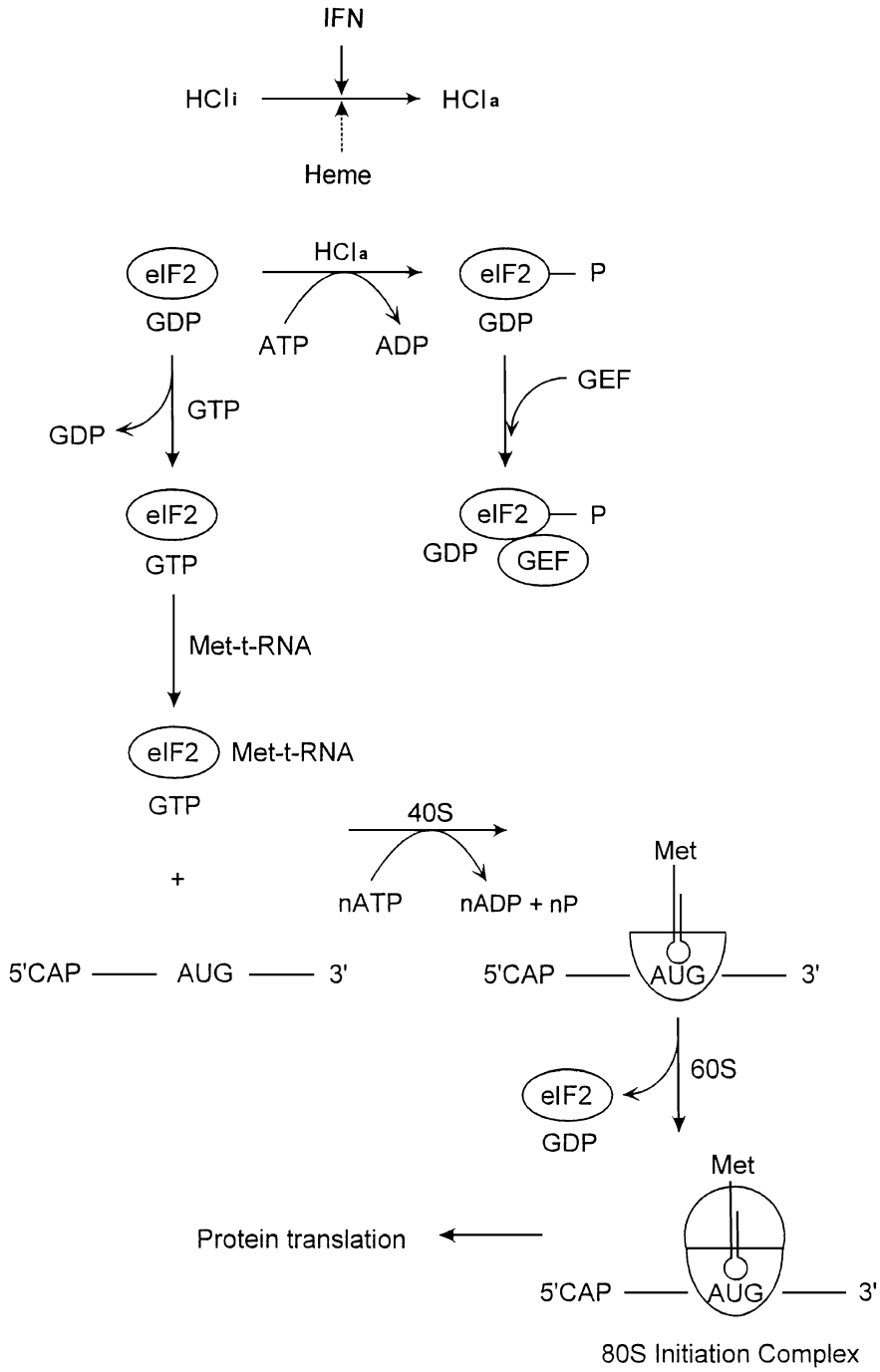


Fig. 64. Example of a mechanism of IFN action. IFN induces the activation of the heme-controlled inhibitor of protein translation (HCI). The latter catalyzes the phosphorylation of the α-subunit of eIF-2, thus permitting its binding of the guanyl nucleotide exchange factor (GEF) to form a dead end complex. Since the normal function of eIF-2 is to promote protein translation with the help of GTP, methionine-transfer-RNA (Met-t-RNA), mRNA, ATP, and the ribosomal subunits 40S and 60S, IFN stops protein synthesis by derailing the initiation process. Flavonoids promote the IFN synthesis. AUG, adenine-uracil-guanine; 5′CAP, 7-methylguanosine. Adapted from Stryer (1995).

IFNs operate in several different ways:

(1) The cell that is invaded by a virus particle is usually doomed, but it perseveres long enough to alert the neighbour cells by emission of cytokines, e.g., IFN. The latter cells respond by fortifying their plasma membrane, thus deterring the viral invasion.

(2) IFN or double-stranded nucleic acids (which can induce IFN synthesis) activate a nuclease that hydrolyses the viral genes (Fig. 65).

(3) IFN activates a protein phosphokinase that phosphorylates and thereby inhibits the action of eIF-2 (Fig. 64). The result is that protein translation in the cell stops. Therefore, newly formed viral genes can no longer be

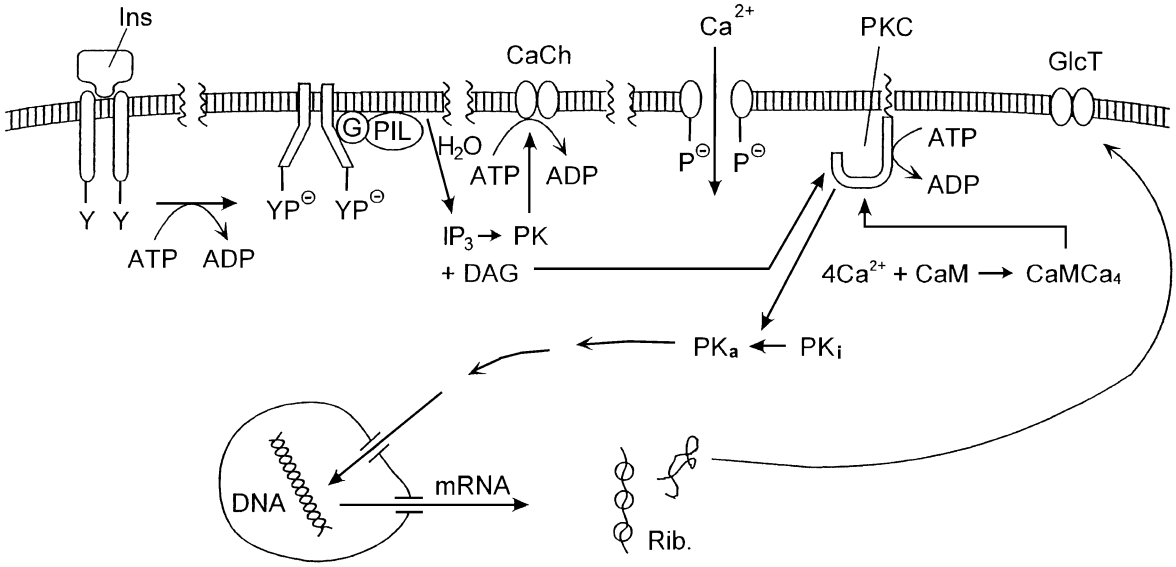


Fig. 65. **a**: Mechanism of IFN action. **b**: Synthesis of tri-2′,5′-adenylate catalyzed by oligo-2′,5′-adenylate synthase (oAS). A, adenine; dsRNA, double-stranded RNA.

covered by a protective protein capsid. Thus, viral propagation and is halted.

(4) IFN induces an antiviral soluble form of the LDL receptor (Fisher et al., 1993) (see Fig. 83).

IFN induces the activation of the protein phosphokinase home-controlled inhibitor of protein translation, which catalyzes the phosphorylation of the α-subunit of eIF-2, thus permitting its binding of guanyl nucleotide exchange factor to form a dead-end complex. Since the normal function of eIF-2 is to promote protein translation with the help of GTP, Met-t-RNA, mRNA, ATP, and the ribosomal subunits 40S and 60S, IFN stops protein synthesis by derailing the initiation process. Flavonoids promote the IFN synthesis (Stryer, 1995).

Inflammation of the liver can lead to jaundice, the presence of bile pigments in the blood. One of these bile pigments, bilirubin, inhibits the oxidative phosphorylation if it can enter the mitochondrion, but this effect is counteracted by flavonoids (Fritz-Niggli, 1968).

17.3. The effect of flavonoids on the condition of diabetes mellitus patients

Flavonoids cannot cure diabetes mellitus because most types of this disease are basically genetic and no single drug can correct an inborn error. However, flavonoids can ameliorate some of the consequences of diabetes mellitus (Vertounnen et al., 1994; Lean et al., 1999; Blostein-Fujii et al., 1999). Their effects are easier to comprehend if some characteristic features of this disease are reviewed first from a biochemical point of view. As the name of the illness suggests, anomalies in carbohydrate metabolism strikes the afflicted if the disease remains untreated. One of these is a high-glucose concentration in the blood and urine. However, diabetes mellitus also causes severe perturbations of lipid metabolism, which can lead to grave and often life-threatening physiological disorders. A major point of attack of the flavonoids is in this sector. Recent evidence also suggests that the anomaly in lipid metabolism is responsible for the dangerous turn of the disease. This may offer some hope for

Table 4
Structure and function of the IFNs

Property	Class I	Class II
Main types	α and β	γ
Other types	τ and Ω	–
Structure	Single chain	Homologous dimer
Source	α: virus-infected cells β: fibroblasts	T-lymphocytes
Effect	Inhibition of virus proliferation	Activation of cytotoxic and NK-T-lys

τ-IFN resembles α-IFN, but is less toxic.

Table 5
Activation of protein genes by IFNs

Proteins induced	Effect
2′-5′-A_n-Synthase	Polyadenylation
p68 kinase	Phosphorylation of p68
Indoleamine 2,3-dioxygenase	Tryptophan degradation
p56	Guanylate binding
GBP/p67	Inhibition of viral replication
Mx 1	
IRF 1,2	Transcription regulation
MHC	Antigen presentation
β_2-Microglobulin	MHC I assembly

Table 6
Diseases treatable with IFN and indirectly with flavonoids

Status	IFN-α	IFN-β	IFN-γ
United States Food and Drug Administration approved	Hepatitis B and C Genital papilloma Virus warts Hairy cell leukemia Kaposi carcinoma	Multiple sclerosis	Granulomatous disease
In clinical trials	Papilloma virus Throat warts HIV Myelogenous leukemia Non-Hodgkin lymphoma	Basal cell carcinoma	Kidney tumors Leishmaniasis

the improvement of the understanding of this disease and in its treatment, since, so far, the attention of medical research has been focused on the carbohydrates (for a review, see Efrat et al., 1994).

The two main types of diabetes mellitus are the juvenile form (Type I, insulin-dependent) and the adult form (Type II, insulin-independent). The juvenile type of the disease begins with an inflammation in the β-cells of the islets of Langerhans in the pancreas. This form of the disease is strongly genetically determined, and may have a viral origin. It is considered a form of autoaggression, i.e., the immune system fails to recognise self-antigens and turns upon its own host. Eventually, the complement-dependent cytotoxic T-lymphocytes destroy the β-cells that have been marked with antibodies. At first, the insulin production increases sharply due to overstimulation of β-cells, and then stops due to exhaustion of these cells. Therefore, there is a lack of inductor (insulin) for the expression of the gene that encodes the glucose transporter in the plasma membrane of all cells. Especially the muscles, the largest organ of the body, suffer from this condition. They lack substrate for the production of ATP, the general energy spender for all anabolic and power-producing processes in the body, and weaken. The patient becomes tired from even light bodily work and eventually the heart stops if the condition is not treated by insulin injections. Insulin, a proteohormone of ~ 50 amino acids, binds to a plasma membrane receptor, e.g., on a muscle cell. This receptor is on the cytoplasmic side of the membrane, a protein phosphokinase of the tyrosine-specific type. It phosphorylates itself with the aid of ATP, undergoes a conformational change, and activates via a G-protein (see Section 12) a PIL, which liberates several second messengers, e.g., inositol phosphates and DAG. The former activate specific protein P-kinases, which open a Ca^{2+} channel in the plasma membrane, thus causing a Ca^{2+} influx, whereas the latter activates (together with Ca^{2+}-loaded calmodulin) one of several PKCs, which starts a P-kinase cascade that propagates through the nuclear pore to the genes. As a result, the gene encoding the glucose transporter is expressed (Hume et al., 1979; Park et al., 1999). The latter soon appears in the plasma membrane, where it catalyzes glucose uptake in the cell from the blood (Fig. 66).

Flavonoids can stimulate an otherwise weak insulin effect in several ways, e.g., by influencing the protein phosphokinases.

When the cells fail to take up glucose from the blood, they resort to other mechanisms of procuring ATP: the glycogen stores in the muscles and the liver are phosphorolysed to glucose-1-phosphate, which is isomerised to glucose-6-phosphate and hydrolyzed to glucose for local demand. When this source of energy is depleted, the proteins are catabolised to amino acids, which are transaminated and desaminated to citric cycle intermediates and pyruvate that can be combusted via the respiratory chain. At first, the plasma proteins are consumed; then the muscle proteins follow; and finally, the proteins in major metabolic organs, especially the liver, are decomposed. Such patients attain a state of malnutrition and become sensitive to infection because they lack the immune proteins. Besides, they tend to develop oedema by osmosis.

The lack of insulin favours the lipolysis of triglycerides in the liver since triglyceride lipase is activated by cAMP, the decomposition of which by the cAMP PDE is stimulated by insulin (Fig. 67). The result is an increase in the concentration of FAs and glycerol. Both are decomposed to acetyl CoA since diabetic patients lack ATP for the reactivation of FAs for anabolism. Since the citric acid cycle has only a limited capacity to combust acetyl CoA, the excess is converted into ketone bodies (Fig. 68). High concentrations of these substances in the blood are typical of diabetes mellitus Type I.

The methylxanthines are components of beverages such as tea and coffee. Flavonoids also inhibit PDE and, therefore, like tea and coffee, should only be given to juvenile diabetic patients with caution.

Although some organs, e.g., brain and heart, can combust ketone bodies, less energy per molecule is derived from these substances than from glucose. Excess ketone bodies are deleterious to the organism because of their acidity. The resulting acidosis may kill weak patients. Hence, measures to restore the proton equilibrium must be taken. Since the flavonoids contribute to the normalisation of the water and electrolyte equilibria, their use could be contemplated.

Recent advances in the understanding of the nature of autoaggression deserve to be mentioned here since they open new approaches to the control of this common ailment. The failure of the T-lymphocytes of Type I diabetic patients to recognise a normal plasma protein in the β-cells of the Islets of Langerhans in the pancreas may mean that contrary to the rule, some of the fetal T-lymphocytes specific for self-antigens have escaped inactivation by anergy or death by apoptosis in the thymus, which normally occurs in the perinatal period. The antigens that cause autoimmunity are often superantigens because they sharply increase the immune response by forming a bridge between the MHC protein presenting the antigenic peptide and the T-cell

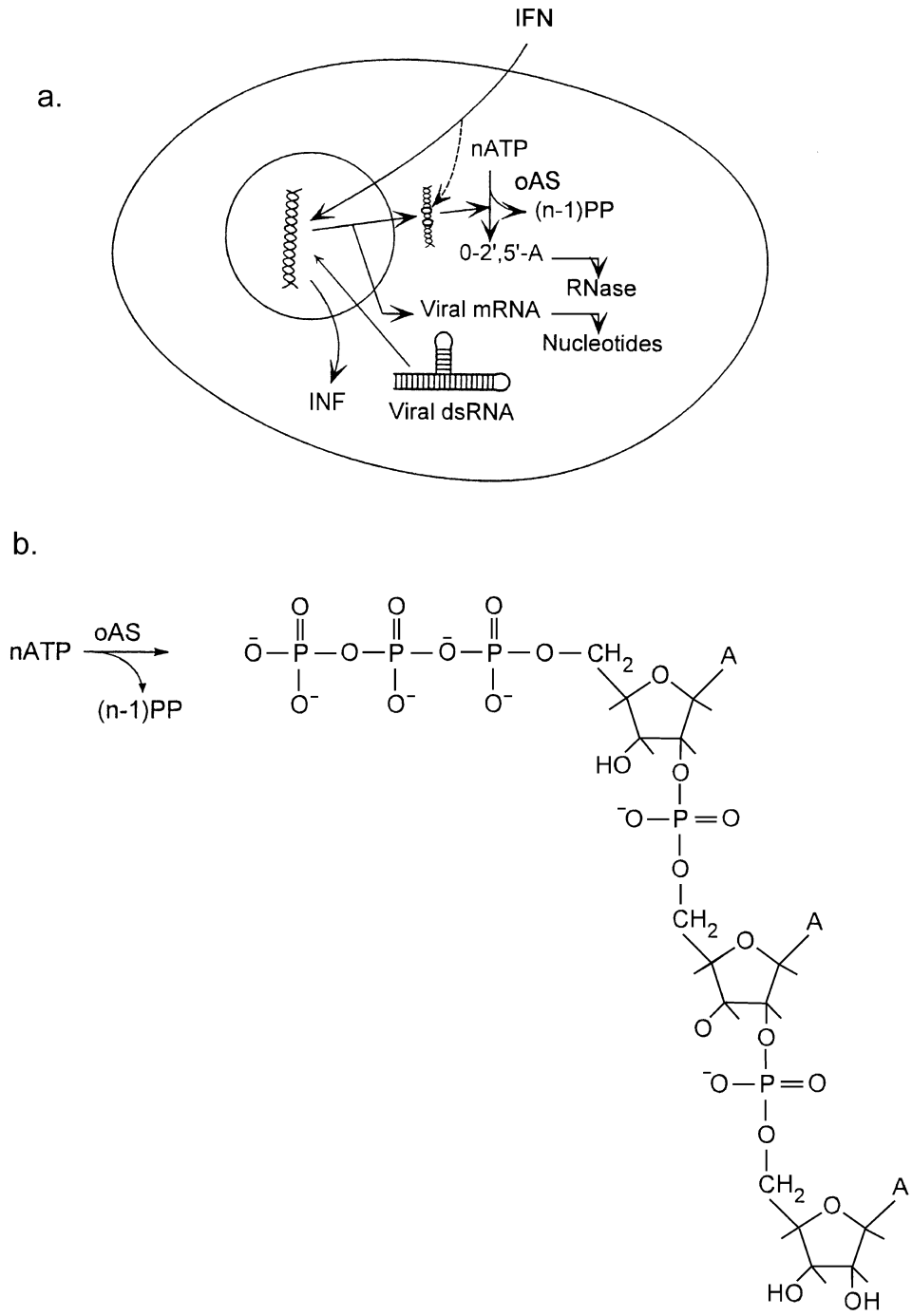


Fig. 66. Sketch illustrating the action of insulin (Ins) at the plasma membrane. A, adenine; CaCh, Ca^{2+} channel; CaM, calmodulin; G, G-protein; GlcT, glucose transporter; IP_3, inositol triphosphate; PK_i, inactive form of phosphokinase; PK_a, active form of PK; Rib, ribosomes; Y, tyrosine; Y-P, tyrosine phosphate.

antigen receptor (Fig. 69) (for reviews, see Rennie, 1992; Bjorkman & Parham, 1990).

The presence of a superantigen bridge between the two cells, in addition to the one provided by the antigenic peptide and the MHC, prolongs the half-life of the complex and, thus, increases the extent of activation of the T-cell. The result is a strong increase in the amount of IL-2 secreted from the T-cell and, hence, in the quantity of antibodies specific for the self-antigen produced by the expanded plasma cell clone. The superantigen may be the product of a gene that has suffered an inborn error, but the mechanism of the escape of the self-destructive T-cell from elimination in the thymus is not understood yet. A viral infection has been suggested.

Interestingly, the self-antigen, which causes pancreatic destruction in juvenile diabetes mellitus patients, recently has been identified as glutamate decarboxylase (Bäkkeskov et al., 1990). This membrane-bound enzyme converts glu-

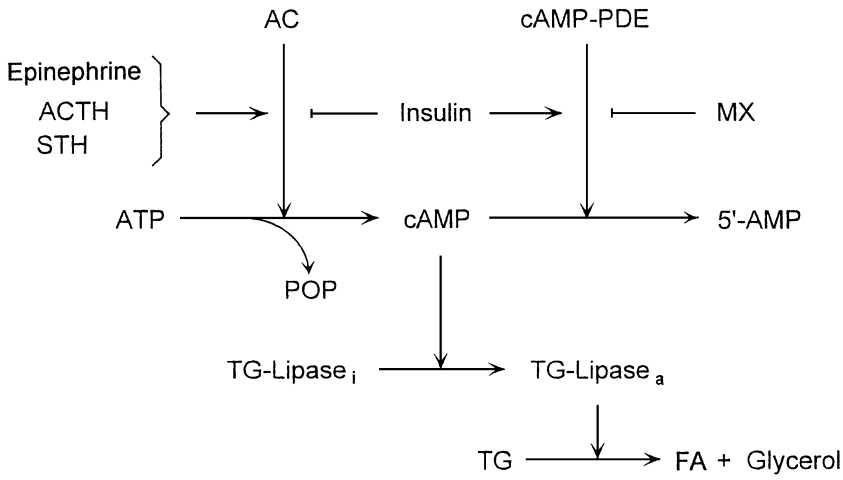


Fig. 67. Sketch of the regulation of lipolysis. →, activation; |−, inhibition; a, active; FA, fatty acid; i, inactive; MX, methylxanthines; POP, pyrophosphate; STH, somatotropin (growth hormone); TG, triglyceride.

tamate to the neurotransmitter γ-aminobutyric acid (GABA), which causes neuronal inhibition. Since the pancreas is furnished with both activating and inhibiting neurons, the action of which normally is balanced, the inactivation of the enzyme, which produces GABA by a self-antigen, causes an uncorrectable activation of the organ (Fig. 70). This hypothesis agrees well with the observation that insulin secretion in the early phase of diabetes mellitus Type I is strongly enhanced. The β-cells literally work themselves to death in order to obey the relentless command to produce insulin. Soon, insulin synthesis stops and complement-dependent cytotoxic T-lymphocytes, guided by the self-antibodies on the glutamate decarboxylase molecules, kill the remaining cells by osmolysis and Ca^{2+} ions, thus opening the way for macrophages, bacteria, viruses, etc., which cause inflammation. Thus, the insight into the pathogenesis of Type I diabetes mellitus has been greatly improved. Therefore, much thought is being given to use this knowledge for the development of new therapies for the disease. Since flavonoids are excellent inhibitors of inflammation by annihilating the free radicals of the oxidative burst and by throttling the production of the eicosanoids, which act as tissue hormones, their use in the early stages of the disease seems to be promising.

Type II diabetes mellitus, which is also called adult onset diabetes, is of a completely different nature than the Type I. It is not insulin-dependent, is provocable by environmental factors, e.g., stress, and is much easier to control than Type I. Therefore, Type II diabetes is rarely fatal in the short term, if it is well managed. This disease arises when the expression of the gene encoding the plasma membrane insulin receptor fails or if it is switched to a related gene encoding a deficient receptor. Since signalling pathways of the phosphokinase type exist, which are initiated by stress hormones, such as epinephrine or ACTH, and which ultimately derepress genes, the pathogenesis of Type II diabetes mellitus can easily be modelled. This disease is treated by a diet that avoids glucose and lipids. A reawakening of the silent gene is not feasible at present, but it is conceivable that a suitable inductor could be found. One of the flavonoids might accomplish this.

The most frequent cause of death by Type II diabetes mellitus is embolism, i.e., occlusion of arterioles or venules, e.g., in the coronary arteries or the brain. This blockage arrests the local blood flow, thus throttling the oxygen supply that within minutes can lead to cell necrosis and possibly to destruction of a major organ. Type II diabetic patients have a thick lipid layer lining the inside of the blood vessels. This layer often reduces the free diameter of the

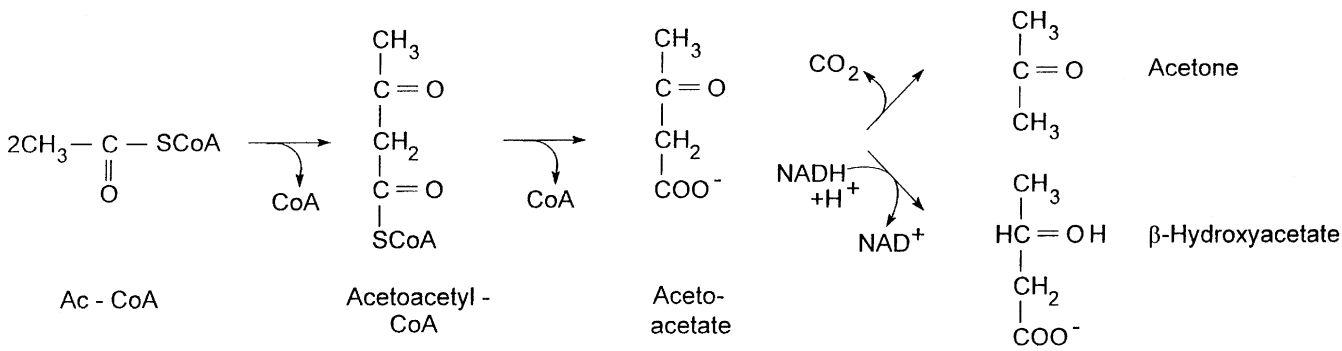


Fig. 68. The mechanism of formation of the major ketone bodies acetoacetate, acetone, and β-ketobutyrate. SCoA, coenzyme A.

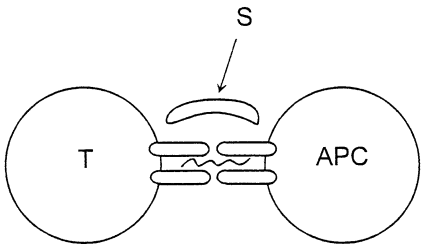


Fig. 69. Stabilization of the T-cell-APC complex by a superantigen (S), a large protein spanning the gap between the antigen receptor and the loaded MHC protein.

vessel by up to 50%. Macrophages in the connective tissue soon discover the anomaly and try to rectify the situation by engulfing some of the lipids. Understandably, that makes them sick, and in stress, they raise the alarm by secretion of IL-1 and other cytokines. The alerted immune cells respond as best as they can by the production of more antibodies against assorted antigens and by the activation of cytotoxic T-lymphocytes. The antibodies form insoluble antigen-antibody complexes and the cytotoxic T-lymphocytes damage endothelial cells lining the vessel, thus initiating the blood coagulation cascade that forms clots of fibrin and thrombocyte shadows (Fig. 71). Epinephrine binds to receptors on the endothelial cells, thus starting a signal chain that includes guanylyl cyclase and NO synthase. The former produces cyclic GMP (cGMP) and the latter NO. NO causes a relaxation of the smooth muscles, thus opening spaces for the entrance of blood cells into the vascular wall. This is the beginning of atherosclerosis, an inflammation that gradually spreads widely (for reviews, see Goldstein & Brown, 1990; Vane et al., 1994; Prince & Gunson, 1993). Like other inflammatory processes, it is treatable with flavonoids (Casalini et al., 1999; Borradaile et al., 1999; Kim et al., 1999; Kobuchi et al., 1999; Haenen & Bast, 1999; Lin et al., 1996). Carbon monoxide has also been suggested as a gaseous messenger molecule and neurotransmitter (Verma et al., 1993; Barinaga, 1993). The NO production is suppressed by flavonoids, e.g., in macrophages (Fushiya et al., 1999; Oldreive et al., 1998; Liang et al., 1999).

An important complication in Type II diabetes mellitus is the formation of cataracts. This process is aggravated by the action of aldose reductase, which reduces aldoses, such as glucose and galactose, to the corresponding hexitols. Since the latter are slowly catabolised and highly water soluble, they can attain high concentrations and exert a strong osmotic activity. Consequently, the hydrostatic pressure increases sufficiently to expand cavities in the lens and to create visual disturbances. This state is enhanced by back pressure due to the reduced lumen of blood and lymph vessels of diabetic patients, which is caused by lipid deposition. Aldose reductase is inhibited by many flavonoids. Hence, their use in ophthalmological clinics is increasing (Leuenberger, 1978; Kader & Sharpless, 1978; Varma et al., 1980; Chylack & Cheng, 1979).

17.4. Local anaesthesia by flavonoids

A beekeeper, wishing to convince skeptic bystanders of the local anaesthetic power of the flavonoids, placed a bee on the back of his hand, an area richly supplied with sensory neurons, and provoked it to sting, which it did. Then the bee was discharged and the beekeeper chewed a piece of propolis. Subsequently, he rubbed the flavonoid emulsion in saliva on the wound. After a few seconds, he claimed that the pain had completely disappeared.

The biochemical process responsible for the local anaesthesia is especially the inhibition of PG COX by the flavonoid (Liang et al., 1999). The catalytic mechanism of this enzyme is a free radical chain reaction involving a tyrosyl radical. The free radicals are scavenged by flavonoids. Further details of the mechanism are discussed in Section 9. The afferent pathway of pain sensation consists of PGs that are carried with the blood to the brain, and due to their lipid nature, easily pass the blood-brain barrier. Subsequently, they bind to surface receptors on neurons containing granula of substance P and bradykinin. These peptides are secreted and bind to receptors on other neurons, which create the pain sensation and somehow project it to the wound (Fig. 72).

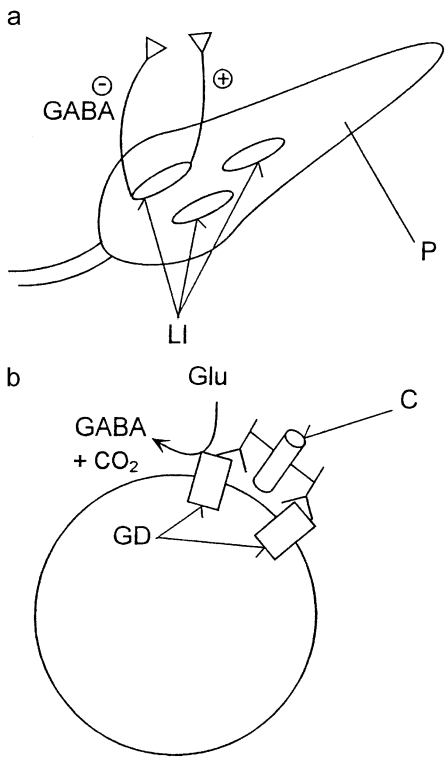


Fig. 70. **a**: Activating (+) and inhibiting (−) neurons influence the islets of Langerhans β-cells in the pancreas. GABA is a negative (i.e., passivating) neurotransmitter. **b**: Glutamate decarboxylase molecules in the plasma membrane in a β-cell catalyze the conversion of glutamate (Glu) to GABA, but is recognized by self-antibodies that guide the complement complex to the destruction of the cell. C, complement complex supported by antibodies penetrates the plasma membrane; GD, glutamate decarboxylase; LI, Langerhans islets; P, pancreas.

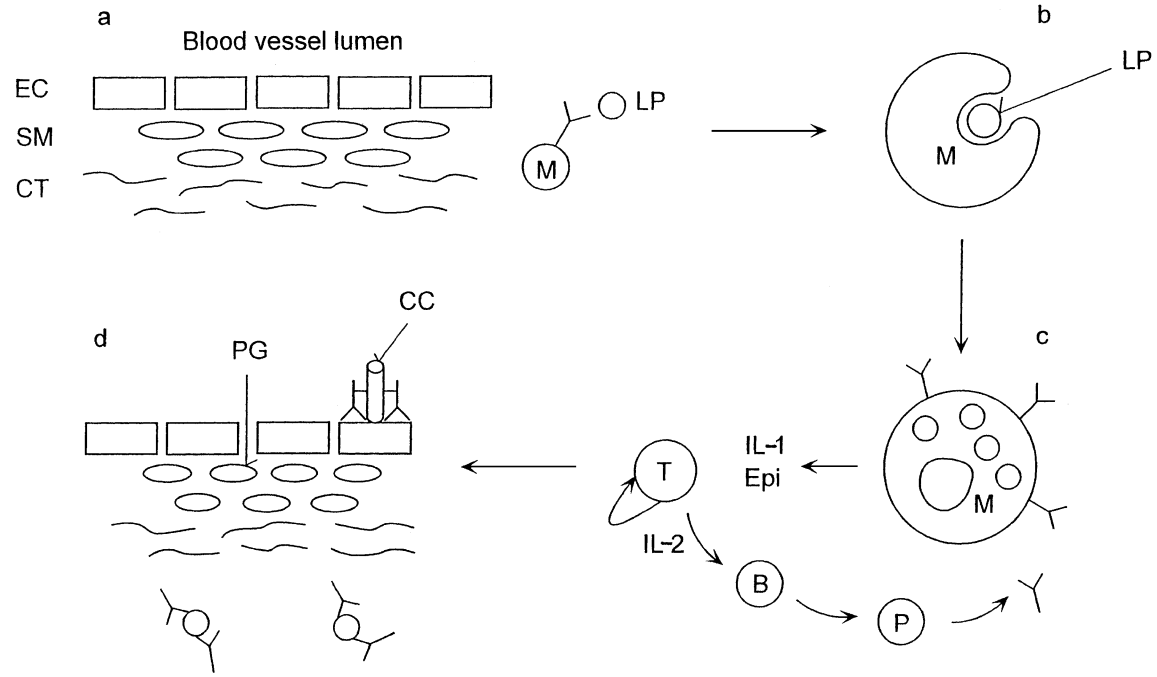


Fig. 71. Sketch illustrating the initiation of inflammation in blood vessel walls. **a**: Macrophage (M) recognizes lipoprotein (LP) by a surface antibody. **b**: M endocytices the LP. **c**: M, which is stressed by fat vacuoles, emits IL-1 and epinephrine (Epi). IL-1 induces IL-2 production in T-Ly, which leads to proliferation of T- and B-Ly. The latter differentiate to plasma cells (P), which secrete antibodies. **d**: The antibodies (Ab) form insoluble Ab-Ag complexes and the Ab mark endothelial cells for attack by the complement complex (CC). Epi binds to receptors on a smooth muscle cell that relax, thus loosening the tissue for invasion by inflammation-promoting cells attracted by chemotactic substances such as PGs. Another important atherosclerosis-enhancing reaction of macrophages to stress is the oxidative burst, the release of active oxygen species that oxidize unsaturated FAs in the membranes. The active oxygen species are free radicals that can be scavenged by flavonoids (Terao & Piskula, 1999). The latter also lower the content of cholesterol and LDLs in the blood. CT, connective tissue with fibrous proteins; EC, layer of endothelial cells; SM, layer of smooth muscle cells.

In Eastern Europe, flavonoids have been used in the treatment of pain for many centuries, and its use is still common practice by both lay and authorised medical practitioners.

Especially in oral surgery, the use of flavonoids for local anaesthesia is widespread (Sakagami et al., 1999). According to an Eastern European source, the anaesthetic potency of flavonoids corresponds to that of codeine, which belongs to the opiates. Unfortunately, that investigation was not secured by the modern standard of double-blind experimentation, random allocation, and controls. Hence, the evaluation is being repeated under optimal conditions. If flavonoids can replace novocaine and other opiate-based local anaesthetics that are currently used in Western dental clinics, then neurological side effects, such as hour-long dizziness after oral surgery, can be avoided. This would improve, e.g., the traffic safety since only few dental patients obey the order of the dentist to abstain from driving a car for an hour after surgery with local anaesthesia.

Another useful application of flavonoids is the treatment of periodontitis, an inflammation of the tissue that surrounds the teeth. This therapy is effective; rapid; easy to apply; inexpensive; and in contrast to the surgical procedure, painless. Also, this effect is now under reinvestigation using Western scientific standards.

17.5. Protein-rich oedema

Oedemas are caused by osmotic perturbations. The protein-rich oedemas are often dangerous. Such oedemas may arise as the result of sepsis, i.e., massive bacterial invasion into the blood. The bacterial cells contain glycolipids on their inner surface that are toxic (endotoxins). These toxins can start an inflammation of the blood vessel wall and other damaged tissue by arousal of macrophages, granulocytes, and vascular endothelial cells that respond by the secretion of cytokines, including eicosanoids, particularly PGs. The result can be massive blood coagulation, anaphylactic shock, and cessation of blood circulation, followed by rapid death. The bacterial cells also secrete toxic proteins called exotoxins, which have effects similar to those of the endotoxins mentioned above. When the bacterial cells are killed, e.g., by antibiotics, their toxins remain active and may even be liberated at a faster rate. This phenomenon limits the effectiveness of antibiotics against massive infections (sepsis).

The PGs bind to specific plasma membrane receptors, which, on the cytoplasmic side, contain protein phosphokinase domains. The latter start a signal chain that extends into the cell nucleus, where a cistron is derepressed that encodes a series of proteolytic enzymes, including elastase and collagenase IV. These proteases hydrolytically cleave

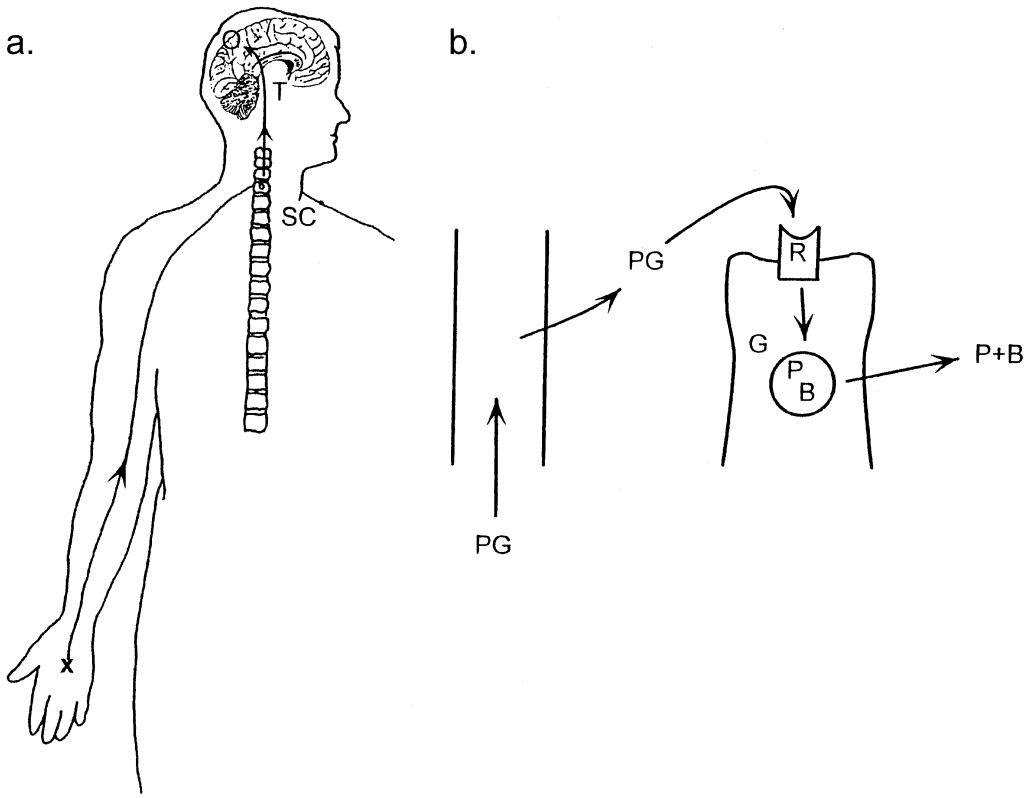


Fig. 72. The pathway of pain sensation. **a**: A bee stings the hand. Its venom contains PLA_2, which hydrolyses arachidonic acid from the plasma membrane of the damaged cell. PG COX converts arachidonic acid to PGs that are carried in the blood to the midbrain. **b**: PG passes the blood-brain barrier and binds to a specific receptor (R) on the surface of a neuron, which initiates a signal chain leading to the exocytosis of granula (G) containing the peptides substance P (P) and bradykinin (B). These neurotransmitters bind to specific receptors on other neurons, which create the pain sensation and project it to the wound. O, occipital lobe; SC, spinal cord; T, thalamus.

fibrous proteins in and around the cell, e.g., collagen, laminin, and fibronectin. The products are peptides of intermediate length, which, in contrast to the proteins from which they originate, are soluble. These peptides are too large to pass the plasma membrane, even after its lockering due to the proteolysis, but they are present in such high concentrations that they osmotically suck large amounts of water into the cells, which then blow up like balloons. Consequently, the tissue swells, sometimes to grotesque proportions.

This is the basis of the drastic disfigurations, which, e.g., are seen in elephantiasis. In that case, a parasitic invasion (by a trypanosome) has caused the inflammation and the subsequent formation of high-protein oedema. Such a condition is immediately life-threatening. Its treatment with flavonoids leads in the course of a few weeks to a striking improvement of the state of the patient (Casley-Smith, 1976; Casley-Smith & Casley-Smith, 1986; Tarayre & Laures-sergues, 1980; Sokolova & Liubartseva, 1979; Gerdin & Svensjö, 1983; Ambrose & de Eds, 1974; Wismer, 1963; Courbat et al., 1966; Sörensen & Hansen, 1970). The excess water is drained by the flavonoids from the swollen tissues into the blood and is excreted through the kidneys. The elastically expanded skin collapses to empty bags, which can be removed by the surgeon. By then, new skin has been formed underneath. The mechanism of regulation of the water homeostasis is described in Section 17.1.

17.6. Loosening of connective tissue

The healthy connective tissue is an effective barrier against the invasion of infectants. The loose connective tissue, which is involved here primarily, consists of a tight network of fibrous proteins, such as collagen, elastin, and chondroitin sulphate. The latter consists mainly of sulphated hexosamines and hexuronic acids arranged in long branched chains and supported by connecting peptide bridges. Dispersed in this mesh lie assorted cells, such as fibroblasts, mast cells, and macrophages, some of which produce the elements, proteins, and heteroglycans of the matrix. In the event of an invasion, e.g., of viruses, bacterial cells, or metastases, these intruders secrete enzymes, e.g., hyaluronidase, collagenase (here especially of the Type III), and elastase, which hydrolyse the polymers of the mesh, thus facilitating the spread of the infection. Hyaluronidase is a simple glycanase that somehow is inhibited by flavonoids (Kuttan et al., 1981; Blumenkrantz & Asboe-Hansen, 1980; Cetta et al., 1978; Zwillenberg-Fridman & Zwillenberg, 1972; Fine et al., 1992). Since certain of their features resemble those of monosaccharides, it is likely that the

flavonoids compete with the substrate for the binding site in the active site of the enzyme. In addition, the flavonoids prevent the loosening of connective tissue by scavenging for free radicals (see Sections 3.6 and 10), and by crosslinking elastin (Cu^{2+}-flavonoid complexes). In the case of the collagenases, we are dealing with metalloenzymes, with Zn^{2+} as an integral part of the catalytic sites. Since flavonoids form strong ligand complexes with heavy metal ions, it is highly probable that such complexes prevent the formation of the transition state. Thus, the flavonoids may be regarded perhaps as therapeutic substitutes of the tissue inhibitors of matrix metalloproteinases (TIMPs), which are so perverted by cancer cells that they bind to their target enzymes without inhibiting them (Khokha et al., 1989; Dowd et al., 1995; Chaumontet et al., 1997). However, their presence sterically hinders the approach of many other inhibitors (Fig. 73).

17.7. The effect of flavonoids on allergy and asthma

Allergy is an idiosyncratic disease. Almost everybody is sensitive to one or more allergens, e.g., to nickel in wrist watch bands, shrimps, strawberries, or pollen, but some individuals react much more violently than others to the same allergen. The reason is that the allergy-sensitive persons, due to their particular genetic constitution, produce some T-lymphocytes that overreact when they are presented with certain antigen structures. Hence, allergy is an inborn disposition to exaggerated immune response to very special allergenic provocations. The allergens are detected by IgE (Takei et al., 1988). They are mounted on the surface of mast cells of the loose connective tissue, especially in

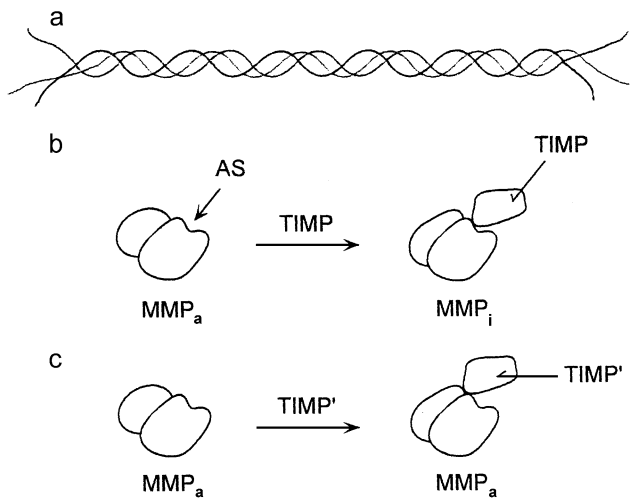


Fig. 73. **a**: Three-stranded, helical portions of a collagen fibril. The loose ends provide sites for the attack by collagenases, whereas the helical rod is rather resistant to enzymatic attack. **b**: Zn^{2+}-containing metalloenzyme inhibited by a molecule of a TIMP. **c**: Metastatic cells can produce TIMP variants, TIMP', that bind near the active site of the enzyme, thus fending off most other inhibitors, but leaving the active site operative. Thus, the mobility of the cancer cells is enhanced. a, active; AS, active site of the enzyme; i, inactive; MMP, matrix metalloproteinase; S, substrate.

connective tissue surrounding small blood vessels. These cells carry on their surface Fc-receptors called Fcε-R (see Fig. 75). The subsequent events take a course similar to the one described in Section 9 for antigens binding to antibodies on the surface of macrophages or B-lymphocytes: two allergen-bearing antibody molecules dimerise, and the dimer diffuses to the coated pit, which collects such items on the adhesive protein clathrin. The loaded coated pit is invaginated to an endosome, which subsequently sorts the receptor molecules from the antibodies and the allergens. The receptors are returned to the surface in a receptosome, whereas the remaining endosome transforms itself to a lysosome by importing hydrolases and a proton pump from the Golgi apparatus. The proton pump acidifies the lysosome, thus activating the hydrolases, which cleave the allergens and the IgE molecules into fragments. The latter are released into the cytoplasm or transferred to the trans compartment of the Golgi apparatus by fusion of the lysosome. MHC proteins that have been glycosylated and trimmed in the Golgi apparatus either pick up allergen fragments in the trans Golgi apparatus or fetch them in the cytoplasm and present them on the surface of the mast cell, where they are recognised together with the MHC protein by a T-lymphocyte with the aid of its antigen receptor. The T-lymphocyte responds by synthesizing and secreting large amounts of IL-2, which causes the clonal expansion of both T- and B-lymphocytes. The latter differentiate to plasma cells that produce large amounts of antibodies against the allergen.

In the mast cell, the tissue hormones histamine and serotonin are bound to the sulphated heteroglycan heparin. The recognition of the allergen somehow causes a conformational change in the heparin, perhaps due to a change in ionic strength or to neutralisation of the sulphate groups by Ca^{2+} ions entering the cell. The result is the abrupt release of a cloud of histamine and serotonin, which is secreted from the mast cell by exocytosis of granules. Simultaneously, but apparently in an event that is not directly connected with the exocytosis of the tissue hormones, a Ca^{2+} channel is opened in the plasma membrane of the mast cell and Ca^{2+} ions enter (Wilson et al., 1991; Alexandrakis et al., 1999).

Serotonin and histamine travel in the blood to specific receptors. Serotonin is a neurotransmitter that in some locations, e.g., the skin, causes capillary dilatation, i.e., reddening of the skin, and in other parts of the body, e.g., the frontal lope of the brain and the lungs causes vasoconstriction that gives rise to headache and apnea in the respective organs. Histamine binds to two types of receptors, H_1 and H_2, of which H_1 is present on the vagus nerve in the stomach, as well as on smooth muscle cells of several glands, e.g., the tear and sweat glands. As a result, gastric juice is secreted in copious amounts, as are tears and sweat. In addition, the airways in the lungs become extensively occluded with mucous of high viscosity.

The treatment of allergic diseases of the respiratory tract, including the lungs, with flavonoids is quite old (Bennett et al., 1981; Frostad, 1977; Easty, 1977; Middleton et al.,

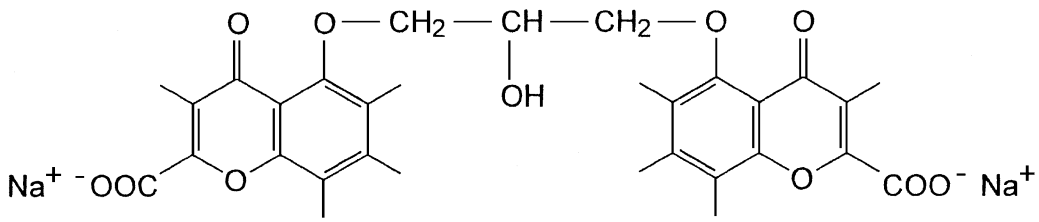


Fig. 74. The structure of disodium chromoglycate (Intal®).

1980, 1989; Middleton & Drzewiecki, 1982; Cheong et al., 1998; Ilek & Fischer, 1998). Already in the 1930s, the synthetic flavonoid disodium chromoglycate was introduced under the name Intal® (Fig. 74) (Frostad, 1977; Velcovsky & Federlin, 1977). This substance was administered as a nasal spray. The nasal epithelium is loose enough to permit the entrance of substances up to and including the size of nonapeptides, e.g., oxytocin and vasopressin. Besides, it is richly supplied with small blood vessels that are reached by the drugs. The latter are then carried by the blood to the target organ. Since the biosynthesis of eicosanoids is strongly inhibited by flavonoids and the leucotrienes, which are eicosanoids, they play an important role in the regulation of bronchial smooth muscle contraction. It is likely that the cells of the nasal epithelium possess surface receptors for leucotrienes. That would explain why flavonoids ease the breathing of patients suffering from allergic apnea (Fig. 75).

It has been observed that flavonoids inhibit the secretion of histamine and serotonin from mast cells that have been activated by allergens. In this way, the allergic symptoms released by these tissue hormones are prevented. However, the mechanism by which the flavonoids accomplish this is yet unknown. Since evidence has been presented that shows that PGs can open ion channels, e.g., K^+ gates, and can also participate in the fusion of membranes, a plausible working hypothesis can be constructed. Quercetin also inhibits the Ca^{2+} pump (Wuthrich & Schatzmann, 1980; Fewtrell & Gomperts, 1977a, 1977b; Bennett et al., 1981; Shoshan et al., 1980; Long et al., 1981), but it activates a Cl^- channel (Niisato et al., 1999). Another line of evidence emphasises other aspects of mast cell metabolism: an allergen crosslinks two IgE molecules mounted in Fcε receptors on the outer surface of the plasma membrane of the mast cell. The Fcε receptors, which on the cytoplasmic side of this membrane are noncovalently associated with the enzymes AC and methyltransferase, transmit an activating conformational signal from the allergen to the enzymes. The latter respond by producing cAMP and by transferring methyl groups to the phospholipids in the membrane. cAMP activates protein phosphokinases (Type A), which phosphorylate microfilaments and proteins in the granula membrane. The former contract, thus moving the granula toward the plasma membrane, whereas the latter rearrange to permit water influx into the granula that subsequently swell.

The additional methylation of the plasma membrane phospholipids causes a conformational change in the Ca^{2+} channels, with the result that Ca^{2+} ions enter the cell. These ions contribute to the swelling of the granula and activate Ca^{2+}-dependent PLs, especially PLA_2, which liberates arachidonic acid that produces eicosanoids under catalysis by COX and lipoxygenase. The other product, phosphatidic acid, participates in the fusion of the granular membrane with the plasma membrane. Subsequently, the granulas are exocytised into the blood, where histamine escapes in exchange for Na^+ ions (Fig. 75).

As mentioned in Section 9.1, flavonoids can act as antigens since antibodies against them have been found in

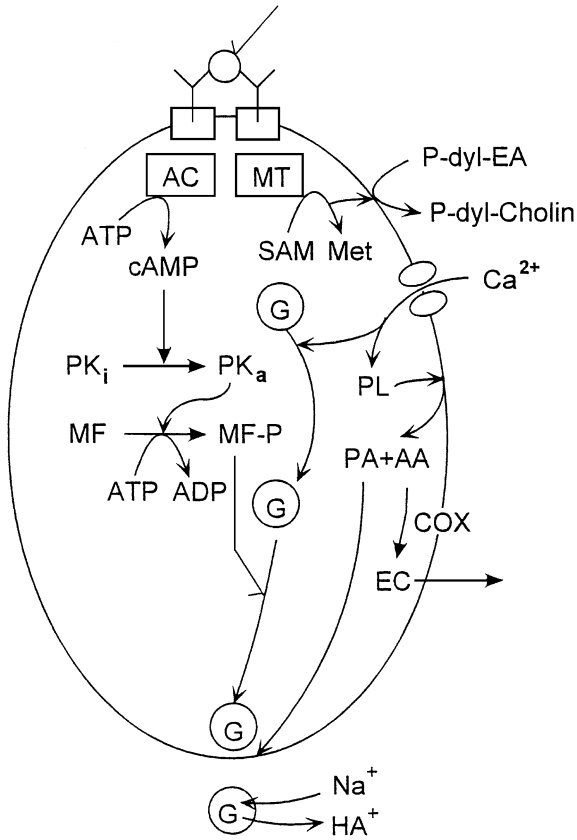


Fig. 75. Mechanism of the release of tissue hormones from mast cell granula. Flavonoids influence the process by inhibiting cAMP-phosphodiesterase and by annihilating free radicals, e.g. in the synthesis of eicosanoids. Adapted from Till and Thielmann (1989). AA, arachidonic acid; EC, eicosanoids; G, granule; HA^+, histamine; Met, methionine; MF, microfilament; MT, methyltransferase; PA, phosphatidic acid; P-dylEA, phosphatidylethanolamine; PK, protein phosphokinase; SAM, S-adenosylmethionine.

human blood. This antigenicity is only possible if the flavonoids bind to a carrier protein in the blood, probably mainly serum albumin. Propolis can also give rise to allergy in a small percent of the population, but this effect may be caused mainly by non-flavonoid components, e.g., pollen. A curious effect of orally administered flavonoids was found by Hollman and colleagues (Steerenberg et al., 1998). They found that the suppressive effect of UV radiation on skin contact hypersensitivity could be reinforced by many flavonoids.

17.8. The influence of flavonoids on cancer

17.8.1. The biology of cancer

Cancer is a group of diseases that are all caused by a disturbance in growth metabolism. The origin is, according to general consensus in the field, a combination of exogenous and endogenous factors, which step by step lead normal cells along the path of transformation to cancer cells (Umezawa et al., 1977). One of the endogenous factors is a genetic disposition that weakens the error control of the DNA transcription, thus enhancing the probability of mutations. One reason is that less efficient variants of the suppressor proteins, e.g., p53 and p16, are produced. The role of these proteins is to serve as brakes for transcription factors, which ensures that the cell cycle does not run so fast that the control and repair enzyme, a DNA synthetase, overlooks base pairing errors, e.g., covalent thymine dimers or chemically modified bases (Lake & Parke, 1972; Yamashita et al., 1999). p53 is actually a transcription factor inducing the expression of the p16 and p21 genes. The product of the latter, Δ, is an inhibitor of the cell division control protein phosphokinases at the G_1/S check point (Harper et al., 1993; Hosokawa et al., 1990). The repair enzyme moves along the DNA double helix, continuously checking that only the base pairs A-T and G-C occur. In case of faults, the enzyme reverses the direction of its movement and removes on its return trip the mononucleotides causing the error. Then, it moves forward again to close the gap with the correct bases (Fig. 76). This essential operation requires a certain amount of time, which is not available if the suppressor factors are deficient. Mammary carcinoma, retinoblastoma, and Wilm's tumor are carefully investigated examples of cancerous diseases in which the p53 suppressor gene has been cleaved. Therefore, the protein expressed has become too small to properly fulfill its assignment.

Flavonoids are also capable of influencing the growth regulation of human cells, but they must be encapsulated to be effective (Molnár et al., 1981; Buening et al., 1981; Lin et al., 1989; Bai et al., 1998). The example described in Section 6.2, the inhibition of the topoisomerase II by flavonoids, shows that quercetin and similar flavonoids can induce a mutation, a single-strand break (Podhajcer et al., 1980; Uddin, 1994; Aruoma, 1999; Astassi et al., 1985), that releases the operation of the repair enzyme (Austin et al., 1992). Most mutations are inconsequential because they hit unimportant points in the DNA sequence, a few are favourable and a few are harmful. The alert of the repair mechanism by a trivial mutation in fact may be favourable because the whole segment then receives a thorough check-up.

Cancer seems invariably to be associated with a derailment of the signal chains regulating growth metabolism (Shibata et al., 1991). Apart from the plasma membrane-associated enzymes, these chains consist almost exclusively of cascades of protein phosphokinases. In normal cells, most of these protein kinases are specific for the side chains of serine or threonine, whereas only a few of them are specific for the phenol group of tyrosine. In contrast, cancer cells have a strong tyrosine-specific protein kinase activity, especially in the cytoplasmic part of plasma membrane proteins, e.g., growth hormone receptors and transport ATPases. Racker and colleagues (Lang & Racker, 1974; Suolinna et al., 1975; Spector et al., 1980a, 1980b; Horisberger et al., 1991) showed that the Na^+/K^+-transport ATPase of a tumor cell line (HELA), in contrast to the enzyme in normal cells, was phosphorylated on tyrosine in the regulatory β-chain. HELA cells have been kept in

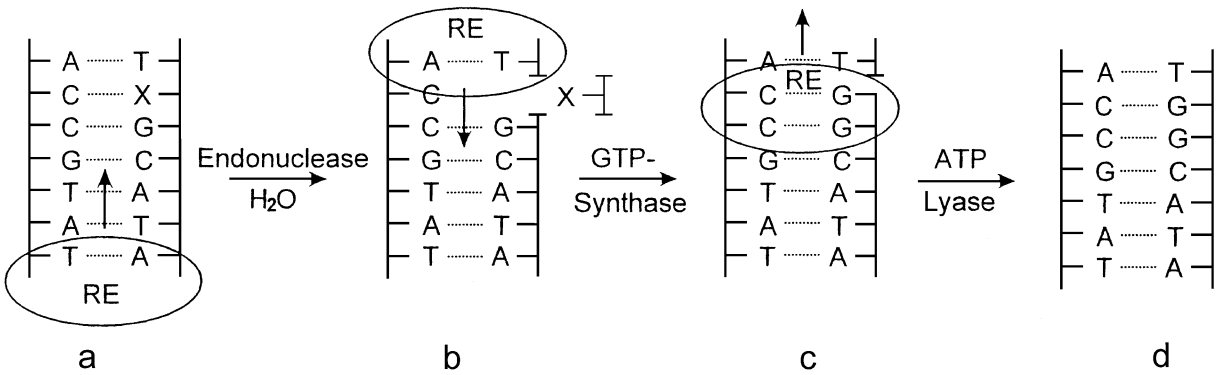


Fig. 76. Repair of a mutation (X, e.g., desaminated G) by the DNA polymerase. **a**: The repair enzyme (RE) moves along the base-paired double strand of the DNA and detects the failing hydrogen bridges between C and X. **b**: The RE slides back over the double strand to cut the faulty base out by hydrolysis. **c**: The RE moves forward again to incorporate the correct mononucleotide (GTP) in the chain by its polymerase action. **d**: A ligase has closed the single-chain gap, using ATP as the energy source.

culture for many years, and are used worldwide for studies of the biochemistry of cancer.

The Na^+/K^+-transport ATPase is an ion pump in the plasma membrane of most cell types. It pumps three Na^+ ions out of the cell and simultaneously takes two K^+ ions up from the blood. Thus, the pump keeps the concentration of Na^+ ions low inside, at the cost of metabolic energy from ATP. The pump is electrogenic, since more cations leave the cell than the number that enters. Unless the cell needs a gradient of the electric potential across the plasma membrane, e.g., for the translocation of substances, it must compensate by other ionic flows. All transport ATPases have in principle a similar structure. In the case of the mitochondrial F_1-ATPase, the active site resides mainly on the β-chain of the oligomeric enzyme. This chain, which is present in three copies arranged as a ring in the plane of the membrane, is visible in the electron microscope. It generates the necessary energy by hydrolysing ATP. The energy released is used to change the conformation of the Na^+/K^+-ATPase such that a Na^+ ion bound on the cytoplasmic side near the active site can enter the blood. The ATPase is activated by the binding of a K^+ ion on the blood side of the membrane. This ion is permitted to diffuse into the cytoplasm as Na^+ ions move in the opposite direction. Each α-chain is accompanied by a β-chain, which regulates the translocation by conformational changes produced by a phosphorylation reaction catalyzed by a protein phosphokinase. Normally, the latter is S/T-specific (S = serine, T = threonine), but after oncogene transformation, i.e., the outbreak of cancer, its role is taken over by a tyrosine-specific protein phosphokinase, with the result that the efficiency of the ion pump is reduced to 15% by tyrosine phosphorylation in the β-chain. Consequently, the Na^+ ion concentration and the ionic strength in the cytoplasm greatly increases. Some Na^+ ions probably are exchanged for protons at the plasma membrane because the pH value in the cytoplasm steeply declines, and many cells have such exchanger proteins. The pH drop is sharpened by the arrest of oxidative phosphorylation, which may be caused by the entrance of protons through the permissive outer mitochondrial membrane that is normally acidified by protons from the respiratory chain. This would lead to an initial burst of oxidative phosphorylation creating large amounts of ATP, which the cancer cells sorely need for their proliferation and subversive activities, but which may destroy the delicate mechanism. At any rate, most enzymes are inhibited by a high proton concentration, and the only remaining source of ATP is soon substrate phosphorylation, which in the glycolytic pathway produces lactic acid, because the coenzyme NADH must be reoxidised to NAD^+ by pyruvate to regenerate NAD^+ for another cycle of substrate phosphorylation at the level of glyceraldehyde-3-phosphate dehydrogenase. High lactic acid concentrations have been demonstrated in many carcinoma cells. Since substrate phosphorylation is an inefficient source of energy, and the cancerous activities have priority, the normal cell functions become starved of ATP and encounter an early death.

Racker and colleagues (Suolinna et al., 1974, 1975; Spector et al., 1980a, 1980b) showed that the flavonoid quercetin removes the phosphate ester from the phenol group of tyrosine (see Figs. 77 and 78). Subsequently, the Na^+/K^+-transport ATPase assumed its normal level of activity and pumped the excess Na^+ ions out of the HELA cells. The oxidative phosphorylation was revived and the pH value in the cytoplasm was normalised (see Figs. 79 and 80). Altogether, the cells showed all signs of having conquered

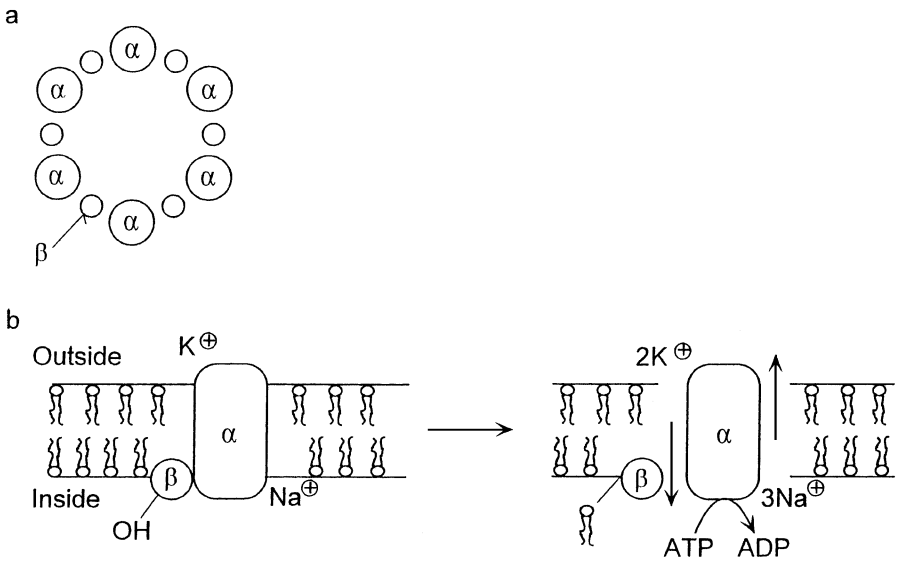


Fig. 77. Model of the Na^+/K^+ transport ATPase in the plasma membrane and a sketch illustrating its operation. **a**: Contour of a hexameric aggregate of α- and β-chains seen by electron microscopy or X-ray diffraction of two-dimensional crystals of the Na^+ pump. **b**: Regulation of the Na^+ pump by phosphorylation of the β-subunit catalyzed by a protein phosphokinase and operation of the pump driven by ATP hydrolysis. The resulting conformational change propels 3 Na^+ ions out of and 2 K^+ ions into the cell.

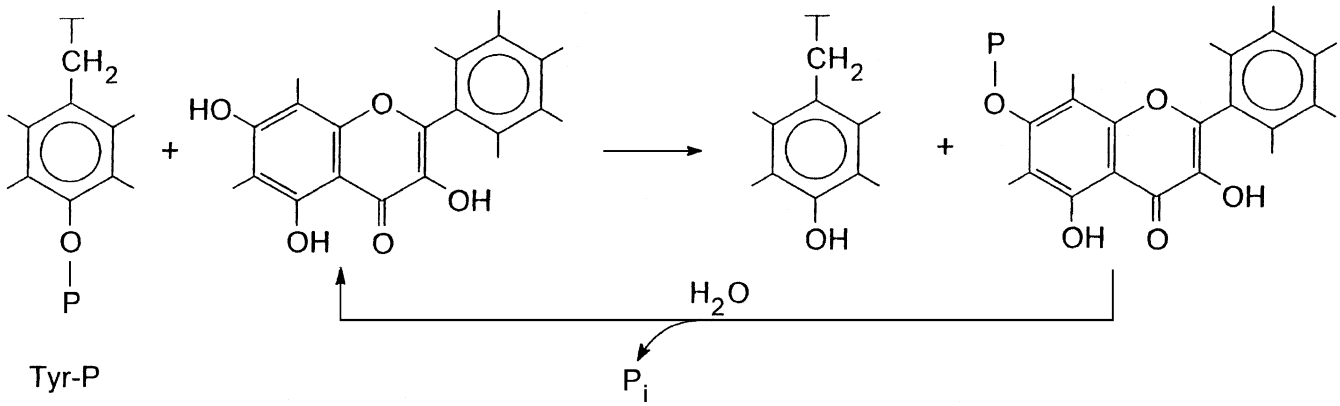


Fig. 78. Hypothetical mechanism of the quercetin effect on HELA cells. Tyr-P, tyrosine-phosphate.

the cancerous disease. Since the regulatory β-chain of the mitochondrial proton pump can replace the β-chain of the Na^+/K^+-ATPase of the plasma membrane, the two enzymes are structurally related. Therefore, the proton pump probably is also regulated by phosphorylation/dephosphorylation reactions that are sensitive to flavonoids (Bertorello et al., 1991; Horisberger et al., 1991; Kimmich & Randles, 1978; Salter et al., 1978; Akiyama et al., 1987). The successful treatment of metastasing cancer cells with a flavonoid derivative has been demonstrated in a mouse model with Warfarin (Ryan et al., 1968).

These experiments were performed in a cell culture, and similar work on experimental animals suffering from carcinoma, to the knowledge of the author, has not been published so far. They are awaited by many medical scientists with hope and anxiety. Unfortunately, Racker died before he could finish his work. Another effect of flavonoids on cancer cells is the inhibition of the glucose transporter in the plasma membrane, which furnishes such cells with glucose for glycolysis (Hume et al., 1979; Salter et al., 1978; Kimmich & Randles, 1978).

A possible mechanism of dephosphorylation of tyrosine phosphate by quercetin (Q) was followed by liberation of inorganic phosphate by hydrolysis. The model is based upon the higher acidity of quercetin hydroxyl groups than that of tyrosine (pK ~10). Phosphate esters are known to be sensitive to acid catalysis.

The formation of metastases by cancer cells is essential to the dispersion of the disease in the body and is a key event in its lethal progression. Tumor cells acquire their

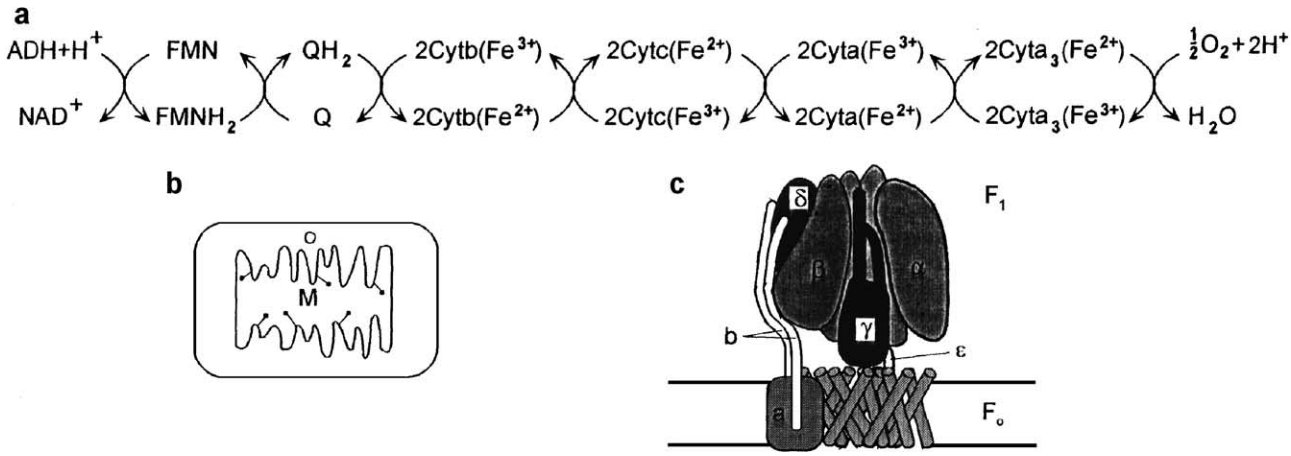


Fig. 79. **a**: The respiratory chain that generates energy for the synthesis of ATP by oxidative phosphorylation. Electrons flow along the chain from the left to the right and are ultimately captured by molecular oxygen, which forms water. The protons are carried by the lipid-soluble ubiquinol (QH_2) from the inside of the inner mitochondrial membrane, where the components of the respiratory chain are located, to the outer compartment (O) of the mitochondrion, where they are accumulated. Upon a yet unknown cue (possibly ADP), the protons are led back to the matrix compartment to drive ATP synthesis. **b**: Sketch of a mitochondrion. The ATP synthase is located on the inside of the inner mitochondrial membrane. It is seen as a rod terminated with a sphere. The outer membrane is penetrable to most small molecules, whereas the inner membrane excludes all substances, including protons, except through regulated pores or on special carriers. **c**: Model of the H^+ transport-ATPase (= ATP synthase). The stalk (F_0) is a hollow cylinder conducting protons, but closed with a regulated lid. The protons arriving at the ATP synthase proper (F_1) force the latter to change conformation, by which the active site on the α-chains (trimeric) becomes operative and synthesize ATP. The β-subunits are regulatory. The γ-subunit can rotate to activate the three active sites in turn. Flavonoids also regulate the activity of mitochondrial enzymes. Examples are hexokinase and ATPase (Graziani et al., 1977; Graziani & Chayoth, 1977). M, matrix or inner compartment.

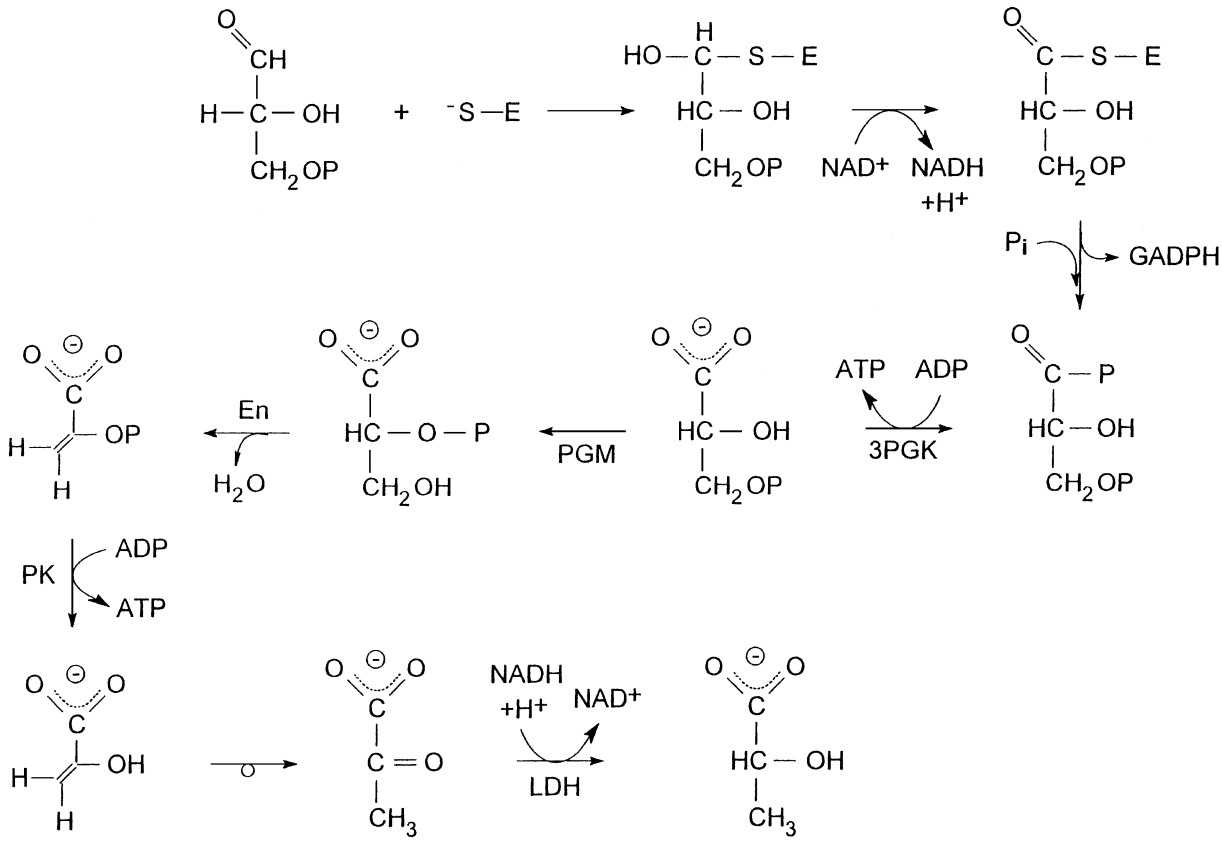


Fig. 80. Substrate phosphorylation in the glycolytic pathway and regeneration of NAD^+. Pyruvate kinase kinase is regulated by flavonoid-sensitive protein phosphokinases. E, glyceraldehyde-3-P dehydrogenase (GAPDH); En, enolase; LDH, lactate dehydrogenase; PGI, phosphoglyceroisomerase; PGK 3-P-glycerate kinase; PGM, phosphoglucomutase; PK, pyruvate kinase.

mobility by an enhancement of the expression of proteinase genes, e.g., those of collagenase and elastase. These enzymes hydrolyse the components of the extracellular matrix, e.g., collagen, but normally are opposed by endogenous protein inhibitors, especially by TIMPs. Unfortunately, cancer cells can subvert the action of the TIMPs by a mutation in the gene of the inhibitor that destroys the inhibiting, but not the binding, interactions, with the result that the hydrolytic activity is not only retained, but also sterically protected against the attacks of most other inhibitors, e.g., drugs. Only small inhibitors can reach the active site of the proteases, but rarely are they sufficiently specific. A possible solution of this dilemma is the administration of flavonoids, since such compounds can increase the resistance of collagen to hydrolysis by collagenases (Kuttan et al., 1981). The relevance of dietary flavonoids to nutrition and therapy has been reviewed by Hollman et al. (1997).

17.8.2. The treatment of cancer by flavonoids

17.8.2.1. Introduction. Few diseases are feared more than cancer because cancerous diseases, after cardiovascular disorders and accidents, kill more people before a normal life span has been reached. Besides, the progress of cancer is often accompanied by great pain and ugly disfiguration of the body. Yet, in principle, cancer is curable if it is discovered early and treated with the best current therapeutic methods. However, radical cancer cure is fraught with considerable life-threatening dangers, loss of organs, pain, and discomfort. Besides, its treatment is expensive. Hence, it is understandable that many cancer patients look for and try milder anticancer therapies that offer some promise of a moderate, long-term life-saving cure. The flavonoids are some of the most promising anticancer natural products that have been tried. Related synthetic substances, e.g., flavone acetic acid, have been subjected to Phase I clinical trials already, and they may soon become adopted into the general repertoire of cytostatic treatment (Plowman et al., 1986; Corbett et al., 1986; Zaharko et al., 1986; Ching & Baguley, 1987; Hornung et al., 1988; Wiltrout et al., 1988; Urba et al., 1988; Ryan et al., 1968; Hladon et al., 1980; Gerritsen, 1998; Smith et al., 1987; Fang & Casida, 1998).

Actually, flavonoids for a long time have been part of the herbal treatment by lay practitioners, but they were recognised only recently as effector substances. Examples of herbal preparations owing their growing recognition as effective anticancer drugs to flavonoids are propolis (Havsteen, 1979, 1983; Grunberger et al., 1988) and Essiac (Snow, 1996).

17.8.2.2. Types of cancerous diseases susceptible to flavonoid inhibition. The inhibition of the growth of cancer cells, both in vitro and in vivo, by flavonoids has been reported in many articles, e.g., by Graziani et al. (1983), Edwards et al. (1979), Kuriki and Racker (1976), Kandaswami et al., 1991, as well as Scambia et al. (1992). These observations pertained to both experimental cancerous diseases in animals induced by chemical mutagens (Markaverich et al., 1990) or viral transformation and to spontaneous human tumors (Ranelletti et al., 1992; Zhu & Liehr, 1994). Since the stepwise path of oncogenesis may depend on the cell type, the cancerous diseases, which proved to be inhibitable by flavonoids, are here in part classified according to the germinal layer from which the normal cells were formed during ontogenesis (Avila et al., 1994; Kupchan et al., 1978; Kupchan & Bauerschmidt, 1971).

17.8.2.2.1. Carcinomas largely consisting of cells of ectodermal origin. Carcinomas largely consisting of cells of ectodermal origin include the skin (Chang et al., 1985; Lama et al., 1998), breasts (Markaverich et al., 1990; Varma et al., 1977; Scambia et al., 1991; Adlercreutz, 1984; Makela et al. 1998), oral epithelium (Makita et al., 1996), the urogenital tract (Malaveille et al., 1998), and lungs (Malaveille et al., 1996).

17.8.2.2.2. Carcinomas largely consisting of cells of entodermal origin. Carcinomas largely consisting of cells of entodermal origin include the gastrointestinal tract (Ranelletti et al., 1992; Hosokawa et al., 1990; Yoshida et al., 1990; Adlercreutz, 1984; Garcia-Closas et al., 1999), mammary glands (Guthrie & Carroll, 1998), the gonads (Scambia et al., 1990a, 1990b, 1992), the uterus (Markaverich & Clark, 1979; Markaverich et al., 1981, 1983, 1988, 1989, 1990, 1992), lungs (Garcia-Closas et al., 1998; De Stefani et al., 1999; Knekt et al., 1997), the prostata (Griffiths et al., 1998, 1999; Denis et al., 1999), the colon (Deschner et al., 1993; Kuo, 1996), the rectum (Graham et al., 1978), and mesenchyme (Makino et al., 1998).

17.8.2.2.3. Tumors of mesodermal origin. Tumors of mesodermal origin include blood cells (Scambia et al., 1990a, 1990b; Varma et al., 1977; Larocca et al., 1990; Hoffman et al., 1989; Molnár et al., 1981; Graziani et al., 1983), bone (Shoshan & MacLennan, 1981; Bissery et al., 1988), and muscle (Shoshan et al., 1980).

17.8.2.2.4. Tumors formed by oncogenic viruses. Tumors formed by oncogenic viruses (Graziani et al., 1981, 1983, 1987). Although this survey cannot be complete, due to the large number of publications on the subject, which are not easily accessible, it is surprising that major classes of tumors, e.g., those of the liver, kidney, bladder, and brain, are not represented. The reason probably is not that such tumors are not susceptible to treatment with flavonoids, but rather, that the cancerous tissues are more difficult to keep in culture and to develop in animal models. Experimental therapy of human cancer patients with flavonoids has just begun (Bissery et al., 1988; Hofmann et al., 1988, 1990).

17.8.3. Biochemical processes of cancer influenced by flavonoids

17.8.3.1. Introduction. The plethora of effects of flavonoids on the metabolism of cancer cells is difficult to rationalise to a few basic, specific mechanisms. The flavonoids interfere with a large number of regulatory pathways, e.g., those of growth, energy metabolism, apoptosis, cell division, transcription, gene repair, neuronal transmission, inflammation, and stress response (Koh & Willoughby, 1979; Khayyal et al., 1993; Richter et al., 1999; Barinaga, 1996; Edwards et al., 1998; Habtemariam, 1997; Harper et al., 1993; Jager et al., 1998). They can act as antioxidants, free-radical scavengers, enzyme inhibitors, hormones (including neurotransmitters), antihormones, or inducers of gene expression.

These effects can be divided into two classes, the electronic and the steric. The high mobility of the electrons in the benzenoid nucleus of flavonoids accounts for both their antioxidant and free-radical scavenging properties, whereas the structural resemblance between the flavonoid aglycone and many substances inherent to the biochemistry of normal biological cells, e.g., nucleic acid bases, coenzymes, steroid hormones, and neurotransmitters, explains their inhibition of enzymes, cytoplasmic/nuclear hormone receptors, and neurotransmitters, as well as gene induction. An example of the latter is the induction of protease genes by coumarin during high-protein oedema (Piller, 1979; Radouco-Thomas et al., 1964; Casley-Smith, 1976; Abate et al., 1990). The high affinity of flavonoids for heavy metal ions provides additional opportunities for interference with the action of enzymes and Zn^{2+} fingers in DNA-binding proteins.

The biochemical pathways, which are influenced by flavonoids, can roughly be classified according to their sensitivities. Generally, the actions mediated by the family of cytoplasmic/nuclear hormone receptors are highly sensitive to flavonoids, whereas enzyme inhibition and similar processes, e.g., at synaptic membranes, require higher concentrations of flavonoids (Reiners et al., 1999).

17.8.3.2. Cytoplasmic/nuclear hormone receptors. The superfamily of cytoplasmic hormone receptors plays a central role in the transmission of steroid hormones and hormones of a similar structure and function to the genes. The hormones, which are carried by this superfamily of proteins from the cytoplasm into the cell nucleus, include steroids, thyroid hormones, vitamin D_3, and retinoic acid. Most of these hormones stimulate growth processes, but some of them also control specialised functions, e.g., electrolyte homeostasis, carbohydrate metabolism, sexual maturation, oxidative phosphorylation, and morphogenesis. All cytoplasmic hormone receptors are single-chain pro-

teins, which are folded into three domains. The N-terminal domain has a trans-activating function, a small centrally located domain binds to DNA, and the C-terminal hydrophobic domain binds the hormone. The structure of the flavonoids suggests that flavonoids also bind to the latter domain. This would explain the ability of various flavonoids, e.g., silymarin (Sonnenbichler et al., 1980; Sonnenbichler & Pohl, 1980; Conseil et al., 1998; Das et al., 1987a, 1987b; Markaverich & Clark, 1979; Kitaoka et al., 1998), to simulate the action of certain hormones.

Since the ligand affinity of the hormone receptors is very high, it is understandable that flavonoids, which are exerting their effect via cytoplasmic hormone receptors, have a much more sensitive influence on the metabolism of the cell than those that are low-affinity inhibitors of enzymes and plasma membrane receptors. The inhibitory effect of flavonoids on the growth of human thyroid cancer cell lines has been demonstrated by Yin et al. (1999).

17.8.3.3. Enzymes. A large number of enzymes involved in oncogenesis are influenced by flavonoids. Most of these enzymes are inhibited by flavonoids, but there are also examples of enzymatic processes that are enhanced by these substances, e.g., the reactions of some phosphatases and oxygenases (see Section 12).

In the case of the phosphatases, the activation seems to be due to the binding of the flavonoid to a heavy metal cofactor, e.g., Zn^{2+}, whereas the effect on oxygenases probably is indirect, e.g., caused by the reduction of a coenzyme, especially folic acid (to tetrahydrofolic acid). Since the pathological effects of flavonoids are insignificant, these substances seem to inhibit pathological enzyme variants preferentially or to favour repair and defense mechanisms. For the purpose of discussing the role of flavonoids in oncogenesis, it is convenient to pay special attention to three branches of metabolism: growth, energy, and detoxification.

17.8.3.4. Growth regulation. The derailment of growth metabolism is a common feature of all types of cancer. The two known pathways for the transmission of environmental signals to the genes are the steroid path via the cytoplasmic receptor (discussed in Section 13) and the protein kinase cascades. The latter are typically started by the binding of protein hormones and mitogens to receptors in the plasma membrane. The events immediately following the binding of the hormone to the receptor include a conformational change across the membrane-activating PLs, GTPases, adenylyl cyclases, and protein phosphokinases. The role of the GTPases is to activate cyclases and PILs, as well as to regulate ion channels. The role of adenylyl cyclase is to produce cAMP, which, in turn, activates a class of protein phosphokinases. The protein kinases phosphorylate either the phenol group of tyrosine (tyrosine phosphate is a high-energy compound) or the alcoholic amino acid side chains of serine or threonine.

Tyrosine phosphorylation is especially important to growth metabolism. It is particularly extensive in oncogenesis. The protein phosphorylation cascades terminate at the transcription complex on the genes and cause an activation of gene expression. Several enzymes, transcription factors, and cell cycle components are activated by phosphorylation, including DNA polymerase II, topoisomerase, and the cell division control protein kinase at the G_1-S check point.

Some flavonoids inhibit tyrosine-specific protein kinases (Levy et al., 1984; Srivastava et al., 1998; Gansauge et al., 1997; Huang et al., 1996), topoisomerases I and II (see Section 12), as well as the cell division control protein kinases. The result is that growth metabolism, including cell division, is slowed. This effect supports the claim of the slow tumor ablating effect of flavonoids (Grunberger et al., 1988). Such a moderate cancer treatment does not seem to agree well with the acute threat to the life of the patient, but it has the distinct advantage that the concentrations of the toxic catabolites of the tumor do not exceed the capacity of the hepatic detoxification mechanisms.

Many flavonoids also inhibit the cAMP and cGMP PDEs (Beretz et al., 1978, 1986; Ruckstuhl et al., 1979). The result is that the cAMP level is raised. Thus, the normal protein kinase chains, which are enhanced by cAMP, may be favoured over the pathological signal chains that, to a large extent, operate via tyrosine phosphorylation. An important enzyme in several protein kinase chains is PKC. This enzyme, which in the resting state is bound to the plasma membrane, is liberated by a Ca^{2+}-activated protease, calpactin. An enzymatically active fragment of PKC, PKM, moves into the cell nucleus to participate in gene activation by phosphorylation of transcription factors. PKC is inhibited by several flavonoids, e.g., quercetin.

Hence, cell growth is slowed. Quercetin neither competes with Ca^{2+} calmodulin nor with 12-O-tetradecanoyl-phorbol-13-acetate for their binding site on PKC, but rather with ATP, since the latter interaction is known from other kinases (Gschwendt et al., 1983; Nakadate et al., 1984; Kato et al., 1985; Chang et al., 1985).

17.8.3.5. Energy metabolism. The demand for ATP by a rapidly growing tumor is high and soon exceeds the supply, which the transformed cell can provide. The major producer of ATP, the respiratory chain, soon fails, possibly due to lack of oxygen, replacement proteins, or citric cycle intermediates. However, glycolysis seems to be more responsive to the oncogenic demands because the metabolic flux through this pathway is increased after regulatory phosphorylation of some of the key glycolytic enzymes. The resulting high rate of production of lactic acid causes a considerable pH drop, which favours lysosomal digestion and inhibits many enzymes. In addition, the oncogenic protein kinases phosphorylate the regulatory β-chain of the Na^+/K^+-ATPase in the plasma membrane, with the result that its activity is drastically lowered (Kuriki & Racker, 1976; Shoshan & MacLennan, 1981; Umarova et al., 1998). Hence, the Na^+

ion concentration in the cytoplasm rises and the ADP concentration drops. Thus, the uptake of glucose, which is driven by a Na^+ ion concentration gradient, is diminished and the efficiency of substrate phosphorylation is reduced. Racker and colleagues (Spector et al., 1980a, 1980b) showed that the addition of a flavonoid, e.g., quercetin, caused the removal of the inhibiting phosphate residue on the β-chain of the Na^+/K^+ pump and the restoration of normal cell function. This effect may be the result of the activation of a protein phosphatase, e.g., by the interaction of the flavonoid with a heavy metal ion cofactor or of the inhibition of a protein phosphokinase (Böhm & Lamprecht, 1959). Most of these experiments were carried out in a cell culture of cervical carcinoma cells, but deserve to be repeated on tumors of living animals. The gastric H^+/K^+-ATPase can also be inhibited by flavonoids (Murakami et al., 1999).

17.8.3.6. Detoxification. Environmental toxins with oncogenic activity, e.g., aromatic hydrocarbons and free radicals, are detoxified by oxidation. The hydrophobic toxins are mostly taken up by lipoproteins and carried to the liver, where they are endocytised. The hepatocytes have a marked ability to express catabolic enzymes in response to the uptake of xenogenic substances. The aromatic carcinogens, exemplified by benzo(a)pyrene, possess a structure suggesting that they can bind to cytoplasmic receptors and be transported into the cell nucleus, where they are likely to induce transcription, e.g., of cytochrome P450 isoenzymes. The latter, and other oxygenases, begin the attack on the aromatic nucleus of the toxin by hydroxylation. These free radical reactions pass through an intermediate epoxide stage in which the toxin can add amino groups of nucleic acid bases, especially the one of guanine. Such alkylation products of the bases suffice to deter the reactions of chromosomal DNA to the extent that the repair mechanisms are overpowered. The result is a mutation that is often followed by the oncogenic transformation of the cell. Flavonoids are known to inhibit neoplastic transformation (Franke et al., 1998).

Flavonoids are structurally sufficiently similar to the hydroxylated aromates to induce the oxidative enzymes, but cause, in addition, the expression of other enzymes, e.g., epoxide hydrolases (see Section 12). The latter enzymes hydrolyse the aromatic epoxides to vicinal diols. Such structures are suitable points of attack of ring opening enzymes. Thus, the flavonoids can facilitate the breakdown of the dangerous polycyclic aromates to relatively safe small decomposition products, e.g., aromatic carbonic acids, that can be excreted with the urine (Bresnick & Birt, 1988; Lasker et al., 1984; Wargovich et al., 1985; Das et al., 1987a, 1987b; Brown et al., 1998). Therefore, smokers need a particularly high content of flavonoids in their diet.

17.8.4. Stress response

17.8.4.1. Mechanisms of stress response. Humans experience a wide variety of physical and psychological stresses from the environment. Some of the more common physical and chemical stress factors are temperature; physical insult; gas concentrations (e.g., O_2 and CO_2); electromagnetic radiation; noise; antigens (e.g., pollen); and dietary components that are irritating due to their acidity, alkalinity, or specific interactions with receptors. Stress, e.g., hyperthermia, is also used in cancer therapy because some types of cancer cells are especially vulnerable to heating (e.g., locally to 45°C for 1 hr), particularly when the treatment is combined with ionizing radiation and cytostatic drugs (Amstad et al., 1991; Applegate et al., 1991; Crawford et al., 1988; Kuntz et al., 1999).

The stress response occurs along several interconnecting pathways, including nervous induction of the catecholamine synthesis, as well as the release of corticoliberin and subsequently of ACTH, which stimulates the production of corticoids in the adrenal cortex. These steroid hormones induce the expression of a variety of genes, including heat shock proteins (HSPs). The latter consist, among others, of chaperonines, inhibitors of steroid binding to cytoplasmic receptors, and glycolytic enzymes. The function of HSPs is to support cell survival by facilitating refolding of denatured proteins, by preventing the overshoot of stress responses, by stimulating lysosomal hydrolysis, and by increasing ATP synthesis. In spite of their name, the HSPs are not only produced in response to thermal stress, but should rather be regarded as a general mechanism of protection against stress. Some of the more prominent HSPs are HSP-90, HSP-70, HSP-117, HSP-40, and HSP-27. The following biochemical functions have been assigned to these proteins: HSP-70 and HSP-90 form a heterodimer that binds cortisol in competition with the cytoplasmic steroid receptor, thus modulating the stress response.

HSP-70 stimulates lysosomal hydrolysis. HSP-90 binds the tyrosine-specific protein kinase, with the result that the cell cycle is slowed and the thermal sensitivity is decreased. Thus, HSP-90 protects cancer cells against hyperthermic therapy.

Since flavonoids resemble catecholamines structurally, they could be expected to interfere with the biosynthesis of these typical stress hormones. Flavonoids actually influence this process, but indirectly, because important enzymes of this pathway are hydroxylases (Hosokawa et al., 1992). Such enzymes need THF as a coenzyme, and THF must be kept in the reduced form by ascorbic acid, which, in turn, is protected from oxidation, e.g., from reactive oxygen species by flavonoids (see Section 10 and Clemetson & Andersen, 1966). In addition, the activity of key enzymes in catechol anabolism is regulated by phosphorylation/dephosphorylation reactions catalyzed by protein kinases and protein phosphatases. Members of both of these enzyme classes are regulated by the binding of flavonoids (see Section 12.3).

17.8.4.2. The inhibition of heat shock protein genes by flavonoids. The specific cytotoxic effect of flavonoids on

cancer cells, which, among others, was observed by Racker and co-workers (Kuriki & Racker, 1976), has been ascribed in part to the induction of apoptosis by these substances. However, observations have also been made of an opposing reaction, which was interpreted as the effect of the products of stress response genes, especially of those encoding HSPs (Wei et al., 1994; Hosokawa et al., 1992; Kudo et al., 1999; Janoutova et al., 1996). The HSPs include chaperonins, e.g., HSP-70, GroEL, and GroES; glycolytic enzymes; and components of the cytoarchitecture. They provide a cell in crisis with energy (ATP) and with catalysts for the refolding of denatured proteins. Thus, the HSP, the expression of which is not only caused by heat shocks, but also by other kinds of perturbation (pH changes, osmotic stress, toxins, etc.), offers the cell a chance of survival, in spite of unfavourable environmental circumstances. However, the effect of HSPs and quercetin (or similar flavonoids) is opposing because quercetin favors apoptosis and HSPs support survival. A previous treatment of the cell with such compounds suppresses the expression of the HSP-70 gene. Hence, the probability of the survival of the cell after a heat shock is drastically lowered. This new insight opens an interesting opportunity for improving the efficiency of the hyperthermic treatment of cancer patients. Hyperthermia is often one component in a multiple-step therapeutic procedure that also includes overloading of the cells with glucose and acidification by hydrolytic products of cell-membrane penetrating substances, e.g., dinitrophenylacetate. It seems, but has yet to be proven, in patients that pretreatment with quercetin would raise the thermal sensitivity of the tumor, thus rendering it more susceptible to the auxiliary agents, acids, and locally applied cell toxins (Koishi et al., 1992; Kim et al., 1984; Li & Werb, 1982; Landry et al., 1982; Johnston & Kucey, 1988; Zimarino & Wu, 1987; Kane et al., 1993; Kawaii et al., 1999a, 1999b; König et al., 1997; Kuntz et al., 1999; Miller et al., 1996; Moyano et al., 1996).

The mechanism of inhibition of the HSP gene expression has yet to be elucidated in detail. However, the specific inhibition of the formation of HSP mRNA by quercetin has been demonstrated (Hosokawa et al., 1992). Hence, inhibition occurs at the level of transcription or earlier because quercetin, flavone, and genistein did not inhibit RNA polymerase II in all cell types tested under a variety of experimental conditions (Nose, 1984; Ono et al., 1990; Simuth et al., 1986). Experiments with specific oligonucleotide probes directed against the initiation sequence (F1), the central section (F2), and the terminal portion of the structural gene of HSP-70 (F3) confirmed that cells pretreated with quercetin, in contrast to untreated cells after heat shock (43°C, 1 hr), hardly expressed the HSP gene. In another experiment, the involvement of quercetin in the activation of the human HSP-70 gene was studied by transfecting cells with a 2.4-kb sequence containing the chloramphenicol acetyltransferase (CAT) gene placed upstream of the HSP-70 gene.

Likewise, a sequence containing the human HSP-70 promoter gene, including the heat shock regulatory element (HSE) was inserted in front of the structural HSP-70 gene in other cells. When the transfected cells were heat shocked in the absence of quercetin, they displayed a high CAT activity, but the latter enzyme activity did not exceed the level in cells that had not been exposed to the heat shock, if the cells had been pretreated with quercetin before the heat shock. This inhibition was studied by a gel mobility shift assay of the heat shock factor (HSF) using extracts of cells heat-shocked in the presence or absence of quercetin. The HSF protein interacts directly with the HSE sequence in the HSP-70 promoter, thus inducing the expression of the latter. The probe used to detect the DNA-binding activity of the HSF was a [^{32}P]-end-labelled oligonucleotide from the HSE segment: HSF was strongly expressed after a heat shock in the absence of quercetin, but not after pretreatment of the cells with quercetin. Hence, the gel-mobility shift test showed that the inhibition of the activation of the HSP-70 promoter was due to the blockade of the activation of this promoter by HSF (Figs. 81 and 82).

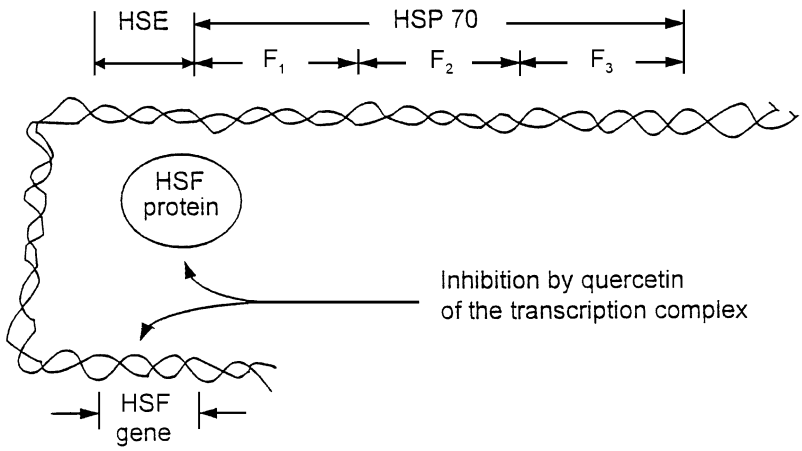


Fig. 81. The genetic activity of HSPs.

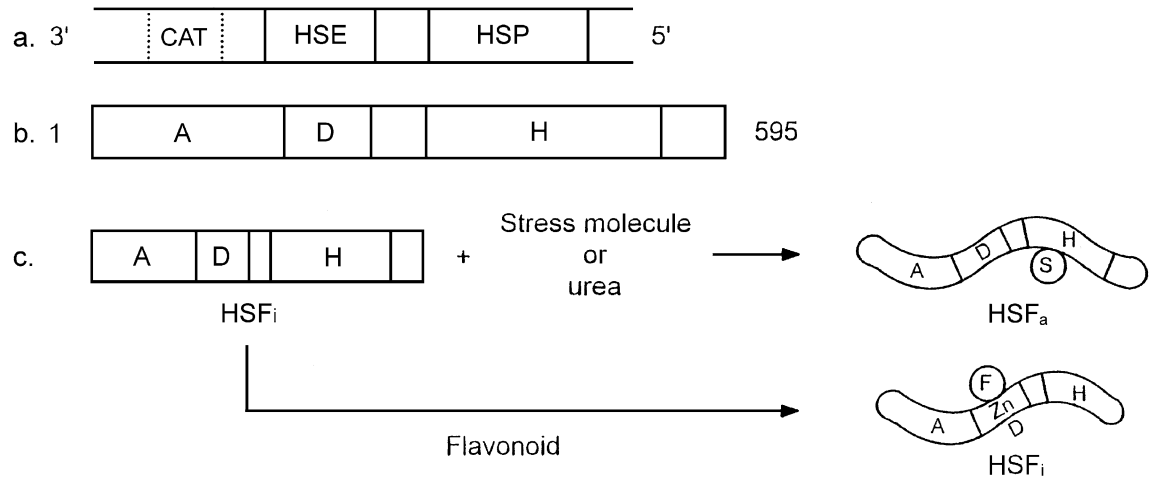


Fig. 82. **a**: Location in the cistron of the HSE in the promoter of the HSP gene and of the experimentally induced CAT gene, which signals the activation of the HSP during expression of the cistron by transfer of an acetyl group to chloramphenicol. **b**: Location of the functional domains in a typical representative (the ER) of the super family of cytoplasmic steroid receptors. These proteins are synthesized in the cytoplasm, but move into the cell nucleus after the specific binding of a hormone molecule to the domain marked by H. Flavonoids are expected to bind to either the hydrophobic, helical domain H, due to the low polarity of the aglycone, or to the Zn^{2+} atom in the D domain, due to their high affinity to heavy metals, or to both (Martin et al., 1978). **c**: Binding of an adequate stress molecule (S) or urea to the inactive form of the HSF (HSF_i) induces a conformational change in the latter to the active form (HSF_a), which can move into the cell nucleus, where it binds and activates the HSE in the promoter of the HSP gene. The binding of a flavonoid aglycone to HSF_i, e.g., to the Zn^{2+}-finger domain (D), prevents the binding of the latter to DNA by a conformational change into another inactive form of HSF, which may even be incapable of passing the nuclear pore. A, activation domain; D, DNA-binding domain.

The HSF can not only be activated by a heat shock, but also by a low pH value, urea, detergents, and Ca^{2+}.

The possibility that quercetin inhibits the binding of the HSF protein to the HSE gene was tested by the addition of quercetin after the heat shock while HSF was in the process of attaching to the HSE. That procedure had no effect. The fact that the effective dose of quercetin is 5-fold higher in vitro than in vivo has been ascribed to compartmentalisation of the cell and to the binding of quercetin to targets from several compartments that are only available in vitro, whereas quercetin in vivo bypasses most of these targets. The activation of the HSF protein in vitro by urea was also inhibited by quercetin, but not that of the detergent NP-40. Although both urea and detergents unfold proteins, they do it differently, and apparently, NP-40, but not urea, denatures the binding site of quercetin. In addition, Mosser et al. (1988, 1990) have shown that glycerol and deuterium oxide prevent the activation of the HSF protein, e.g., by heat shocks. These agents are known to stabilise protein structures.

The nature of the interaction of quercetin with transcription factors is further illuminated by the action of this compound in its native environment, the kingdom of plants. One example is modulation of the expression of the plant nodulation genes of *Rhizobium sp.* by a transcriptional regulatory factor in complex with quercetin (see above). The kinetics of the inhibition of the binding of the HSF protein to the HSE by quercetin showed that the effect of the flavonoid began after a lag period of about 15 min if quercetin was added to a whole cell suspension after or during the heat shock. In contrast, the effect of quercetin was immediate, if the cells had been preincubated with the flavonoid. This difference was interpreted as the result of the slow transport of the flavonoid across the plasma membrane through the cytoplasm to the steroid receptor, and in complex with the latter, to the transcription complex in the cell nucleus. The gel mobility shift assay also showed that quercetin not only prevented the binding of HSF to HSE, but also dissociated HSF-HSE complexes that had been formed already before quercetin was added.

Hence, quercetin changes the HSF to a form that is incapable of binding to the HSE (Figs. 81 and 82).

Whereas in *Saccharomyces cerevisiae* the HSF protein is already bound to the HSE under non-shock conditions, but is activated to enhance transcription by the heat shock, the situation in *Drosophila* and in human cells is different. In the latter cells, the HSF does not bind onto the HSE until the heat shock has occurred (Larson et al., 1988; Sorger & Pelham, 1987, 1988).

The activation of the HSF induced by stress consists of two steps: a conformational change of the HSF protein, which creates the specific DNA-binding site, and phosphorylation (Larson et al., 1988; Sorger & Pelham, 1987). These properties resemble those of the superfamily of steroid receptors so much that the question arises whether the HSF belongs to this family. In that case, it may even be regulated by a hydrophobic ligand such as a steroid. Since flavonoids are carried by such proteins, a competition between a stress-induced hydrophobic ligand and a flavonoid molecule could be envisaged. The discriminating effect of the flavonoid could then be the induction of a different conformation than the one released by the adequate stress ligand (Edington et al., 1989). The former would then destroy the HSE-binding

fold, whereas the latter would form it. Since the geometric restrictions by specific DNA-protein interactions are severe, the two kinds of conformational changes may be quite similar and the binding sites of the regulating ligands may be partially overlapping.

If this model is correct, the reaction to stress may be self-sustaining, i.e., autocatalytic. The reason is that both the HSF and the product of the gene, which is activated via the HSE, belong to the same protein superfamily. Their mutual support in that case must be interrupted by a different mechanism before the reaction is exaggerated to the detriment of the cell. In the case of cancer, one could envisage the therapeutic application of this flavonoid-assisted pathway to cell death. Flavonoids are known to kill cancer cells selectively by slow apoptosis (see Section 17.9), but the point of their attack has not been identified yet. Alternatively, or in addition, they may kill the cancer cells via the HSE, as described above. The advantage of such a procedure is that it is slow enough to commensurate with the time scale of detoxification of the debris (especially of biogenic amines) in the liver.

Another aspect of the perturbation of the HSF structure by the binding of a flavonoid is the susceptibility of the former to proteolysis. It is known that the cell nucleus contains a protease that digests cyclin molecules when they have been marked by phosphorylation. Since HSF is phosphorylated, the possibility exists that the HSF is also decomposed and that the binding of the flavonoid influences the susceptibility of the HSF to digestion. The simplest way to answer such questions is to clone the HSF and to express sufficient amounts of this protein to conduct chemical and crystallisation experiments. Besides quercetin, the flavonoid genistein also inhibited the activation of HSF, but not by preventing its binding to DNA (Price & Calderwood, 1991). Instead, genistein influences a second stage of HSF activation, the phosphorylation, because genistein inhibits the activity of protein phosphokinases (Akiyama et al., 1987; Osada et al., 1988).

Since HSPs are involved in many cellular processes, e.g., the cell cycle, differentiation, apoptosis (Wei et al., 1994), development, and oncogenic transformation, studies of the regulation of HSF activity are important. The inhibition of this process by flavonoids is the only known specific reaction yet. Flavonoids, therefore, can be used to analyze the processes mentioned. Besides, the flavonoids have practical applications, e.g., as enhancers of the thermal sensitivity of cancer cells prior to hyperthermic therapy. The role of HSPs as antagonists to the induction of apoptosis by quercetin is complex.

When the cancer cells were treated with an antisense oligodesoxynucleotide to the HSP-70 initiation codon and 4 downstream exons, HSP-70 was not formed, and the subsequent addition of quercetin increased the fraction of apoptotic cells (hypodiploid cells from 20 to 80%). In a control sample containing the corresponding sense oligonucleotide, the fraction of apoptotic cells was comparable with the effect of quercetin alone, 20%. The sense oligonucleotide alone only produced an insignificant fraction of apoptotic cells ($< 2\%$). Hence, HSP-70 antagonises the apoptotic effect of quercetin on cancer cells. Since HSP-70 aggregates in the nucleoplasma, the nucleolus, and the cytoplasm following a heat shock, but is prevented from doing so by a pretreatment with quercetin, the apoptosis initiated by quercetin may be accelerated by the failure of the chaperonine function of HSP-70. Thus, proteins that must translocate intracellularly to reach their sites of action, e.g., in the mitochondria, would fail to refold correctly after unfolding to pass a membrane. Consequently, the mitochondrion would lack the chromosomally encoded proteins that are imported normally, and vital functions, e.g., oxidative phosphorylation, would cease.

Quercetin also cooperates with cytostatic agents and inhibitors of protein biosynthesis in the induction of apoptosis. Cycloheximide, an inhibitor of protein translation, increased the fraction of apoptotic cells from $40 \pm 2\%$, the level ascribable to quercetin alone, to $76 \pm 2\%$, whereas actinomycin D, an inhibitor of transcription, raised the fraction of apoptotic cells to $91 \pm 5\%$. In the control samples, the cells in medium alone contain $2 \pm 1\%$ apoptotic cells, whereas the cells with cycloheximide contained $14 \pm 2\%$ of such cells. Actinomycin D alone produced $19 \pm 3\%$ apoptotic cells. Hence, the effect of quercetin synergises with that of protein inhibitors (Ferriola et al., 1989; Hockenbery et al., 1993). In contrast, agents modulating the activity of PKC., e.g., the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate, the PKC inhibitor H-7, and the Ca^{2+} chelator EGTA, failed to increase the influence on the apoptotic index. However, *cis*-diaminedichloroplatinum (II) and nitrogen mustard enhanced the antiproliferative effect of the inhibition of PKC (Hofmann et al., 1988; Scambia et al., 1990a).

Interestingly, the endonuclease inhibitor aurintricarboxylic acid also failed to synergise with the apoptotic effect of quercetin. Hence, effects of quercetin lie in the early phases of apoptosis and not in the final committed steps, including nucleolysis between nucleosomes (Fig. 80) and proteolysis catalyzed by enzymes of the interconverting enzyme (ICE) type (caspases).

17.8.4.3. The influence of flavonoids on mammary carcinoma cells. Some tumor cells of the female breast are characterised by their ability to bind estrogens circulating in the blood to specific plasma membrane receptors and to respond by performing some of the steps of differentiation, which have been cancelled or reversed in the course of oncogenic transformation. Since this process reduces the malignancy of such cells, or even restores them to normality, the ability of mammary carcinoma cells to bind estrogens specifically has been regarded as a property indicating a favorable prognosis. This sensitivity to sexual steroids does not seem to be shared by other epithelial tumors, not even by carcinomas of other female reproductive organs. Thus, the mechanism appears to be

part of a normal physiological process that has been interrupted by the oncogenic transformation. The epithelial cells of the female breast can express at least two estrogen receptors (ERs), EBS-I and -II, which possess the same binding specificity, but differ in affinity for the ligand. The lower-affinity receptor ($K_d = 10-20$ nM), EBS-II, is inducible by flavonoids (Scambia et al., 1993; Verma et al., 1988; Ranelletti et al., 1988). Quercetin is especially efficient in inducing EBS-II (Markaverich et al., 1988; Astassi et al., 1985; Bracke et al., 1999; Rodgers & Grant, 1998; Guthrie & Carroll, 1998; Caltagirone et al., 1997).

The estrogenic effect of some flavonoids originally was discovered by observing the behavior of sheep that had eaten fermented clover containing silybin (Sonnenbichler et al., 1980; Sonnenbichler & Pohl, 1980). It was ascribed to the fact that the steric positions of the hydroxyl group in this flavonoid are almost identical to those of estrogens. Flavonoids, due to their nonpolarity or in complex with serum albumin, can pass the plasma membrane and can attach to the cytoplasmic steroid receptor. Accordingly, flavonoids are carried into the cell nucleus to the transcription complex at the genes controlling the expression of ERs and perhaps also of other proteins participating in the growth and function of the mammary gland.

The ability of quercetin to induce the EBS-II receptors in a dose-dependent fashion was found in cell lines of both ER-positive and ER-negative tumors. The latter observation is particularly interesting since the prognosis for the type of tumor lacking EBS-II is poor. The time course of receptor induction after quercetin treatment, at least in the two cell lines examined, is biphasic because the initial appearance of the receptor, which peaks after 24 hr, is followed by its disappearance after 72 hr and its reappearance and lasting expression. This kinetics of expression, which is difficult to interpret, has also been found for other hormone receptors of the plasma membrane of reproductive organ cells (Ferrandina et al., 1992; Bagavandoss & Midgley, 1988; Scambia et al., 1991).

The analysis of Scatchard plots revealed that the specific binding affinity of the quercetin-induced EBS-II receptors did not differ from that of the constitutively expressed receptors, but the average number of such receptors per cell doubled.

The induction of EBS-II receptors by quercetin required gene transcription and protein synthesis, since the effect was prevented by actinomycin D and cycloheximide. The inhibition by quercetin of the proliferation of breast cancer cells appears to occur at several levels of metabolism and by different mechanisms (Kawaii et al., 1999a, 1999b). When the cells are exposed to low concentrations of quercetin, cell proliferation is inhibited to a greater extent than when the cells were pretreated with this substance, but at higher concentrations, a pretreatment of the cells with quercetin does not affect their sensitivity to the agent. Thus, exposure of the cells to low concentrations of flavonoids (< 1 μM) appears to restore the normal growth metabolism by inducing EBS-II receptors, whereas high quercetin concentrations inhibit a variety of cellular enzymes (see Section 12). The EBS-II receptor-inducing effect of quercetin is shared by a number of other flavonoids, but for most of them, is less pronounced. Thus, both the ability to increase the number of EBS-II receptors and the receptor affinity varied in the following order:

$$\text{Quercetin} = 3',4',7\text{-trimethoxy-quercetin} > \text{ipriflavone}$$

whereas rutin and hesperidin failed to show any effect. The reason seems to be steric hindrance caused by the glycoside in position 3 of the benzopyrone ring system.

17.9. The influence of flavonoids on cardiovascular diseases

Cardiovascular disease is the most frequent cause of death in developed countries. Advances in the treatment of such diseases, therefore, have the potential of yielding a high return on the research effort. Recent evidence shows that flavonoids have an important effect on the intricate regulatory processes, which are derailed by these diseases, and that they can markedly improve the condition of patients suffering from various stages of this progressive illness (Hollman et al., 1999b; Hertog et al., 1993; Brouillard et al., 1997).

A common denominator of these diseases is an inflammation of the vascular wall that is called atherosclerosis, but the tendency to develop the illness is strongly dependent on the individual genetic disposition and upon environmental factors, such as the composition of the diet, smoking, alcohol consumption, and psychological stress. Flavonoids interfere at several points in the pathogenesis of cardiovascular disease, and each will be discussed separately.

The general course of inflammatory processes has been described in Section 17.2. The discussion of the reactions will only be expanded if they are of particular importance to cardiovascular diseases and their response to flavonoid treatment (Yochum et al., 1999; van Acker et al., 1997).

17.9.1. The genetic disposition

Some individuals are born with resistant vascular walls, but others with weak vascular walls. That is probably an important reason for the surprising observation that some people can smoke heavily throughout most of their lives and age without developing cardiovascular diseases, whereas others are very sensitive to smoke. For the same reason, it is very difficult to define general criteria for safety limits, e.g., the cholesterol concentration in the blood. A good starting point for the evaluation of the vascular health of a patient, therefore, is the family record. If the parents of the patient and their ancestors reached an advanced age before they died, then the vascular walls of the patient are probably fairly robust and the normal upper limit of 250 mg choles-

terol/100 mL blood may be applied. Conversely, if the family record indicates that caution is necessary, then dietary restrictions and other measures must be introduced much sooner. One of the indications, which requires special attention to avoid cardiovascular crises, is the existence of a familial coagulopathy, especially one that enhances the tendency for blood coagulation.

Other inborn dispositions that increase the risk of cardiovascular diseases are morphological anomalies in the heart valves. They often require surgery, and the reaction of the patient to this intervention sharply increases the stress on the vascular system.

Other risk factors, e.g., alcohol consumption and heavy smoking, are probably partly determined by the genetic constitution and are partly conditioned by the social environment (Hladovec, 1979a, 1979b). The inborn error seems to cause the partial inactivation of the enzyme, which liberates the endogenous opiate enkephalin. Since ethanol improves the activity of this enzyme, presumably by causing an activating conformational change, the consumption of alcoholic beverages calms such patients. However, alcohol also has an effect on vascular tone, the mechanism of which is yet unclear, but which increases the risk of vascular occlusion, i.e., embolism. Alcohol seems to activate a converting enzyme (ICE) of angiotensin by inducing a conformational change, and a relative to this ICE initiates the final (fatal) phase of apoptosis (see Section 17.1).

17.9.2. The role of flavonoids in the dietary component of cardiovascular stress

Experience teaches that a high cholesterol level and a high ratio of saturated and monounsaturated to polyunsaturated FAs in the blood predisposes patients to vascular diseases, whereas a high dietary content of vegetables and fruits has the opposite effect (Bok et al., 1999; Fotsis et al., 1997). The lipids of marine animals, which contain many ωC3-unsaturated FAs, are especially effective in protecting against cardiovascular crises. The latter seldom occur in people living in Arctic countries, e.g., Inuits. A possible reason is that the distribution of double bonds, the first of which occurs at the third carbon atom from the hydrophobic end, confers a high resistance against the peroxidation of these FAs. Moreover and more importantly, ωC3-unsaturated FAs are transformed by COX to a thromboxane (Tx), Tx3, which is different from the usual form, Tx2. Tx3 has a lower propensity than Tx2 for the induction of the formation of the white clot (aggregate of thrombocyte shadows).

The influence of the flavonoids on the endogenous regulation of cholesterol biosynthesis has already been dealt with in Section 17.3. However, another critical aspect of cholesterol homeostasis is the uptake of lipoproteins containing dietary phospholipids, triglycerides, and cholesterol esters into cells, especially hepatocytes. The lipoproteins contain a protein surface structure that is recognised by a specific plasma membrane receptor (Fig. 83). However, both the apolipoprotein and its receptor can be defective due to an inborn error, with the result that the efficiency of the transmembrane transport of the lipoprotein is drastically reduced. Hence the half-life of the lipoprotein in the blood is greatly increased in that case. Finally, lipoproteins are engulfed by macrophages or collapse due to proteolytic attack, thus emptying the lipids into the blood and precipitating insoluble steroids. The unsaturated FAs, both in the liposomes and in the plasma membranes, are oxidised to peroxides by active oxygen species from macrophages in stress caused by the ingested lipids. Thus, the receptors are incapacitated. Whereas the flavonoids are hardly able to correct the genetic faults, they efficiently destroy the aggressive oxygen forms, as previously described in Section 10. Since vegetables and fruits are rich in flavonoids, their anti-atherosclerotic effects

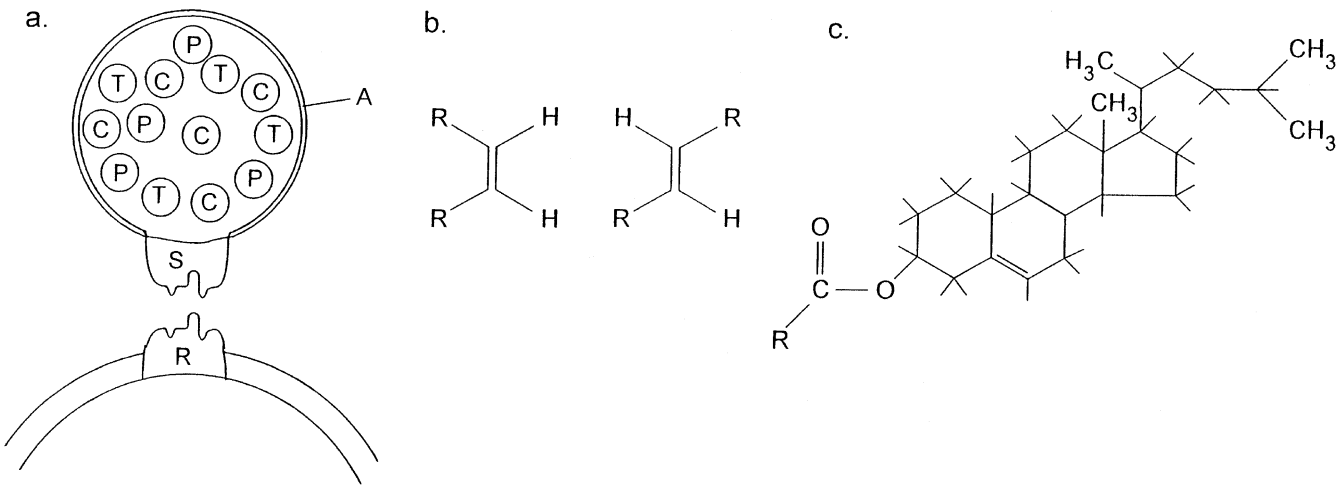


Fig. 83. **a**: Lipoprotein docking with the specific recognition structure (S) of the apoprotein (A) in a receptor (R) on the outside of the plasma membrane. The bag-shaped apoprotein contains triglycerides (T), phospholipids (P), and cholesterol esters (C). **b**: The cis- and trans-configurations of a double bond. **c**: Cholesterol ester.

can be explained easily (Filipovic et al., 1972; Borradaile et al., 1999; Attaway & Buslig, 1998; Aviram & Fuhrman, 1998; Hladovec, 1979a, 1979b).

More difficult is the construction of a rationale for the advantage of polyunsaturated FAs over monosaturated and saturated ones, apart from the fact that some of the polyunsaturated ones are essential, i.e., required for the biosynthesis of membranes. One would expect that the unsaturated FAs would be particularly sensitive to destruction by peroxidation. However, they have an additional effect, because if they replace the usual palmitic acid into cholesterol esters, then they counteract the incorporation of cholesterol esters into the plasma membrane. The reason is steric because many of the double bonds are in the cis configuration, which because of its rigid angular structure, disturbs the membrane structure. A high content of cholesterol esters in the membrane increases the viscosity of the latter, which hampers the surface diffusion in the membrane that is essential to capping, endocytosis, and other important cell functions. Thus, the protection of the unsaturated FAs by flavonoids indirectly protects the cell from malfunction (Leontéva et al., 1979; Younes & Siegers, 1981).

17.9.3. Flavonoids in the management of ischaemia/reperfusion damage

Heart surgery, e.g., the replacement of valves or the heart, the establishment of a coronary bypass, or the closing of a leakage through the A/V septum, requires that the heart is stopped for at least 0.5 hr while the blood is oxygenated and circulated by a heart-lung machine. This part of the treatment imposes a stress on the organism, in addition to that arising from the surgical lesions to the tissues:

(1) The extracorporal circulation of the blood of the patient through the heart-lung machine causes increased haemolysis and rigidification of the blood cells due to the contact of the latter with nonphysiological surfaces (plastic, glass, and metal).
(2) The heart that is to be operated upon or to be transplanted must be cooled to 4°C in Euro-Collins solution, or its equivalent, in order to stop its contraction cycles and to minimise its catabolism in the ischaemic state. Although this period can often be reduced to ~30 min, the lack of oxygen activates the catabolic activities of the lysosomal enzymes, which cause appreciable and irreversible damage to the myocardium, even at 4°C.
(3) The reperfusion of the operated heart, which immediately follows the completion of the corrective measures and the resumption of the physiological circulation, inevitably causes extensive oxidative damage due to the formation of large amounts of active oxygen species.

In addition, the surgical tissue lesions cause the secretion of stress hormones, such as epinephrine and ACTH, which release a strong vascular contraction in the coronary arteries and the lungs, thus enhancing hemolysis and the risk of embolism (Metzner et al., 1982).

The nature of the interaction of the surfaces of the blood cells with those of artificial materials in medical appliances is still poorly known, but it is subject to much research. The blood cells that are most sensitive to such damage are the thrombocytes, which upon contact with foreign surfaces, undergo viscous metamorphosis. In the course of this process, they secrete several substances, e.g., ADP and Tx, and they expose phospholipid Pf3 on their surface. These compounds enhance the formation of both the white (aggregates of thrombocyte shadows) and the red (fibrinogen-enclosing erythrocytes) clot, thus increasing the risk of embolism and haemolysis. The participation of Tx, which is an eicosanoid, could be inhibited by flavonoids (Mabry & Ulubelen, 1980; Taskov, 1977; Hladovec, 1977, 1979a, 1979b; Beretz et al., 1978, 1981, 1982, 1986; Danon & Elazar, 1968; Cazenave et al., 1981; al Makdessi et al., 1999). The extent of the beneficial effect of such a treatment to patients receiving extracorporal circulation remains untested. Erythrocytes are known to change their shape grossly when they are forced through narrow passages by the force of convection. However, as long as the biochemical mechanism by which they accomplish this feat remains unknown, attempts to interfere with this risky haemolysis-prone process seems futile.

The ischaemic damage to the cooled heart with the arrested contraction cycle is partly due to lysosomal enzymes that are only active in the acidic pH range. However, flavonoids have been shown to inhibit the proton pump that acidifies the contents of lysosomes (Lee et al., 1982). Although the mechanism in this particular case has not been proven yet, the structural similarity of the transport ATPases seems to be sufficient to permit an educated guess by analogy. The Na^+/K^+ pump of the plasma membrane of transformed uterine epithelial cells is greatly influenced by the flavonoid quercetin, which restores normal regulation of the pump by phosphorylation/dephosphorylation reactions on the β-subunit of this pump. Since it is very likely that this proton pump is also controlled by protein phosphokinases and their associated phosphatases, known inhibitors of the latter, some of which are flavonoids, might be applied.

In the reperfusion phase, the active oxygen species, which rapidly cause damage, especially to the unsaturated FAs in membranes, are currently counteracted by the injection of the enzyme SOD. Although that treatment has some desirable effects, it is insufficient to protect the vascular epithelium. Since flavonoids scavenge free radicals very efficiently (see Section 10), an addition of such substances to the blood immediately after the resumption of the corporal circulation would be expected to bring about an improvement of the therapy (Zhou & Zheng, 1991).

In the first postsurgical days after organ transplantation, the patients invariably suffer from insufficient blood supply, not only to the transplanted organ, but also to the lungs (Robbins, 1977). The reason is primarily regarded as an insufficiency in the biosyntheses of the endothelial-derived

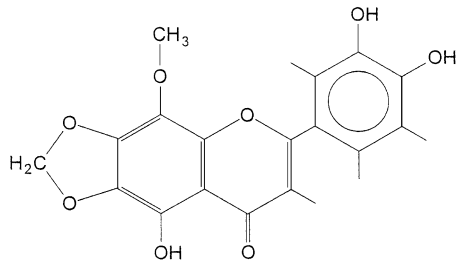


Fig. 84. The flavonoid (6,7-dimethox-8-methyl-3′,4′,5-trihydroxyflavone) used in the experiments with the contractility of aortic smooth muscles.

relaxation factor (Pedersen, 1977). The latter, which is believed to be identical with the cellular mediator ·N=O (NO), is easily destroyed by H_2O_2, which is formed by many oxidative reactions initiated by the state of stress. The product, pernitrous acid, is a very aggressive acidic oxidant that causes much tissue damage. However, Girard et al. (1995) have shown that a synthetic flavonoid promotes the endothelial-derived relaxation factor-induced relaxation of rabbit arteries in vitro through its superoxide scavenging activity (Law et al., 1999; Ohshima et al., 1998) (Fig. 84). Hence, similar experiments with intact experimental animals, which have received an organ transplant are needed. If the circulation in the threatened organs in the animals receiving flavonoids exceeds the one in the control animals, then experiments with flavonoids on informed patients scheduled to receive organ transplants would seem to be appropriate (Pearson et al., 1979; Tomova & Taskov, 1978; Guendjev, 1978).

17.10. The effect of flavonoids on gastrointestinal ulcers

Gastrointestinal ulcers is a very common disease in the developed countries, and its occurrence becomes more and more frequent in parts of the world in which the economy is emerging into a state satisfying modern standards. In the pathogenesis of this disease, psychological stress is usually, if not invariably, participating since the stress hormones, e.g., epinephrine, norepinephrine, and ACTH, cause a vasoconstriction in the integument and the periphery, whereas they dilate the vessels of the muscles, the heart, and the brain, thus preparing the organism for fight or flight. However, if the duration of this state is prolonged, then the reduced blood supply to major organs, e.g., stomach, intestine, liver, kidneys, and skin, fails to satisfy their demand for oxygen, antibodies and other agents that are required to maintain a healthy condition. The stress hormones also increase the glandular secretion, e.g., of gastric acid and corticoids. The highly concentrated hydrochloric acid (0.1 M) secreted by the parietal cells of the gastric mucosa in the fundus denatures proteins in plasma membranes and catalyses the hydrolysis of polysaccharide moieties of proteoglycans in the protective mucous coat covering the luminal surface of the stomach and the upper intestine to a perilous extent during prolonged stress. When the walls of the blood vessels supplying this area with oxygen, nutrients, and protective substances are sufficiently weakened, then slight mechanical insults easily cause ruptures, resulting in leakage of blood into the tissue. Such events start inflammation and repair processes in which eicosanoids, e.g., PGs, participate.

Stomach ulcers and bleedings, which are diagnosed by pain in the region and blood in the faeces, are often treated with aspirin (acetylsalicylic acid) or cimetidine (Barasoain et al., 1980). The former inhibits the PG COX, whereas the latter may stimulate the secretion of the gastric inhibitory peptide, which inhibits the production of gastric acid (see Section 9 and Fig. 85).

However, both of these drugs have serious side effects because the high doses of acetylsalicylic acid, which are often used, cause acidosis, a potentially dangerous condition, that especially perturbs kidney and brain functions. Besides, aspirin, in contrast to flavonoids, inhibits COX2, which is required for tissue repair (see Sections 3.6 and 12.3). Cimetidine and related drugs (Fig. 85) have neurological side effects, e.g., on the heart. Therefore, flavonoids are favorable, effective, and usually innocuous substitutes for the classical therapeutic agents (Kyogoku et al., 1979; Sasajima et al., 1978; Pfister et al., 1980; Goel et al., 1988; Angel et al., 1994; de la Lostra et al., 1994; Di Carlo et al., 1994; Middleton et al., 1989; Ares & Outt, 1998; Martin et al., 1998; Fewtrell & Gomperts, 1977a, 1977b). Reports have also appeared that claim that flavonoids protect against gastric cancer (Garcia-Closas et al., 1999).

Similar to aspirin, acylated flavonoids may transfer their acyl group to the side chain hydroxyl group of serine in the active site of COX (Tubaro et al., 1989).

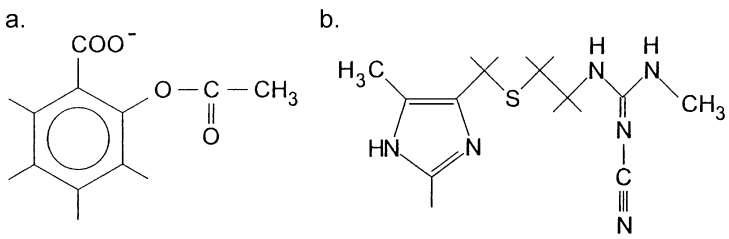


Fig. 85. The structures of some inhibitors of gastric secretion. **a**: Acetylsalicylic acid (aspirin). **b**: Cimetidine (1-cyano-2-methyl-3-[2-(5-methyl-4-imidazolyl)methylthio-ethyl]guanidin). Cimetidine blocks the H_2 receptors for histamine, thus inhibiting the secretion of gastric acid and indirectly preventing thrombolysis in open wounds.

The reservation about the harmless nature of flavonoid treatment refers to incidents of flavonoid allergy that occur in a small fraction of the population (see Section 8), but that can easily be recognised soon enough to spare the patient from the unpleasant symptoms.

Another aspect of gastrointestinal ulcers recently has attracted much attention in the medical profession, i.e., the discovery of the frequent involvement of the bacterium *Helicobacter pylori* in their pathogenesis (Hentschel et al., 1993). A young Australian medical scientist, who had isolated this organism from the pyloric flora of patients suffering from gastrointestinal ulcers, suspected its participation in the pathogenesis of these ulcers. He ingested a purified culture of *H. pylori* colonies, and promptly developed an ulcer. This highly suggestive evidence is supported by the conditions in the tissue that has become sensitised by the increased gastric secretion induced by psychological stress. The stress-induced reduction in blood supply to the integument and the organs sharply diminishes the immune response that normally counteracts bacterial and other infectants. Moreover, the copious flow of gastric acid erodes the gastric mucosa and its mucous layer, thus facilitating the entry of bacteria, which are particularly tolerant of the acid, into the tissue, where they secrete exotoxins that initiate inflammation. *H. pylori*, which tolerates a low pH value, therefore fits excellently into this micro-environment. Since flavonoids kill many bacterial strains (see Section 17.12), it is likely that the many reports by lay medical practitioners of the successful treatment of gastrointestinal ulcers is due in part to this effect. However, not much scientific proof has appeared yet to support the claim that flavonoids are also effective against *H. pylori* (Bae et al., 1999).

17.11. The effect of flavonoids on rheumatic diseases

Rheumatic disease is a group of illnesses, the aetiology of which has not been completely elucidated yet. However, many aspects of this disease are known and form the basis of its management. The propensity for rheumatic disease is determined by one's genetic constitution. A hallmark is that the regulation of both the immune system and the dense connective tissue is perturbed. Besides, transient conditions, e.g., draught or infection, can release episodes of rheumatic pain.

According to current opinion, autoimmunity and inflammation are important components of rheumatoid arthritis (RA). Autoimmunity is a condition that is also not fully understood yet, but it is believed that certain genetic constitutions produce T-lymphocytes, which are particularly prone to participate in the process, and it is suspected that viral infection plays a role. The reason is the existence of certain structures in the T-cell antigen receptor that have a strong affinity for large proteins, so-called superantigens (Fig. 69). The latter permanently crosslink the antigen-loaded MHC protein of APCs to the T-cells, thus over-activating lymphokine production. The superantigens may be products of the diseased genome, of viruses, or of bacteria. For further details, see also Section 9.

Since rheumatic disease is associated with painful joints, one might guess that the antigen specificity of the T-cells, which are locked in a permanently activated state, by a superantigen happens to be directed against an epitope characteristic of the cartilage in a joint, e.g., a part of chondroitin sulphate. The hypothesis of the initiating role of a superantigen and of autoimmunity in the pathogenesis of rheumatic arthritis can explain the subsequent development of the disease rather well, because there is a coupling between the regulation of cellular immunity and that of bone metabolism. A common denominator is the cytokine IL-1, but there may be others. IL-1 is produced by macrophages and acts as a growth hormone on T-lymphocytes, which are induced to proliferate and to secrete IL-2, that, in turn, is a mitogen to several cell types, including T- and B-lymphocytes, macrophages, granulocytes, and blood vessel endothelial cells. In many respects, osteoclasts resemble macrophages because both produce and secrete IL-1 and are phagocytic. Although it remains to be demonstrated, it seems likely that osteoclasts also respond to IL-2 secreted from immune cells. If this is true, then the enhanced osteoclastic activity leads to the destruction of the heads of the long bones in the joints. The hip joint is especially at risk because it carries almost the entire body weight. In RA, such a destruction is observed. It is followed by repair processes, which are uncoordinated with the mechanical requirements defined by the stress gradients. The topological activation pattern of the osteocytes leads to the formation of bony spikes in the head of the bone. These spikes cause tissue lesions, bleeding, inflammation, and pain. It is logical that this activation pattern corresponds to the location of the epitopes, and hence, to autoimmunity in the joint.

If this hypothesis is essentially correct, then the beneficial effect claimed by many lay medical practitioners of orally consumed flavonoids would include the elimination of the PGs that mediate the pain (Becker et al., 1981; Karlson et al., 1993). An additional effect of the flavonoids may be to activate cytotoxic T-lymphocytes, which kill cells presenting the harmful foreign antigen. Flavonoids do activate cytotoxic T-lymphocytes, but the antigen, against which the lymphocytes are directed, remains unidentified.

Patients with RA who develop AIDS surprisingly show complete remission of their arthritis. This indicates the importance of the $CD4^+$ cell (T-helper cell) in the pathogenesis of RA, and supports the case for an immunosuppressive therapy for this disease (Brostoff et al., 1991).

Rheumatoid factors are idiotypical proteins that appear to be actively and specifically involved in the etiology of rheumatic disease. They circulate in the blood and can be assayed for with specific antibodies. Their concentration is considered a measure of the progression of the disease. RFs are auto-antibodies that react with other antibodies of the patient. They are characterised by a lack of terminal galactose on the associated glycoside. RFs may be of the

classes IgM, -G, -A, or -E, but IgM prevails in RFs. They are not disease-specific, and may even play a physiological role by clearing immune complexes. Most RFs are directed against Igs, cellular antigens, or collagen II.

17.12. Bacterial infection

The use of flavonoids against bacterial, protozoan, and fungal infections has two purposes: (1) to kill the bacterial or fungal cells and (2) to counteract the spread and the effects of the bacterial toxins (Harborne et al., 1976; McClure, 1975; Lopes et al., 1998). Many, but not all, of the bacterial strains commonly encountered by humans are killed by flavonoids (Bagaev, 1978; el-Gammal & Mansour, 1986). However, the mechanism by which this is accomplished is not known yet. Since eicosanoids do not appear to be formed by bacteria, the primary targets of the flavonoids, the PG COX and the related enzyme lipoxygenase, do not come in question since only eucaryotic cells, including plants, possess such enzymes. Neither does another important target, the cAMP PDE, since bacteria, like other procaryotic cells, do not possess this enzyme. However, they do contain metalloenzymes, the heavy metal atoms that form strong ligand complexes with flavonoids, e.g., phosphatases. Therefore, the bacteriocidal effect of the flavonoids may well be the result of a metabolic perturbation. Ion channels, which are components of both bacterial and animal cells, are especially sensitive points of inhibition and likely targets of flavonoids. In animal cells, these channels are regulated by phosphorylation/dephosphorylation reactions. Fungi, which often accompany bacterial infections, may be killed by flavonoids due to any of the two mechanisms mentioned above. All infectants, including viruses, may be eliminated through the immunostimulatory effect of flavonoid treatment (see Section 9.2 and Ohnishi & Bannai, 1993; Conti et al., 1998).

Apart from the active role that the flavonoids play in the destruction of infectants, they fortify loose connective tissues by inhibiting some of the enzymes that can hydrolyze their proteoglycan and protein meshwork. This mesh sterically hinders the diffusion of infectants through the tissue. One example is the inhibition of hyaluronidase by flavonoids. Thus, the latter contribute to the immobilisation and encapsulation of the infectants. In that state, the infec-

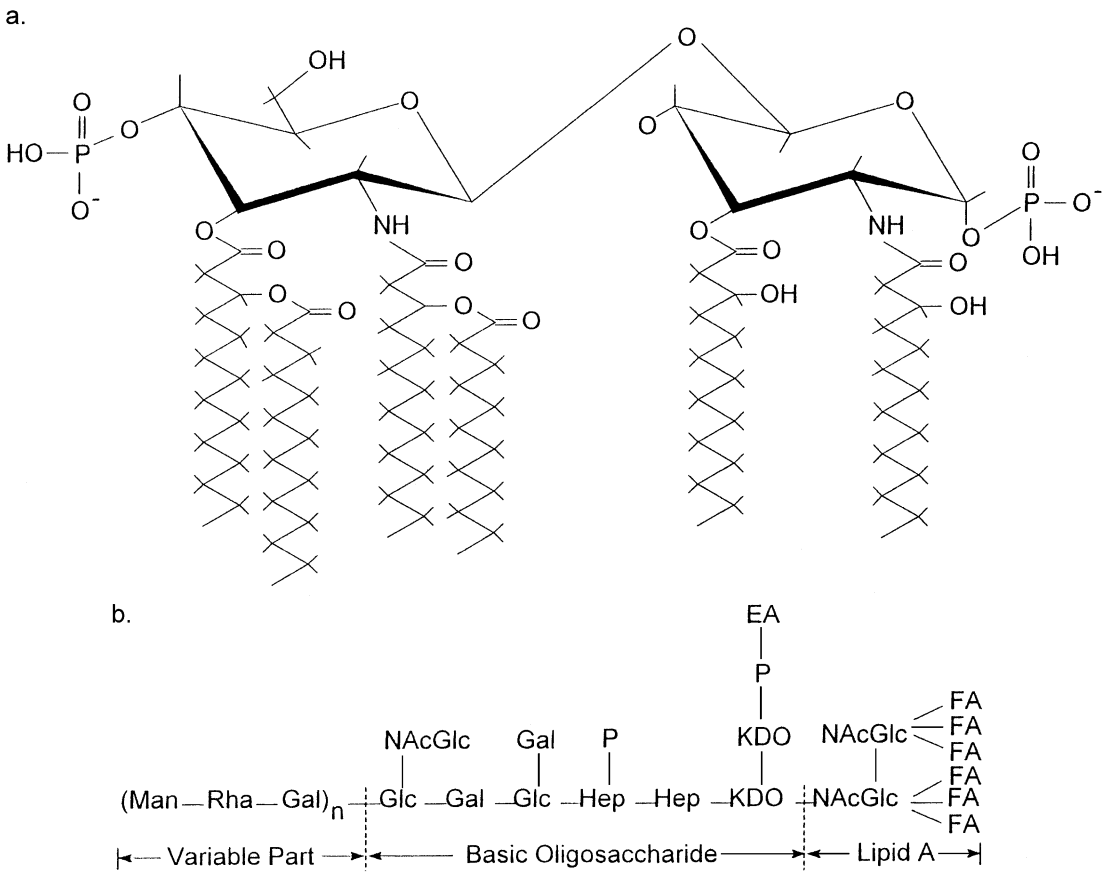


Fig. 86. **a**: Structure of lipid A of an endotoxin from *Escherichia coli J-5* (Brade et al., 1993). The monosaccharide moiety is D-glucosamine. **b**: Typical section of lipopolysaccharide (O-antigen of Gram-negative bacteria). The specificity of the O-antigen resides in the variable terminal trisaccharide repeats, whereas the toxicity arises from interaction of lipid A with CD14 of macrophages that release TNF-α, IL-1, and IL-6 (Buddecke, 1994). Abe, D-abequose (3,6-didesoxy-D-galactose); EA, ethanolamine; Gal, D-galactose; Glc, D-glucose; Hep, heptose (L-glycero-D-mannoheptose); KDO, 2-keto-3-desoxyoctanoic acid; Man, D-mannose; NAcGlc, *N*-acetyl-D-glucosamine; P, phosphate; Rha, L-rhamnose (6-desoxy-L-mannose).

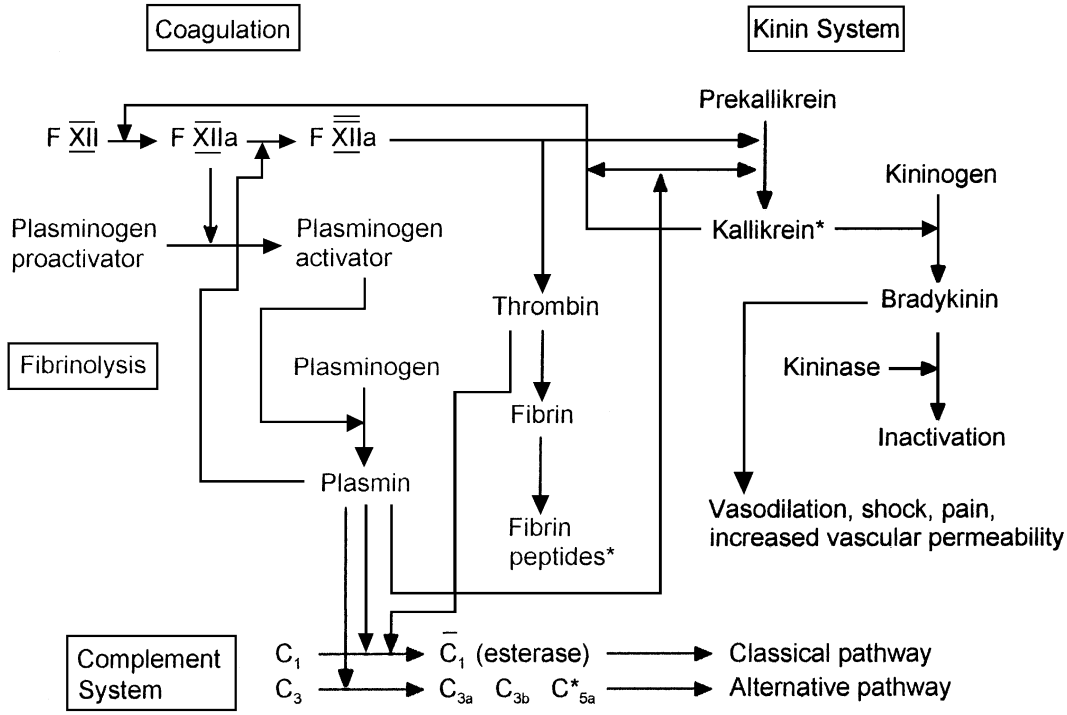


Fig. 87. Interaction between biochemical systems participating in inflammatory processes: blood coagulation, fibrinolysis, the complement cascade, and the kinin system. Flavonoids counteract the secretion of tissue kallikrein and of serotonin and histamine from mast cells, granulocytes, and macrophages. They scavenge free radicals, e.g., superoxide, from macrophages and granulocytes; inhibit Tx synthesis in thrombocytes; alleviate pain; and reduce vascular leakage (Hugnet et al., 1990; Scheller et al., 1990; Cotelle et al., 1992; Paya et al., 1992). *, chemotaxis-evoking peptide.

tants will gradually be decomposed by scavenging and repair processes.

The bacterial toxins are divided into endotoxins and exotoxins. The former are toxic glycolipids (Gram-positive bacteria) or Teichonic acids (Gram-negative bacteria), which are components of the outside face of the bacterial membrane (Fig. 86). This membrane is surrounded by a matrix of proteoglycans and peptidoglycans (Buddecke, 1994). When these glycans are attacked by enzymes in the blood of the patient, endotoxins are exposed. They are engaged by the immune cells and the antibodies, with the result of complement activation, anaphylaxis, fibrinogenesis, and eicosanoid formation (Figs. 87–95). The latter process starts with the activation of PLA_2, which liberates arachidonic acid. The toxicity of the lipid A moiety of the

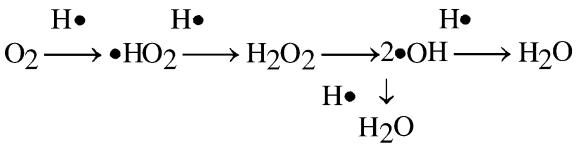


Fig. 88. Formation of free radicals in the respiratory chain by successive reduction of molecular oxygen by hydride radicals from the enzyme complexes I and II. They also arise in oxidative decomposition reactions, especially in the liver, and by irradiation with energy-rich electromagnetic or particle radiation. Free radicals react due to their high reactivity with immediate molecular neighbours. They are eliminated enzymatically in peroxisomes and by scavengers, e.g., cysteine, glutathione, ascorbate, tocopherol (vitamin E), ubiquinol, and flavonoids (dietary or therapeutic).

lipopolysaccharide A, the most important endotoxin, is due to an interaction with the CD14 receptor of macrophages. The latter initiates the production of the TNF-α, as well as of IL-1 and IL-6 (Buddecke, 1994). Myrecetin and other flavonoids suppress the TNF-α-mediated NF-κB activity by down-regulating the activity of the kinase kinase IκB kinase (Tsai et al., 1999; Manthey et al., 1999; Wakabayashi, 1999; Isai et al., 1999).

Complement activation by glycolipids is antibody-independent, i.e., it follows the alternative pathway. Also, viruses can unleash this mechanism. Although the activated complement system can kill some normal cells if they are marked with an antibody, it is also protective for higher animals because it destroys many infectants. Thus, patients suffering from an inborn defect in the complement system are more often troubled with bacterial infections than persons without such genetic errors.

As described in Section 9, the preparatory, enzymatic stage of the complement cascade releases peptides with

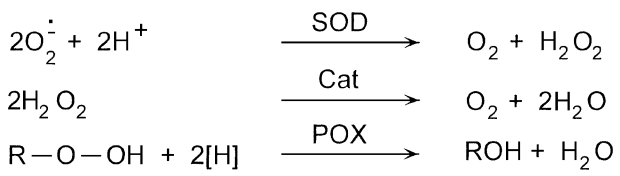


Fig. 89. Destruction of superoxide ($\cdot O_2^-$), H_2O_2 and hydroperoxides by peroxisomal enzymes. Cat, catalase; [H], nascent hydrogen; POX, peroxidase.

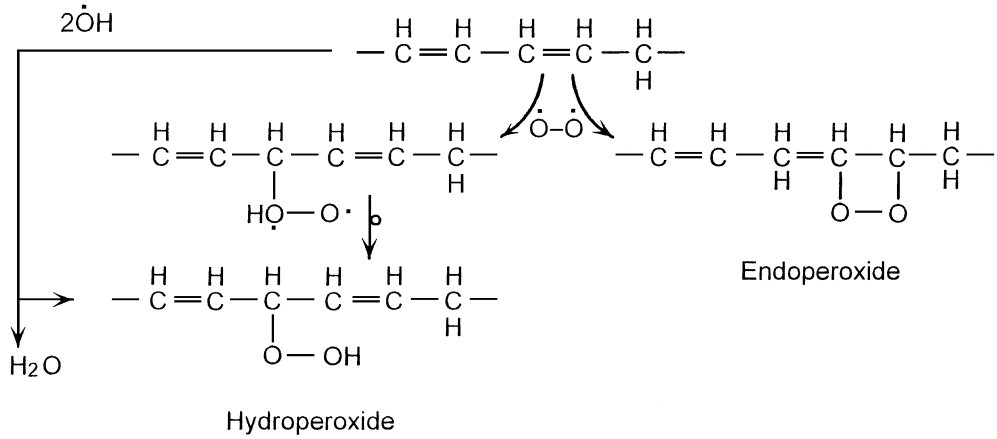


Fig. 90. Oxidation of unconjugated unsaturated FAs in membranes by the oxygen diradical under formation of endoperoxide and hydroperoxide, as well as by an ·OH-forming hydroperoxide.

anaphylactic and chemotactic effects. The entry of bacterial cells into the blood, a phenomenon called sepsis, therefore, can have grave consequences to the patient. The anaphylotoxins can cause a fatal hypovolumic shock by extensive vasodilation, and immune cells are summoned by chemotaxis. The bacterial endotoxins, which are exposed after cell death, e.g., due to antibiotics, irritate endothelial cells and stimulate macrophages to secrete IL-1 (and IL-6), which subsequently activates T-cells to produce IL-2, which, in turn, clonally expands both T- and B-cells. The latter are also differentiated by IL-2 to plasmocytes, which produce antibodies against bacterial epitopes. The result is the formation of insoluble antigen-antibody complexes. Moreover, most cells, granulocytes, and macrophages are provoked by bacterial endotoxins to degranulate, thus releasing histamine, serotonin, serine proteinases, and active oxygen species into the blood. The proteases initiate fibrinogenesis, which indirectly stimulates the blood coagulation cascade by exposing the phospholipid Pf3 on the thrombocytes. If bacterial sepsis is treated with glucocorticoids, the expression of an immune response inhibiting transcription factor, NF-κB, is increased to counteract the rise in blood viscosity resulting from the antibody-antigen complexes (Scheinman et al., 1995; Saliou et al., 1998; Schreck et al., 1991). However, if glucocorticoid treatment is exaggerated or prolonged, the tendency to bleeding will increase and the immune defence will be weakened (Till & Thielmann, 1989).

Therefore, the use of flavonoids as a substitute for, or a supplement to, glucocorticoids is an attractive possibility. The flavonoids also inhibit eicosanoid synthesis, but they do not negatively influence cellular immunity. On the contrary, they support it by activating the T-lymphocytes and IFN synthesis. Although the lack of Tx following the administration of flavonoids would be expected to reduce thrombogenesis, and although flavonoids (as γ-pyrones) structurally resemble coumarin (α-pyrones), no change in blood coagulation status has been observed after therapy with flavonoids. This is a surprising observation, which may be due to favourable compensatory effects.

17.13. The antiviral properties of flavonoids

Viral infection is one of the most difficult diseases to combat, since viral particles consist of only a few building blocks, which they share with all living organisms. Yet, viral particles are equipped with powerful tools to aid their invasion into cells and shrewd plans for the corruption of the cellular metabolism. The great similarity between the building blocks of viruses and those of biological cells renders the development of antiviral drugs difficult because healthy uninfected cells will take up these drugs and die. In fact, many antiviral drugs may be more dangerous to the patient than to the infectant. An additional difficulty in combating viral infections is the high mutability viral genomes. As a result, the detailed structure of the proteins in the protective capsid or membrane surrounding the viral genome continually changes. Therefore, attempts to combat viruses with specific antibodies against surface epitopes have often been futile.

The infectious material of a virus is a nucleic acid, either an RNA or a DNA. These nucleic acids contain the viral genes, which in the case of RNA viruses, are overlapping. That saves space and confuses the enemy. The viral nucleic acids may be present in a virus particle in one or two copies. In the latter case, the strands are complementary. These nucleic acids, which are rolled up into a spherical bundle, are surrounded by a protective protein capsid that, to simplify the coding, consists of an aggregate of a small, simple proteins. Larger viruses have in addition an outer lipid membrane, in which glycoproteins are imbedded (Fig. 96). These glycoproteins recognise weak points on the target cells, at which the virus particle can enter. In the case of HIV infection, this weak point is the K^+ channel of T_4-lymphocytes (helper T-Ly), which is associated with a chemokine receptor (Dragic et al., 1996). Moreover, some

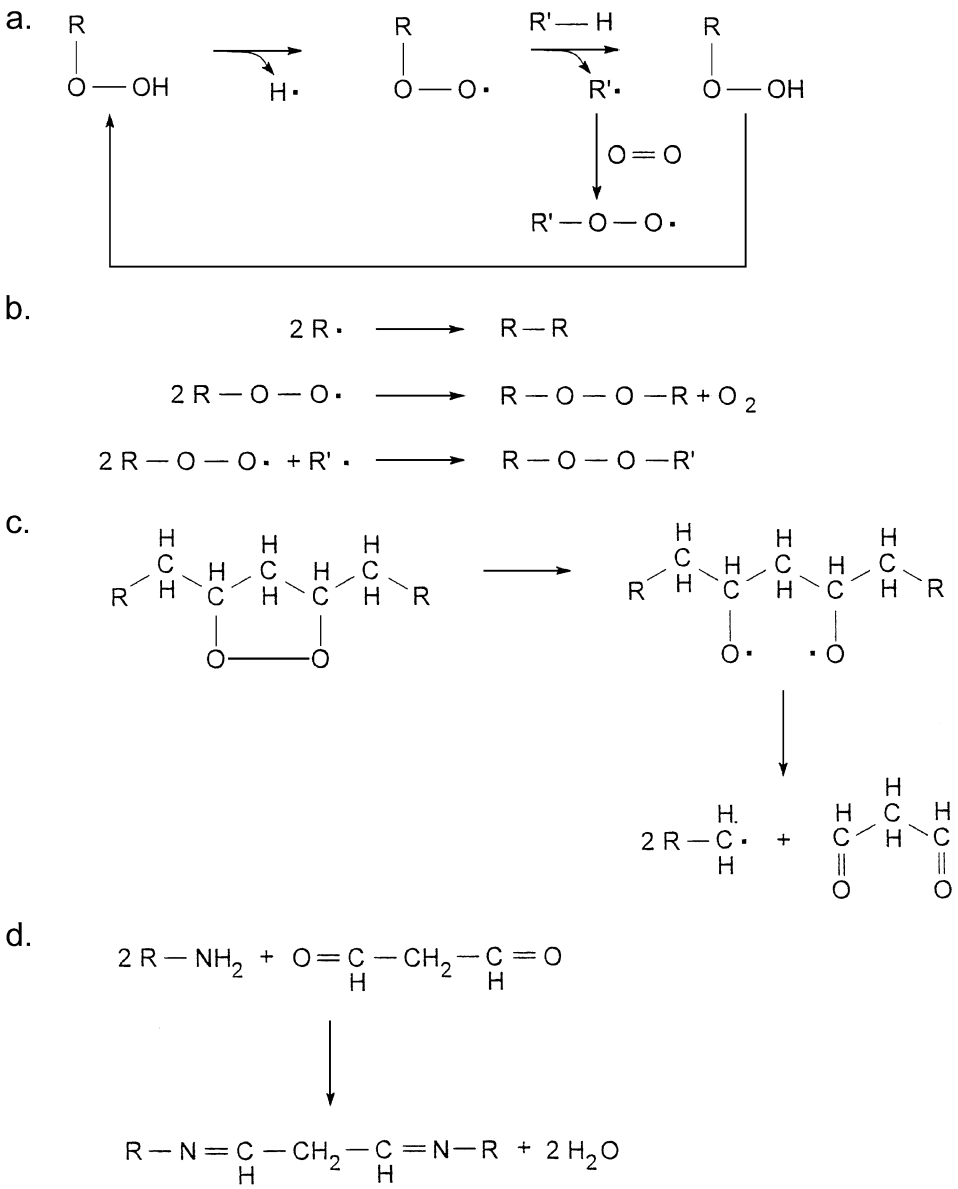


Fig. 91. **a**: Propagation of the peroxidation in a chain reaction. **b**: Termination of free radical reactions in the absence of O_2. **c**: Cleavage of an endoperoxide of an aliphatic chain under formation of MDA. **d**: Crosslinking of two proteins by MDA. Consequences of free radical reactions: isomerisation of C = C double bonds, cross-linking of FA residues by peroxide bridges and of proteins by malonic acid bridges, displacement of double bonds in fatty acids, cleavage of FAs to carbonic acids, formation of water by removal of H· from FAs and proteins, and changes in the membrane structure and its properties (polarity, viscosity, receptor affinities, etc.). Rates of peroxidation: ER > mitochondrion, nucleus > lysosomes > plasma membrane. Flavonoids intercept such reactions by scavenging the free radicals (Cavallini et al., 1978; van Rijn & van den Berg, 1997).

viruses carry enzymes, e.g., neuraminidase or lysozyme, on their surface with which they can hydrolyse protective structures in the plasma membrane of the victim's cells.

The viral genome must possess at least three different genes named pol (polymerase), gag (a gene-regulating protein), and env (envelope) (Fig. 97). In the RNA viruses, which are the most dangerous because they are potentially oncogenic, the polymerase is a reverse transcriptase that can form a DNA chain complementary to an RNA, thus breaking the formerly central dogma of molecular biology that DNA makes RNA, which, in turn, makes protein.

Once the virus particle has passed the plasma membrane of the target cell, it fuses its lipid membrane with a lysosome. Then, the proteases in the latter hydrolyze the protein capsid around the viral genome and the nucleic acids somehow escape into the cytoplasm. They reach the chromosomes through a nuclear pore. If it is an RNA virus, the pol gene delivers the reverse transcriptase that another virus may have provided during a previous infection of the host cell, and synthesises a DNA-strand complementary to the viral RNA (Fig. 97). The viral DNA is then incorporated into the cellular genome. The DNA viruses do not need the

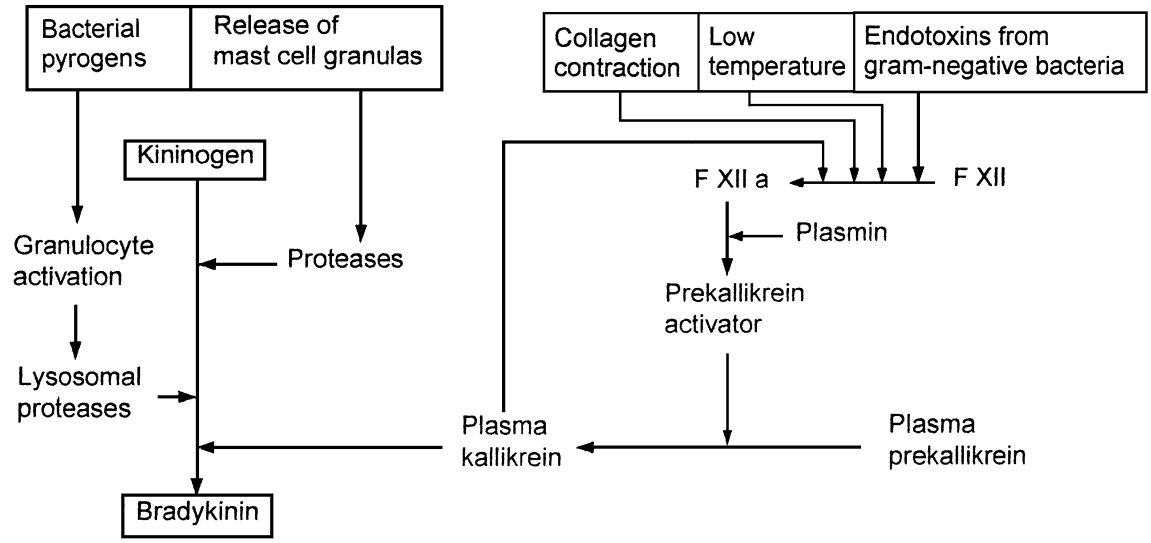


Fig. 92. The mechanism of kinin liberation: F XII (Hagemann factor) and F XIIa (activated F XII), which also enhances fibrinogenesis, fibrinolysis, and complement activation. The latter influences inflammation by liberation of chemotactic peptides. Flavonoids inhibit the degranulation of mast cells and granulocytes, as well as the rupture of the latter (Till & Thielmann, 1989).

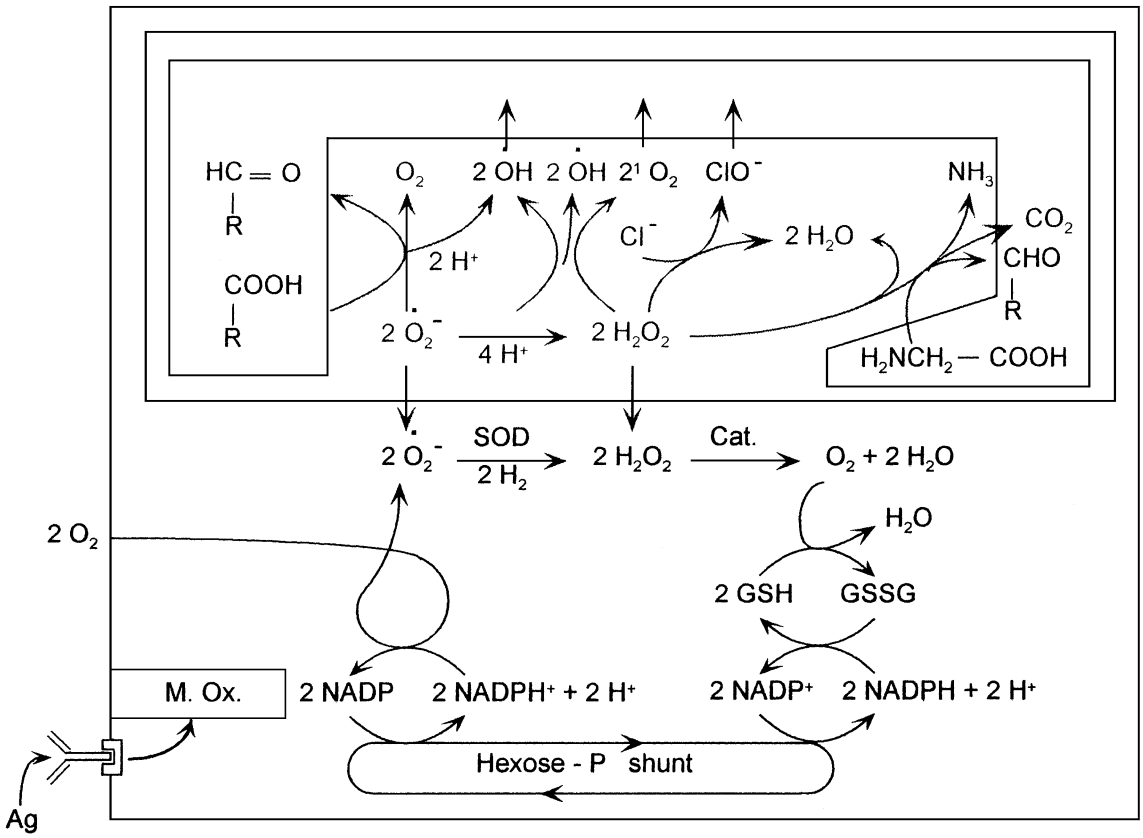


Fig. 93. Oxidative metabolism of phagosomes in phagocytes. Phagocytic cells, e.g., macrophages and granulocytes are, in inflammation, activated by antigens, lectins, auto-antibodies, etc. The receptor activates the membrane-bound NADPH-oxidase producing superoxide ($\cdot O_2^-$), which with H_2O_2 forms a hydroxyl radical ($\cdot OH$) and $\cdot O_2^-$. Likewise, $Cl^- + H_2O_2$ forms hypochlorite (ClO^-). The results are peroxidation and chlorination, which destroy infectants. H_2O_2 is inactivated by catalase and peroxidase using GSH and NADPH as reductants, as well as by amino acid oxidation. The reductants are generated by the hexose phosphate shunt. It is also used to reduce O_2 to $\cdot O_2^-$. Ag, antigen; cat, catalase; GSSG, oxidized GSH; $^1O_2$, singlet oxygen (activated); OX, membrane oxidase; POX, myeloperoxidase, Flavonoids scavenge the free radicals, thus preventing extensive destruction (Pincemail et al., 1988; Das et al., 1984). Adapted from Till and Thielmann (1989).

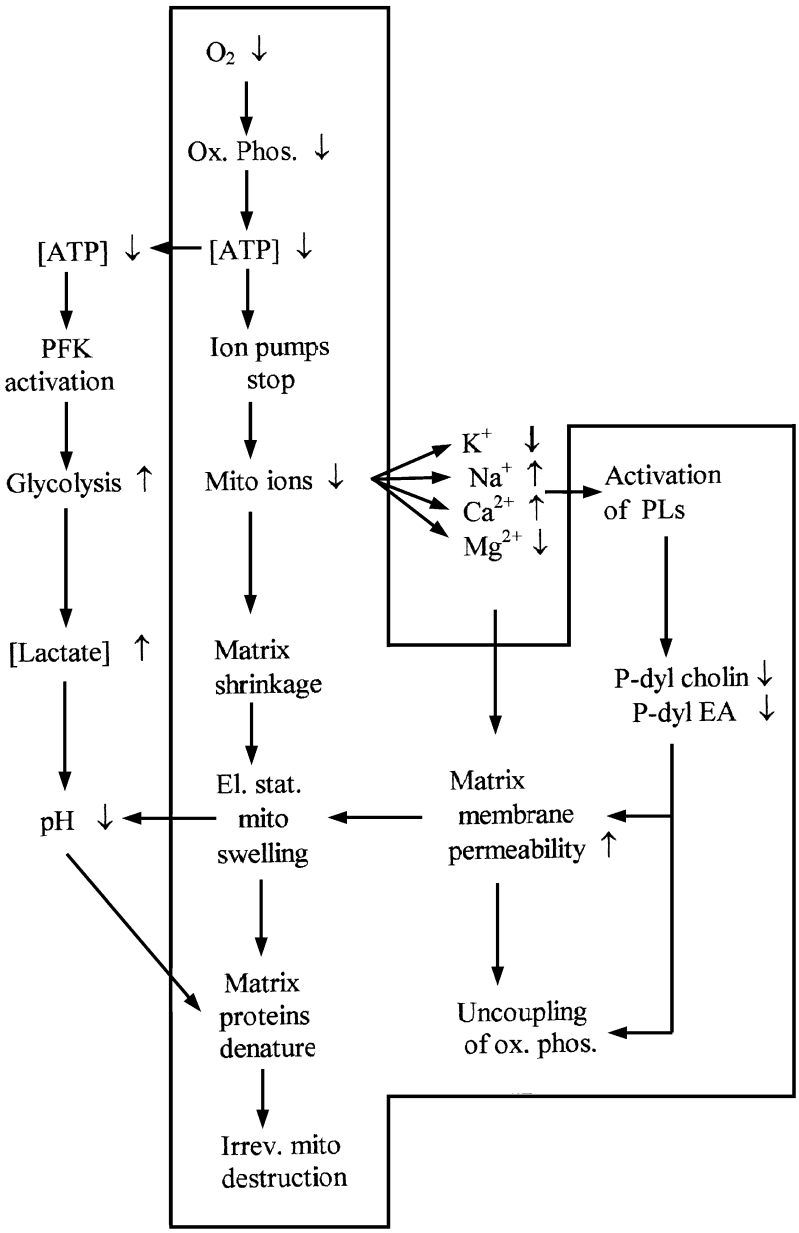


Fig. 94. Hypoxic damage. Via protein phosphatases, flavonoids can restart ion pumps and inhibit phospholipases, thus reducing hypoxic damage (Till & Thielmann, 1989). The area inside the box represents the mitochondrial compartment (mito). The area outside the box represents the cytoplasm. Ox. phos., oxidative phosphorylation; El. stat., electrostatic; Irrev., irreversible; P-dyl EA, phosphatidylethanolamine; PFK, phosphofructokinase.

reverse transcription, but insert their genomes directly into the chromosomes of the cell with the aid of enzymes from the host.

Now the integrated viral genome can enter into one of two states, lysogenic or lytic. In the former state, the viral genes may remain silent for a prolonged period, but upon adequate provocation, perhaps a second infection or an irradiation, it becomes active, i.e., it enters the lytic state. In the latter, the viral genome takes command over the metabolism of the cell. It uses the enzymes and the substrates in the cell to provide energy and building blocks for new viral particles. These processes deprive the cell of its essential substrates. Therefore, the infected cell starves and suffers an early death, while scores of new virus particles leave the dying cell. Alternatively, the infected cell becomes "immortalised" and provides a source of new viral particles during its extended life time.

Evidence has been presented that shows that substances closely related to flavonoids inhibit the fusion of the viral membrane with that of the lysosome (Miller & Lenard, 1981) (Fig. 98). Veckenstedt and colleagues have also observed antiviral effects of flavonoids (Veckenstedt & Pusztai, 1981; Veckenstedt et al., 1978, 1987; Guttner et al., 1982; Choi et al., 1999). Therefore, the many claims from lay medical practitioners of the prophylactic effect of flavonoids against viral attack have substantial support (Béládi et al., 1977,

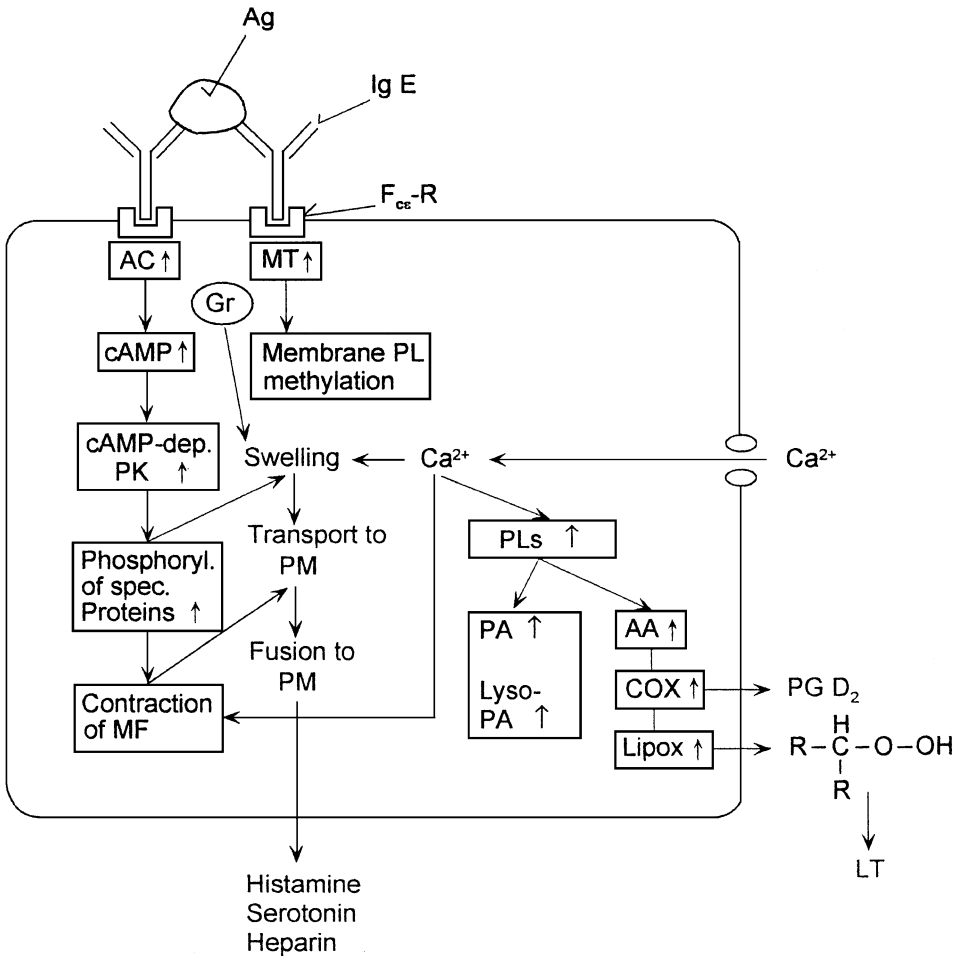


Fig. 95. Mechanism of mast cell activation: Stereospecific dimerisation of receptors for the Fcε-fragment of IgE (Fcε-R) by a polyvalent allergen (Ag), lectin, anti-IgE-antibody, or anti-idiotypic antibody. Lectins are proteins recognizing monosaccharides. Activation by binding of the anaphylactic complement peptides C_{3b} and C_{5a} to their respective receptors or by Ca^{2+} influx caused by lymphokines, toxins, pharmaca, bradykinin, etc. is also possible within 1 min (in vitro). Flavonoids inhibit degranulation, COX, PL, and lipoxygenase (Lipox) (Till & Thielmann, 1989). AA, arachidonic acid; cAMP-dep., cAMP-dependent; Gr, granula; LT, leukotriene; MF, microfilament; PA, phosphatidic acid; Phosphoryl. of spec., phosphorylation of specific; PK, protein kinases; PM, plasma membrane.

1979; Baird et al., 1979; Debiaggi et al., 1990; Ono et al., 1990; Kaul et al., 1985; Wleklik et al., 1988; Mucsi et al., 1992; Mucsi & Pragai, 1985; Amoras et al., 1992a, 1992b; Verma, 1973; Uda et al., 1997; Thaisrivongs et al., 1996; Hagen et al., 1997; Tait et al., 1997). The mechanism of the inhibition remains unclear, but it seems that PGs participate in the fusion of cell membranes. Since flavonoids inhibit their formation, a rationale can be constructed for the protective effect of flavonoids against viral diseases (Nagai et al., 1995a, 1995b; Carpenedo et al., 1969). Besides, the lysosomes can only be acidified and activated by their proton pump, and that can be inhibited by flavonoids (Horisberger et al., 1991; Bertorello et al., 1991; Graziani et al., 1983). However, if the viral nucleic acids have reached the cytoplasm, then it is very difficult to prevent their integration in the cellular genome. In contrast, viral genes, which are imprisoned in their protein capsid, are harmless.

Some flavonoids, e.g., quercetin, have been shown to be inhibitors of the reverse transcriptase of RNA viruses (Ono et al., 1990; Wang et al., 1994; Wacker & Eilmes, 1978; Veckenstedt & Pusztai, 1981; Amoras et al., 1992a, 1992b). This property is very desirable since the currently used inhibitors, e.g., arabinosides and acyclovir, of this enzyme are extremely toxic, not only to virally infected cells, but also to normal cells.

An additional advantage of the flavonoids is that they induce the production of IFNs (Veckenstedt et al., 1987; Wleklik et al., 1987). As previously described in Section 9, these substances have several antiviral effects, including fortification of the cellular membrane; induction of nucleases, which attack the viral genome; and modification of the phosphorylation pattern of the protein translation eIFs, which stop the biosynthesis of all proteins, including those of the virus.

Unfortunately, viral damage of the fetal genome, which is believed to occur in the second prenatal trimester in the case of schizophrenia, cannot be corrected by flavonoids after the outbreak of the disease in early adulthood because important

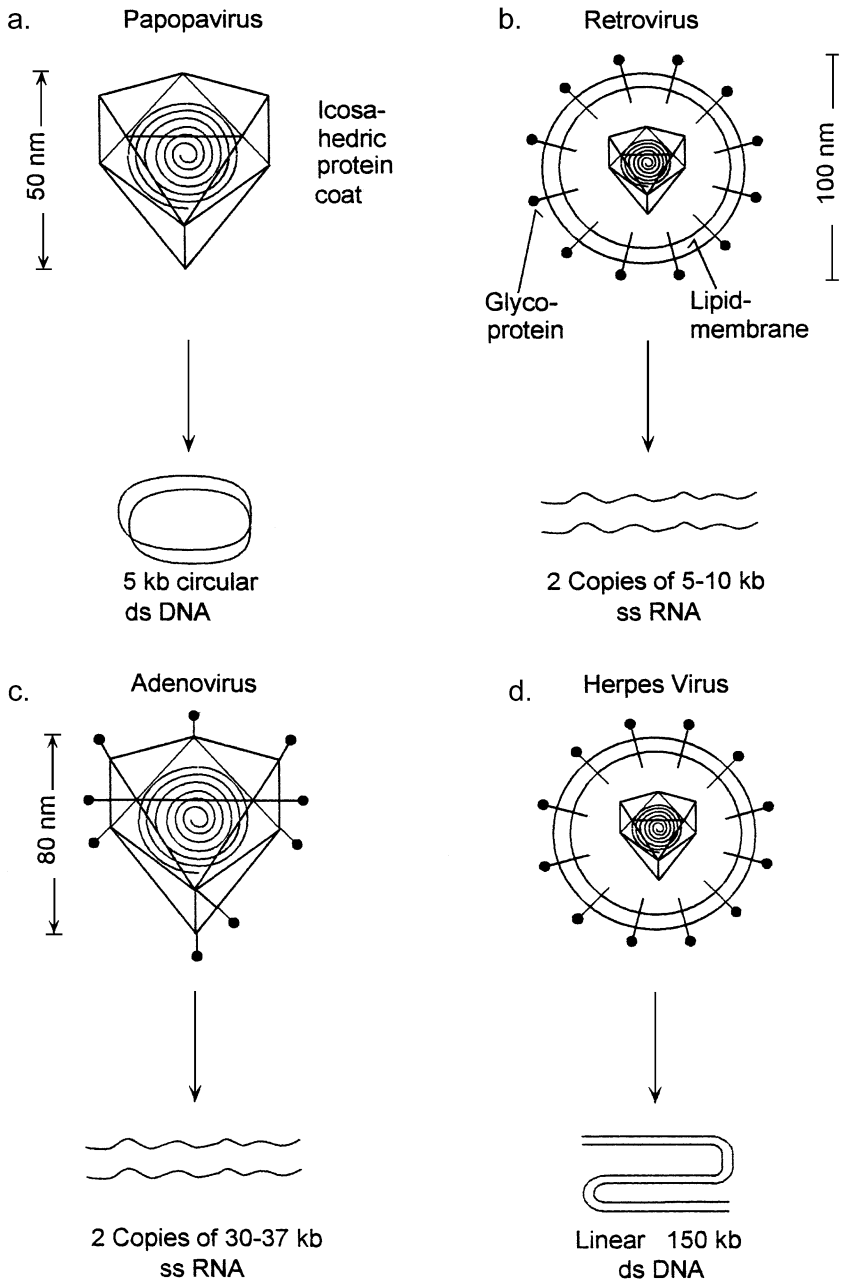


Fig. 96. The structures of some typical virus particles with icosahedral protein capsid. **a**: Polyoma, SIV40. **b**: Rous Sarcoma virus. **c**: Influenza. **d**: Epstein-Barr virus, HIV. ds, double strand; ss, single strand.

brain tissues have failed to develop. Gene therapy of early gametes appears to be technically feasible, once the error has been found.

17.14. Morbus alzheimer's

Morbus Alzheimer's is a disease from which a large proportion of the population suffers, perhaps 10% of those older than 80 years. A genetic predisposition to Morbus Alzheimer's has been located on chromosome 1 (Levy-Lahad et al., 1995). The disease often strikes after the age of 65, but in some cases, it occurs even earlier. Alzheimer's disease (AD) causes the loss of memory, and is extremely debilitating. In medical terms, AD is a selective disruption of neuronal cortico-cortical connections. Its diagnostic hallmarks by the examination of samples from post mortem brain sections are fibrillary tangles and senile plaques in neurons (see Fig. 99). These structures mainly contain the τ-protein and the β-amyloid (Aβ) precursor protein, respectively. The abnormal processing of the τ-protein and the precursor of Aβ is known. It is caused by a genetic peculiarity, which predisposes certain families for AD. In contrast, the reason for the accumulation of the insoluble fibrillary tangles and the senile plaques is not known (Harrington &

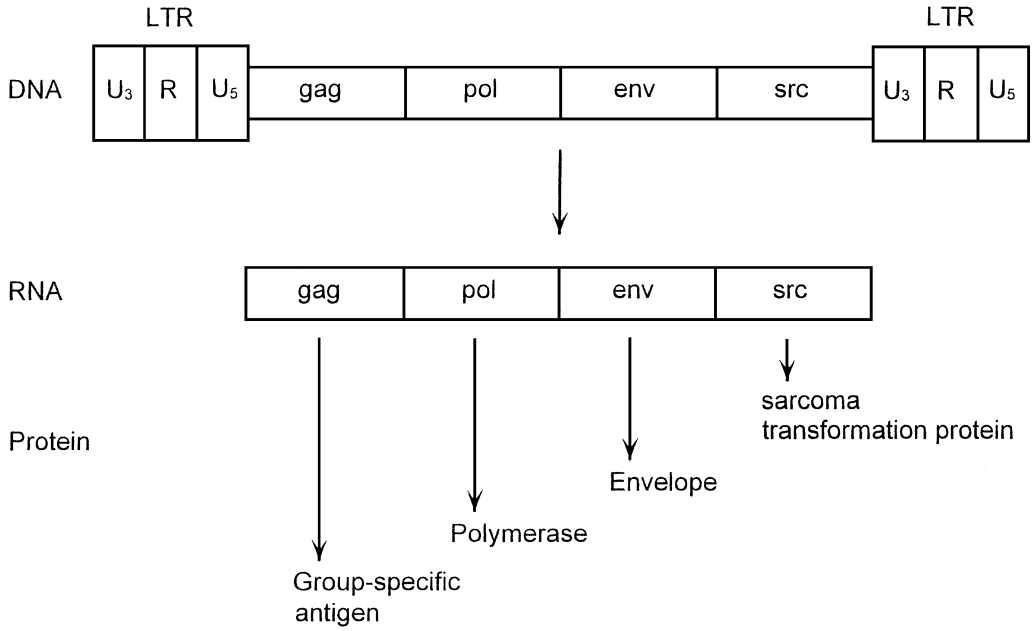


Fig. 97. Structure of the provirus (DNA) form of the genome of an RNA virus produced by the action of reverse transcriptase (Ono et al., 1990). The latter is inhibited by many flavonoids. U_3 and U_5 are different base sequences and R stands for repeats (Till & Thielmann, 1989). The src product is a tyrosine-specific protein phosphokinase. env, viral coat protein (envelope); LTR, long terminal repeat; pol, reverse transcriptase (polymerase).

Colaco, 1994; Games et al., 1995). An additional anomaly has been found recently in a protein lodged in neurons. The physiological consequence of the latter genetic disposition is not known yet, but the altered protein carries the characteristics of an ion channel (seven-transmembrane segments) (Levy-Lahad et al., 1995; de Rijk et al., 1997).

The τ-protein is a small protein, the role of which is to stabilise molecular aggregates of microtubulin (Mandelkow & Mandelkow, 1993, 1998). The latter forms long, hollow microtubules extending from the plasma membrane to many organelles. The energy for the construction of the microtubules is supplied by GTP, which is hydrolyzed to GDP and phosphate. According to current thought, the microtubules are leading small molecules directly from their source to the receiving organelle powered by the ATPase kinesin (Gupta, 1990; Stryer, 1995; Karlson et al., 1993). Organelles can change positions in the cell by the same mechanism. In the nucleus, the mitotic spindles, which sort and separate the chromosomes during cell division, consist of microtubuli held together by the τ-protein (Fig. 100). The origin of the affliction caused by the $A\beta_1$ protein seems to be that small individual variations in the amino acid sequence of the precursor protein alter the point of cleavage by a protease, which normally liberates the Aβ protein. The physiological role of the $A\beta_1$ protein, a proteoglycan, is not known (Baumann et al., 1993). However, extracellular Aβ aggregates can be toxic, e.g., by disturbing synaptic ion channels or by provoking glial cells to excret neurotoxins, especially activated oxygen species and H_2O_2.

A new development in the field is the discovery of the importance of glycation and oxidation of the proteins in the fibrillary tangles and the senile plaques to their insolubility. The latter property subsequently leads to the accumulation that destroys synaptic function (Barger & Mattson, 1995).

The glycation of blood proteins, e.g., hemoglobin, has long been used as an indicator of the state of diabetic

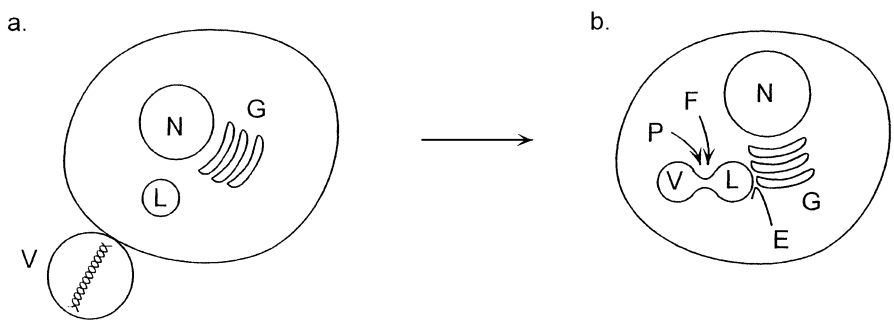


Fig. 98. **a**: Invasion of a cell by a virus (V). **b**: Inhibition of the fusion of the viral membrane with that of a lysosome and of the lysosomal acidification (activation) by flavonoids. E, zymogens in the process of transfer from G to L; F, flavonoid inhibition point; G, Golgi apparatus producing lysosomal enzymes. L, lysosome; N, nucleus; P, H^+ transport ATPase.

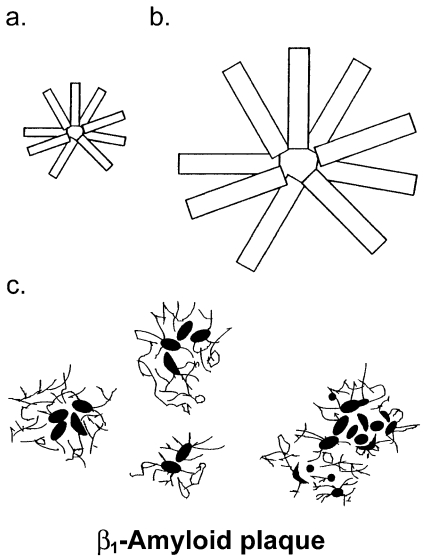


Fig. 99. Structures characteristic of Morbus AD. **a**: Fibrillary tangle of the τ-protein. **b**: Senile plaque of the $A\beta_1$ protein. Sketch of principle. **c**: Sketch of microscopic picture.

patients. If the blood glucose level for extended periods has been high, then the proteins can become irreversibly cross-linked by reactions initiated by the carbonyl group of the monosaccharides. It reacts with primary amino groups, especially the ε-amino groups at the end of the side chain of lysine. The first product formed is a Schiff base (Fig. 101), which can easily be decomposed, but if an Amadori reaction follows, then irreversible cross-linking of the biopolymers can occur. Schiff-base formation is known in the breweries as the Maillard reaction (Koenig et al., 1977). It causes a brown colorisation of the products. The antioxidant effect of flavonoids on hemoglobin glycation has been demonstrated by Asgary et al. (1999).

When these adducts undergo the irreversible Amadori rearrangements to Heyns products, oxidative steps are involved that produce acetyl-lysine and pentosidine. The latter is routinely measured in the blood of diabetic patients. The carbonyl group may arise from proteins, lipids, nucleic acids, or carbohydrates by auto-oxidation. The result in vitro is a protease-resistant and detergent-insoluble product, which seeds the aggregation of soluble $A\beta_1$. There is good reason to assume that similar processes also occur in vivo. Since they are promoted by free radicals, they probably could be slowed or perhaps even prevented by flavonoids (Hensley et al., 1994; Jones & Hughes, 1982; Oyama et al., 1994; Hughes & Wilson, 1977; Paladini et al., 1999). Therefore, it is advisable for elderly people to consume considerable amounts of vegetables and fresh fruit, which are known to be rich in flavonoids.

Another contributor to the crosslinking of proteins is malonic dialdehyde (MDA). It is a product of the peroxidation of unsaturated FAs, a reaction that, due to the intermittence of free radicals, can be inhibited by flavonoids (Cavallini et al., 1978). Hence, the precautions against presenile debilitation should include a reduction of the dietary intake of fat, proteins, and carbohydrates of low molecular weight. Those who manage to stay lean at an advanced age, therefore, often feel fit and remain more healthy.

17.15. Wound healing

Cell damage, inflammation, and regeneration are widespread and frequent events. They are among the most common pathological phenomena. Although these processes can be initiated by a host of different agents, they tend to proceed along the same pathways. Flavonoids can be used very effectively in this complex system of biochemical processes. The latter can only be understood after a review of the cytotoxic agents, their molecular mechanisms, the metabolic derailments, and the action of the signal substances, the mediators, e.g., chemotactic substances, tissue hormones, and components of the immune system. This survey must be restricted to the essentials, since a thorough treatment of this vast subject would exceed the frames of this review. Particularly interested readers are referred, therefore, to other publications, e.g., Till and Thielmann (1989), Kim, R. B. et al. (1999), Kim, H. K. et al. (1999), and Kolaczkowski et al. (1998).

17.15.1. Cellular reactions to damage

Frequent causes of cell damage are physical (e.g., mechanical forces, radiation, heat, and frost), chemical (e.g., acids, bases, free radicals, organic solvents, environmental toxins, and oxygen deprivation), or biological (e.g., parasite infection, and autoimmunity). The point of attack on a cell is usually the plasma membrane, and its reactions tend to be restricted to a narrow range of processes, in spite of the disparity of the provocations (Till & Thielmann, 1989).

17.15.2. Peroxidation of lipids by free radicals

Free radicals are aggressive substances or ions carrying an unpaired electron (see Section 3.6). They are formed in physically restricted locations, such as peroxisomes and mitochondria, within the cell as the result of normal metabolic processes and as products of the radiolysis of water. The cell survives the toxicity of the small amounts of free radicals that are formed in the course of normal metabolism by their confinement to organelles; by the catalytic action of SOD; and by the sacrifice of endogenous reductants, such as cysteine, GSH, ascorbate, tocopherol (Vitamin E), and ubiquinol. However, when free radical production in pathological situations becomes excessive, then additional antioxidants and radical scavengers should be given to minimise the damage to the membranes, enzymes, and genes. Flavonoids are excellent agents for this purpose, but they have to be taken regularly and in considerable amounts due to their short half-lives in the body (1–2 hr) (Yamauchi et al., 1992; Krol et al., 1990; Packer et al., 1999; Hassig et al., 1999; Lien et al., 1999; Ohshima et al., 1998; Vinson, 1998).

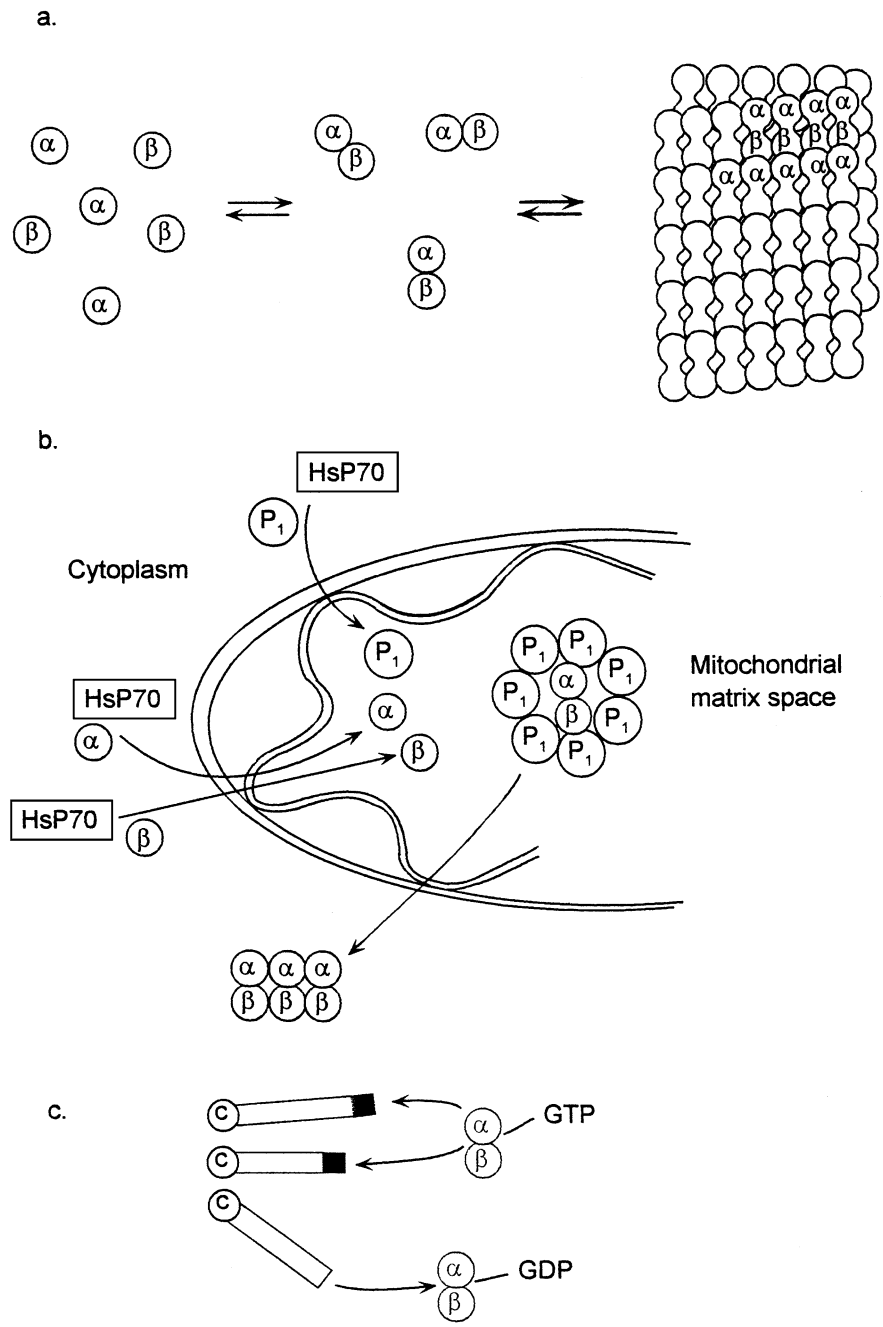


Fig. 100. **a**: Construction of a microtubulus with the aid of the τ-protein and GTP. Alternating α- and β-tubulin subunits joined in heterodimers to form an 18-mer sheet, which folds into a tube. **b**: Assembly of microtubuli at the mitochondrion. HSP70 carries α- and β-subunits, as well as P1 (an HSP), into the matrix space where the αβ heterodimers are formed. The latter are transferred to the cytoplasm (Gupta, 1990). **c**: microtubuli grow by binding of tubulin-GTP, but are decomposed when GTP is hydrolyzed to GDP (Stryer, 1995). C, binding site for tubulin on the centromer.

Lipid peroxidation of cell membranes is considered a key reaction in cell damage. The consequences are perturbations in the transport processes across membranes and secondary effects such as cross-linking of fibrous proteins, e.g., collagen and Aβ, ageing and formation of the neurofibrillary tangles, as well as the senile plaques of Morbus Alzheimer's (see Section 17.14). Apparently, cGMP is involved in macromolecular transport across microvessels of the brain (Joó et al., 1983). This is interesting because the enzyme that catalyzes the formation of cGMP from GTP, guanylate cyclase, is a hemoenzyme and, hence, subject to the influence of flavonoids.

The oxygen diradical ·O=O·, which can be formed in all aerobic tissues, initiates a series of reactions that may be considered as a model of membrane damage. This radical primarily attacks the α-methylene carbon atom of unconjugated double bonds in FAs of membrane lipids (Fig. 101). During the formation of the hydroperoxide of the FA, the

latter transiently assumes the character of a free radical, symbolised by R·, but such species are also products of the direct interaction of a FA with the hydroxyl radical ·OH (Fig. 102). Such radicals arise by the radiolysis of water and in the course of inflammation by the interaction of H_2O_2 and superoxide, $\cdot O_2^-$:

$$H_2O_2 \rightarrow O_2 + OH^- + \cdot OH$$

FA free radicals react easily with oxygen in a chain reaction to form hydroperoxides. This chain is terminated when the supply of oxygen is exhausted by the reactions:

$$\cdot R + R\cdot \rightarrow R - R$$

$$R - O - O\cdot + R\cdot \rightarrow R - O - O - R$$

$$R - O - O\cdot + \cdot O - O - R \rightarrow R - O - O - R + O = O$$

The endoperoxides decompose under the formation of free radicals and MDA. The concentration in blood serum of the latter substance is taken as a measure of the extent of the damage due to lipid peroxidation (Fig. 103).

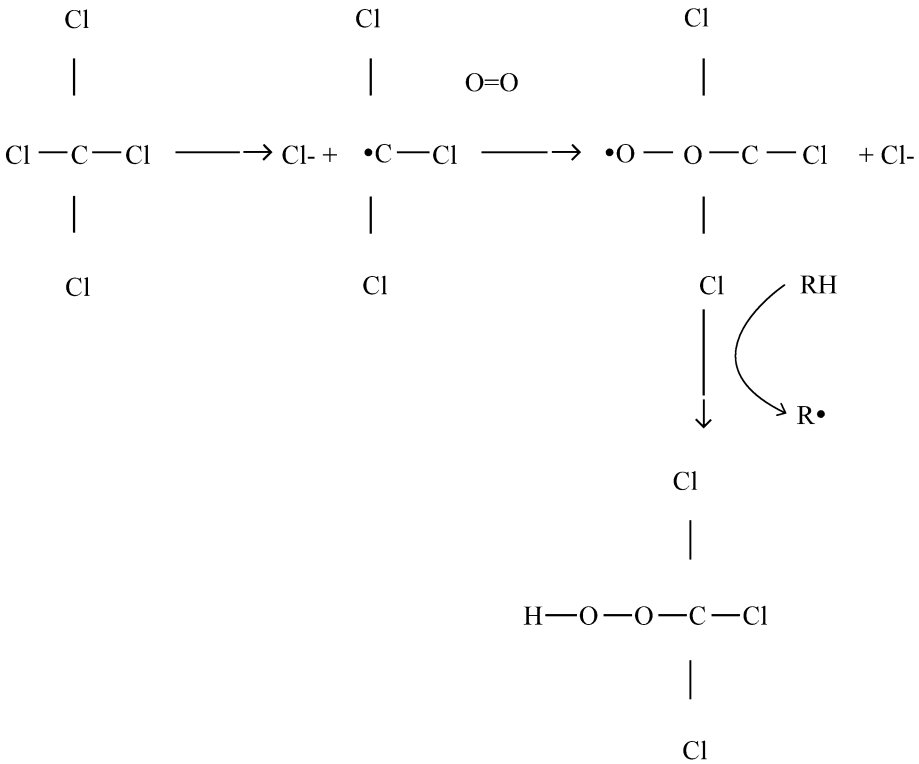
Malondialdehyde can crosslink protein chains, thus hampering the biological function, e.g., of membrane proteins. Such reactions contribute to the ageing of cells (Fig. 104).

Lipid peroxidation is catalysed by heavy metal ions and by UV light. The process leads to cis → trans rearrangements of double bonds; crosslinking of neighbouring lipid or protein chains; displacement of double bonds; cleavage of lipid chains under formation of terminal carboxyl groups; and the appearance of water inside bilayer membranes, resulting in changes in the physical characteristics, such as viscosity and dielectrical constant, as well as in the position, reactivity, and mobility of membrane proteins. In addition to their radical scavenging and antioxidative properties (Hempel et al., 1999; Montanari et al., 1998; Ursini et al., 1994; Tsuda et al., 1996), flavonoids also possess a strong ability to remove the catalytic heavy metal ions (see Section 17.16), and many of the flavonoids probably can activate the membrane bound PLA_2 by induction of a conformational change in the enzyme-membrane complex (Grainger et al., 1989). In this case, the substrate is part of the membrane, but since such complexes are difficult to crystallise in the native state, i.e., in the absence of detergent molecules, it is yet unknown which particular rearrangements are involved in the activation of PLA_2. However, since many such enzymes require a heavy metal ion, e.g., Zn^{2+}, as cofactor, the stimulating effect of a flavonoid aglycone may be due in part or entirely to its ligandation to the cofactor.

The vulnerability of the organelles to lipid peroxidation varies considerably. It is most pronounced for the endoplasmic reticulum, less so for the nucleus and the mitochondria, still less for the lysosomes, and the least for the plasma membrane. Accordingly, this sequence reflects the relative rates of peroxidation and the metabolic changes for which it is responsible (Wang & Joseph, 1999).

17.15.3. Metabolic changes caused by organic solvents

We follow, as an example, the progression of the injury caused by carbon tetrachloride to hepatocytes. Such molecules are decomposed by a free radical mechanism that is analogous to the one described in the previous section:



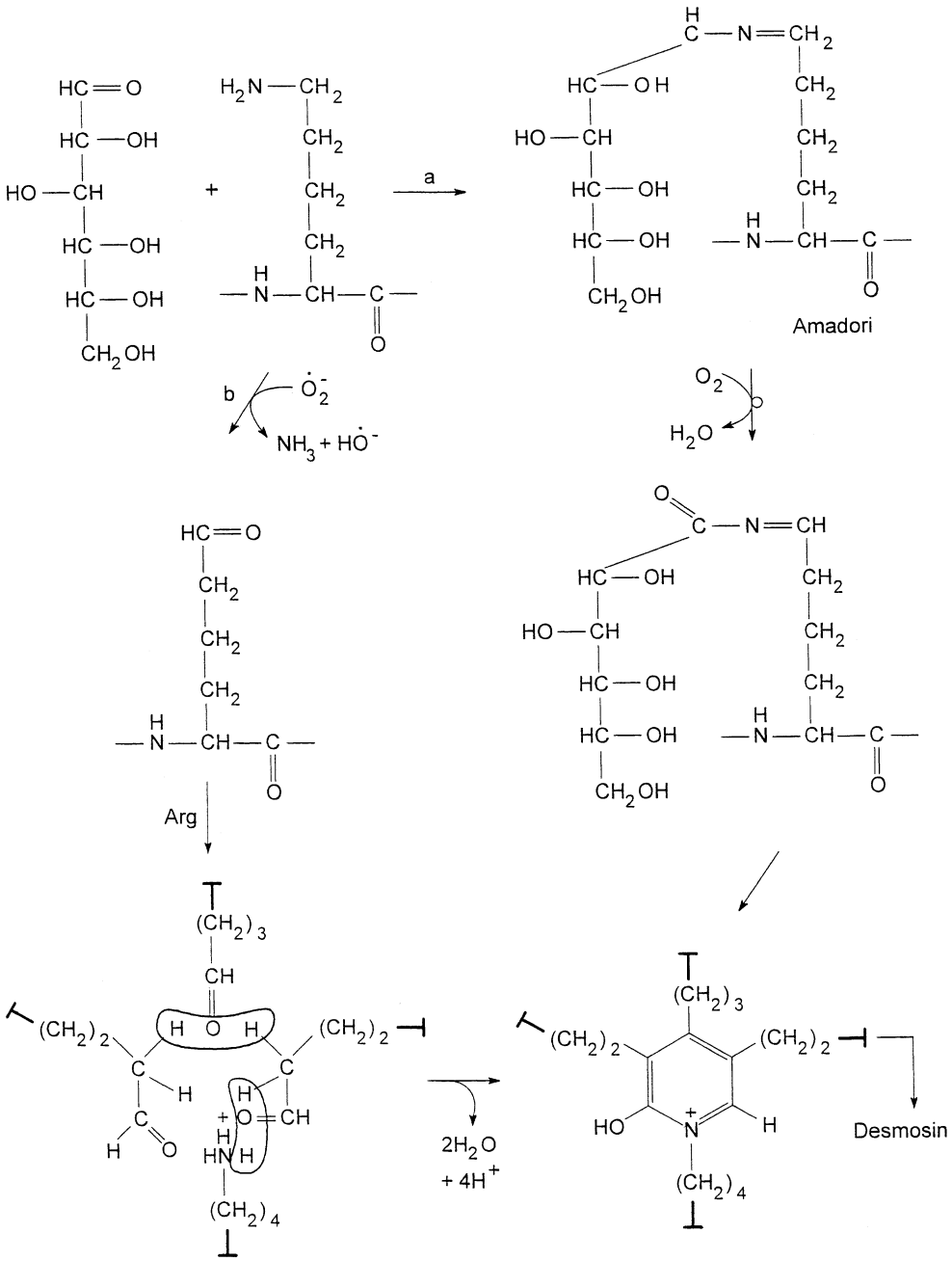


Fig. 101. Protein glycation and AGE product formation. The free radicals can be scavenged by flavonoids (Games et al., 1995; Karlson et al., 1993). a: Schiff base formation followed by Amadori rearrangement and condensation to a covalent hydroxypyridinium bridge. Oxidative desamination of arginine by reactive oxygen species generated by $\cdot O^2$, NaDPH, Fe^{2+}, and monoamine oxidases. Condensation to a pyridinium crosslink.

The destructive side reactions causing damage to proteins and the metabolic machinery are often fatal, but they can also be counteracted by flavonoids in this case (Perrissoud & Weibel, 1980). The progress of the cellular deterioration is illustrated in Fig. 105.

As long as the lysosomal membranes remain impermeable to the lysosomal enzymes, the cell has a chance to recover. In the early stages of the intoxication, mitogens are released. Hence, the degenerative process is paralleled by a regeneration of the damaged organ (see Section 9). Even intoxicated cells can summon enough ATP to complete a final cell division the early phase of the deterioration.

17.15.4. Hypoxic cell damage

A lack of oxygen leads to free radical chain reactions that are just as harmful to cell biochemistry as those that arise in the presence of an excess of oxygen or by intoxication. The oxygen deficiency may have a respiratory cause, but more often, it is the result of deficient blood flow (ischaemia). Ischaemia can be the result of atherosclerosis, shock, or vascular rupture (e.g., by aneurism or apoplexy). The

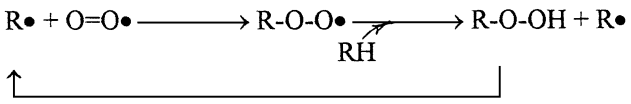


Fig. 102. The formation of hydro- and endoperoxides by the reaction of unconjugated, polyunsaturated FAs with the oxygen diradical.

primary victim of a lack of oxygen is the respiratory chain, i.e., the most important supplier of ATP. The short-term demand for this substance in the cell then increases the substrate phosphorylation that causes lactic acid accumulation and a pH drop, which leads to lysosomal dissolution and irreversible damage. The mechanisms involved are described in Fig. 106.

Unfortunately, a low oxygen tension still suffices to maintain the free radical chain reactions, including lipid peroxidation. While ATP is still available, the XO system is capable of starting such reactions as shown in Fig. 107. However, this initiation can be suppressed by flavonoids that scavenge the free radicals and inhibit XO. Reperfusion, e.g., after conclusion of the transplantation of an organ, also activates the XO system (Fig. 107). Hence, a prophylactic administration of flavonoids also seems to be appropriate in this case (Ito et al., 1995). Another reason for the use of flavonoids in connection with organ transplantation is that an important platelet-activating enzyme, ATPase, is inhibited by reactive oxygen species. This effect accounts for the enhanced tendency for thrombotic episodes in transplantation patients (Lin et al., 1993). However, antioxidants such as flavonoids keep this enzyme active and strongly reduce the risk of hyperacute tissue rejection in experimental animals. The relevance to human patients has yet to be proven (Roush, 1995).

17.15.5. Tissue regeneration

Some flavonoids are claimed to support the regeneration of tissues after damage (Rohwedder, 1987; Ronzère et al., 1981). However, this process is so complex and incompletely understood that the assignment of specific mechanisms to the effects of flavonoids is only possible for a few of its aspects. For millenaries, flavonoids have been used as sterilising, local anaesthetic and regeneration-promoting constituents of ointments and smears, e.g., produced from propolis or honey, to cover, protect, and heal open wounds. The bactericidal and antiviral properties have been described already in Sections 17.12 and 17.13, whereas the analgesic effect has been explained in Section 17.4. All experiments on pain relief through flavonoids, to the knowledge of the author, have been conducted so far on pain from insults to tissue at or near the body surface. The more difficult problem of the deep pain from viscera or bone, e.g., due to a tumor or to surgery, is at present usually treated by opiates given orally or by perithecal infusion at the ganglions near the spine. It is yet unknown whether an intravenous injection of a water-soluble flavonoid, e.g., hydroxyethyl rutosides (see Section 17.1), can replace or reduce the need for the administration of opiates, but it is an interesting possibility since flavonoids do not give rise to significant side effects or dependence.

17.15.6. The anabolism

The reconstruction of the damaged tissue requires the coordinated action of a large number of biochemical systems, the nature of which depends on the presence or absence of contaminating toxins in the wound. Flavonoids can kill or pacify many bacteria, viruses, and other toxins (Guttner et al., 1982; Metzner & Schneidewind, 1978; Bankova et al., 1988). Moreover, they counteract further decomposition of connective tissue, e.g., by collagenases and elastase, since they inhibit PG COX (COX or PGH_2 synthase; see Section 12.2), which produces eicosanoids that via a plasma membrane receptor and a signal chain, induce the expression of protease genes (Wahl et al., 1990; de la Puerta et al., 1999; Mantle et al., 1999). However, the flavonoids perhaps should be given transiently and not continuously over an extended period, since some of the auxiliary, non-anabolic processes, e.g., the chemotactic attraction of phagocytes, that combat invading toxic agents require eicosanoids as guides.

Another auxiliary system, which operates in the wound, is blood clotting. In its venous branch, fibrinogenesis is not significantly affected by flavonoids, although structurally related coumarin derivatives (e.g., Marcumar) are classical inhibitors of this process. They prevent the reduction of the oxidised form of vitamin K (Fig. 108) (Dowd et al., 1995). The arterial branch of thrombogenesis, however, is influenced by flavonoids, since it depends upon an irreversible aggregation of thrombocyte shadows, which, in turn, is strongly favoured by TxA_2 that also causes local vaso-

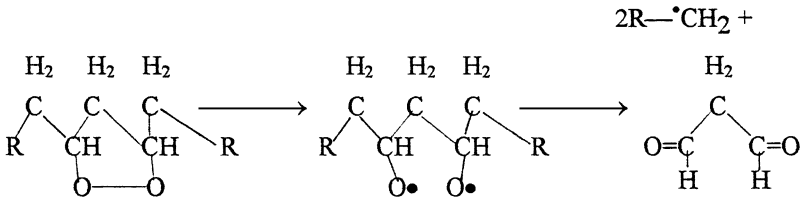


Fig. 103. Formation of malonic dialdehyde.

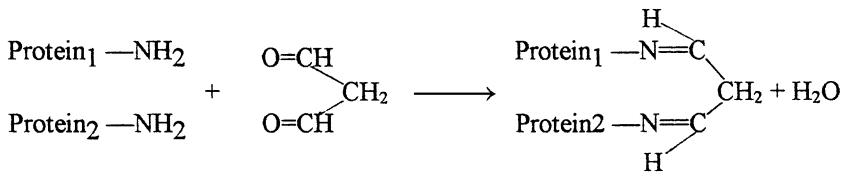


Fig. 104. Crosslinking of proteins by malonic dialdehyde.

constriction, which prevents excessive bleeding. The effect of TxA_2 on vascular tone is counteracted by prostacyclin (PGI_2) from endothelial cells, since PGI_2 causes vasodilation by raising the cAMP concentration (Fig. 109). Flavonoids are known to reduce platelet aggregation (Beretz et al., 1981; Bourdillat et al., 1988; Goker et al., 1995; Kimura

Duration of Exposure	Metabolic Changes in Organelles
Early phase	Endoplasmatic Reticulum:
	Vacuoles arise, RN-ase is activated, Polysomes disintegrate → Protein synthesis Inactivation of: detoxification enzymes G6P-ase, amylase → CHO-dysregulation P-lipid synthesis stops → TG storage
	Mitochondria:
	Outer membrane ruptures cristae collapse → ATP accumulates in the matrix space due to transporter failure → Lack of ATP in cytosol
~4 hr	
Late phase	Respiratory chain stops, P/O sinks → Serious lack of ATP min. at 12-24 hr
	Cytoplasma:
	Increased glycolytic activity → Lactic acid accumulation ↓ pH drops → further membrane damage ↓ Transmembrane ion gradients collapse
	Lysosomes:
	Destruction of the membrane → Liberation of lysosomal hydrolases ↓ Dissolution of cellular structures ↓ Cell death

Fig. 105. Cellular disintegration in the liver following exposure to chlorinated hydrocarbons. CHO, carbohydrate; G6P-ase, glucose-6-phosphate phosphatase; RN-ase, ribonuclease; TG, triglyceride.

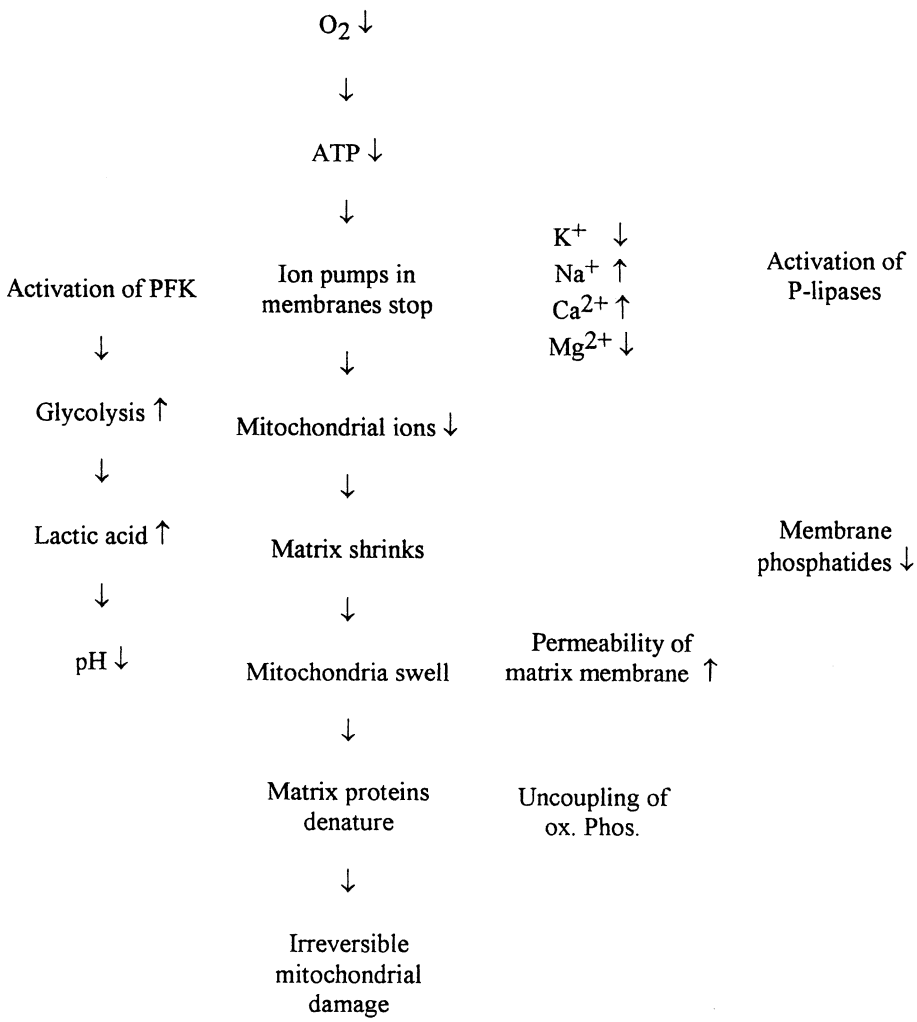


Fig. 106. Deterioration of the energy metabolism due to lack of oxygen. The processes in the boxes take place in the cytoplasm. All other processes shown occur in mitochondria. Arrows pointing downwards signify decreasing concentrations or functions and those pointing upwards increasing concentrations or functions. Ox., oxidative; PFK, phosphofructokinase; Phos., phosphorylation.

et al., 1993; Pace-Asciak et al., 1995; Porcellati et al., 1990; Xiao et al., 1995; Graziani & Chayoth, 1979; Graziani et al., 1981).

Cell damage and the viscous metamorphosis of platelets liberate all known inflammation mediators. Among these, the chemotoxins in the early phase play an important role in

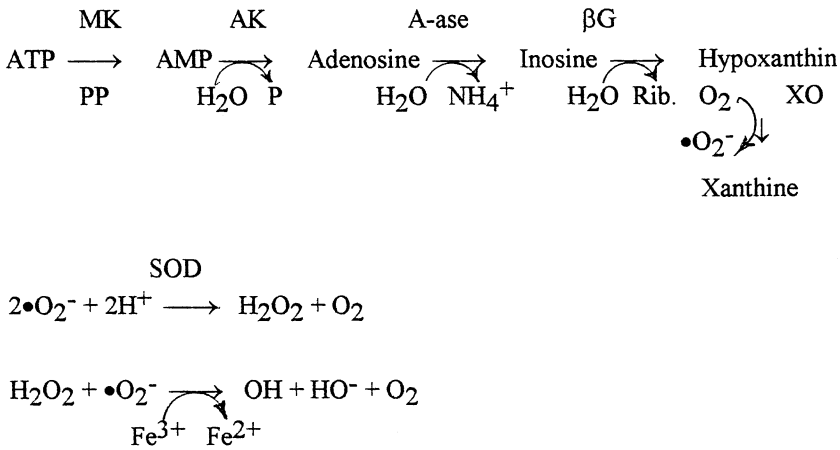


Fig. 107. Production of hydroxyl radicals by the XO system. AK, alkaline phosphatase; A-ase, adenase; βG, β-glycosidase; MK, myokinase.

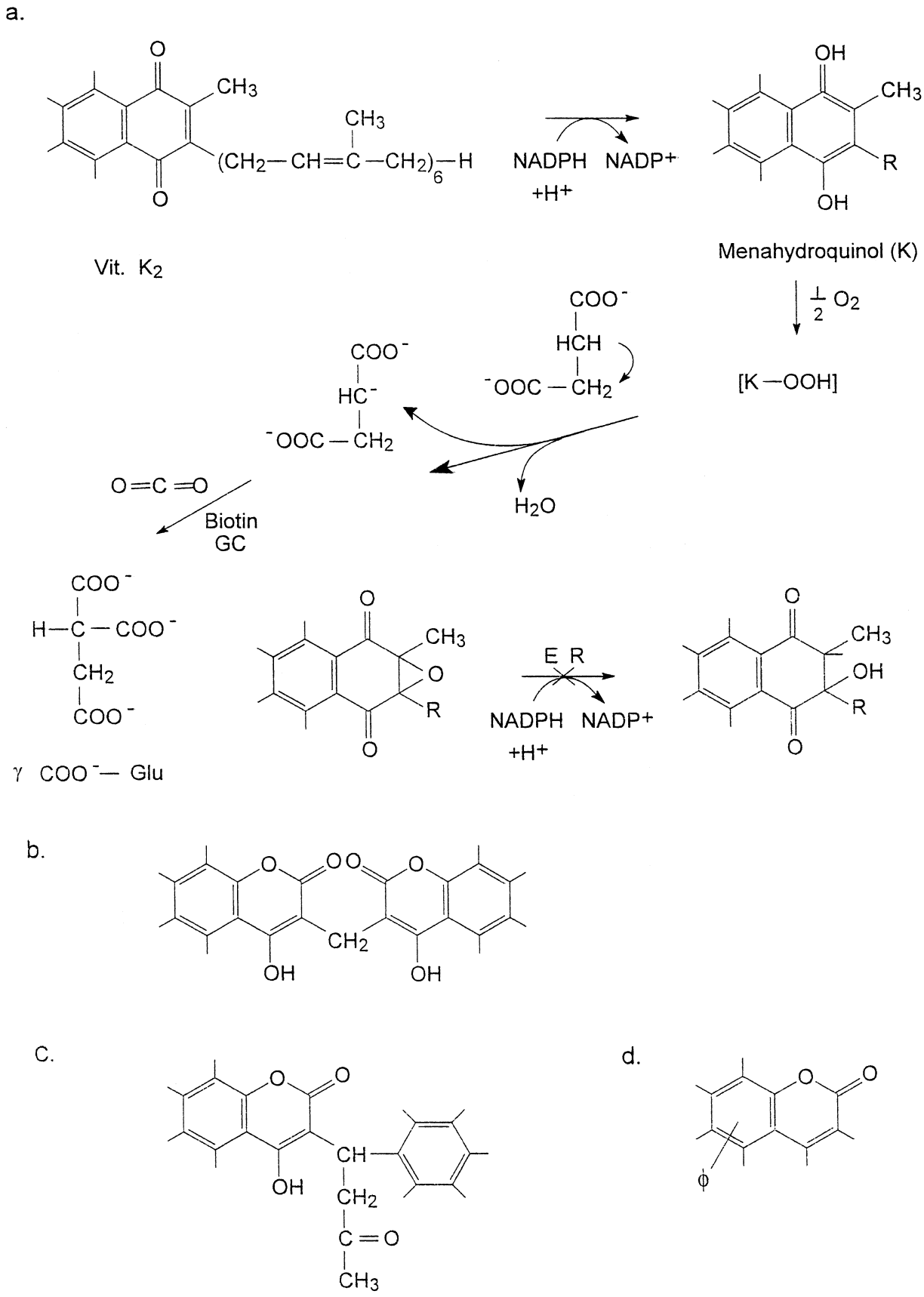


Fig. 108. **a**: Inhibition of the restoration of the active form of vitamin K by interaction of flavonoids or warfarin (at the cross) with the epoxide reductase. **b**: Dicoumarol. **c**: Warfarin. **d**: Marcumar. ϕ, phenyl; γ-COO^-—Glu, γ-carboxy-glutamate; GC, glutamate carboxylase; ER, epoxide reductase.

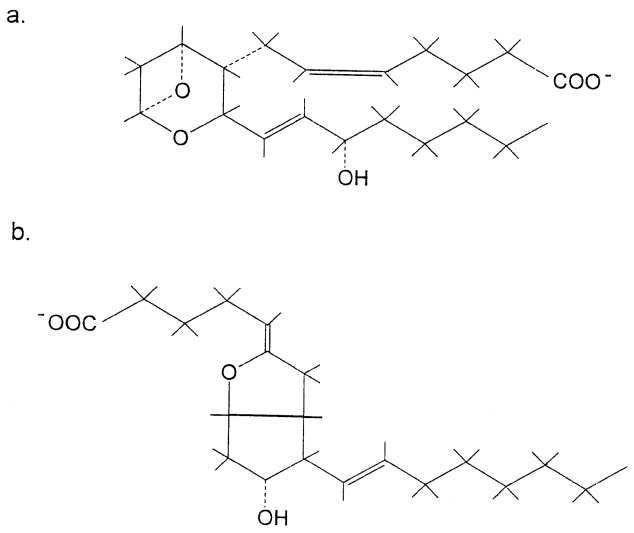


Fig. 109. Eicosanoids involved in the regulation of blood clotting in the arterial branch of the vascular system. **a**: TxA_2. **b**: PGI_2.

attracting phagocytes, which remove the cell debris. Subsequently, connective tissue, fibroblasts, and endothelial cells are allowed to enter the scene. This order of appearance is believed to be controlled by a chemotaxis inhibitor protein released from granulocytes. Vascular relaxation induced by epinephrine and NO (see Section 17.3) and the stress-induced increase in vascular permeability (due to IL-1β → PG → proteinase gene signal chain) mentioned in Section 17.1 increases the supply of substrates for regeneration.

Important chemotactic attractants for the collagen-producing fibroblasts are fibronectin and its fragments, collagen and its peptides, as well as the platelet-derived growth factor, lymphokines such as LDCF-F, tropoelastin, complement factor C_{5a}, and leucotriene B_4. The endothelial cells also produce other matrix components. Fibronectin, which is synthesised in the liver and secreted into the blood, binds to fibrin, i.e., it is concentrated in the lesion. Lysosomal hydrolases liberate peptides from fibronectin, which attract fibroblasts by chemotaxis. Fibronectin also binds collagen of all types, as well as endocytes, fibroblasts, and smooth muscle cells, by hydrophobic interactions. The endothelial cells are aligned in this way along the collagen fibrils. Plasma fibronectin is nonadhesive due to its special conformation, but the latter is changed upon its binding to collagen or interaction with sulfated proteoglycans such as heparin.

The wound is covered by epithelial cells due to the interaction of the latter with collagen IV. This process has a mitogenic effect on these cells, which within 1 hr begin to replicate. The zone of proliferation at the edge of the wound progresses at a rate of ca. 1 mm/day and at first forms a thin layer, which subsequently thickens. Within 12 days, angiogenic factors, like the basic form of the fibroblast growth factor, begin the formation of capillaries that improve the supply of nutrients to the wound. When the wound has been closed with a scar, cell proliferation diminishes and myofibrils contract the scar. Infected wounds are repaired more slowly. This emphasises the importance of maintaining the sterility of the wound and justifies the old remedy of applying a smear containing flavonoids, e.g., honey or propolis ointment, to the lesion (Rohwedder, 1987). Metallothionein, to which a growth-stimulating activity is ascribed, is more strongly expressed in the presence of the flavonoid genistein (Kuo & Leavitt, 1999).

17.16. Heavy metal detoxification

Modern civilisation uses large quantities of heavy metals for buildings, transportation, energy transformation and distribution, instruments, and medical purposes. The structural and electronic properties of these metals are indispensable, but the unintended dissipation of heavy metals in urban areas and in the environment in the form of polluted air, water, and soil has grown to an extent that threatens all biological life. All living organisms require small amounts of heavy metals and can protect themselves against moderate quantities of these elements, but they are intoxicated when certain concentration limits are exceeded. Furthermore, a heavy metal intoxication is difficult to treat. These elements are easy to absorb and, therefore, by orders of magnitude more toxic when they are present in the ionic than in the free, elementary form. However, once the free elements have entered the organism, they are carried to the liver where they are stored or brought to fat deposits. In both cases, they are slowly oxidised and leaked into sensitive parts of the system, e.g., haemotopoietic tissue, where they inhibit δ-amino laevulinic acid synthase and ferrochelatase, with the result that porphyrin synthesis stops. Such deposits, e.g., of elemental mercury, are very difficult to mobilise due to the hydrophobicity of the storage sites. The structure and properties of the flavonoids predict that they have a good chance of reaching and mobilising heavy metals in such sites. Flavonoids are known to form strong ligand complexes with heavy metal ions (see Section 16 and Thompson et al., 1976), but to the knowledge of the author, so far no experiments of this effect have been conducted outside the laboratory to solve such problems. The growing awareness of the environmental hazards, e.g., from motor exhaust, chimney smoke, and polluted drinking water, increases the pressure on the responsible authorities to develop new methods of decontamination and containment of such agents. Hence, we can hardly afford to leave promising approaches, such as the one offered by the flavonoids, untried.

17.17. Hypercholesterolemia

One of the leading causes of death in industrialised countries is cardiovascular disease, especially ischaemic heart disease and apoplexy. These potentially fatal conditions are usually caused by atherosclerosis, which, in turn, is

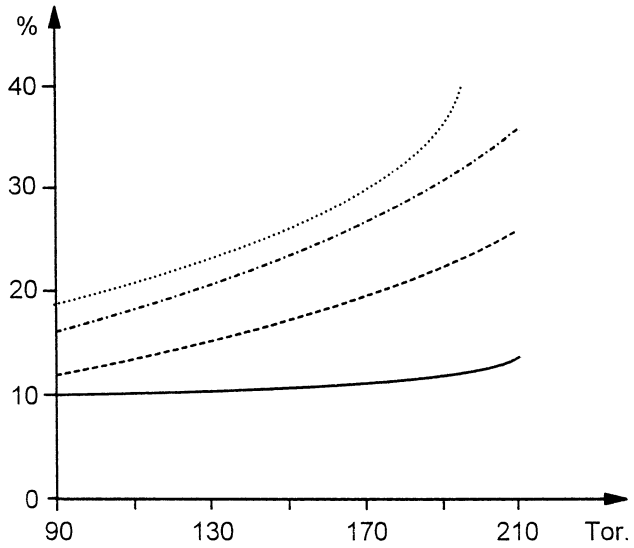


Fig. 110. The dependence of the probability of incurring ischaemic heart disease within a decade for males in the age group of 30–62 years. —, 4.6; – - - - - - - -, 5.7; –·–·–, 7.2; and · · · · · · · · ·, 9.1 mM/L of cholesterol in blood serum (Till & Thielmann, 1989).

strongly correlated with hypercholesterolemia, hypertension, and smoking (Fig. 110) (Brinkworth et al., 1992). The former of these three risk factors is the most serious (see Section 17.1).

Fortunately, flavonoids have proven to be capable of lowering at least two of the three cardiovascular disease-predisposing conditions effectively (Igarashi & Ohmuma, 1995; Leontéva et al., 1979; Kazakov, 1980; Sharma, 1979; Aviram & Fuhrman, 1998). However, before the proven and probable mechanisms of action of the flavonoids on the metabolism of cholesterol can be explained, some basic principles of the role and pathology of cholesterol must be presented.

Cholesterol is an indispensable component of many biological membranes, e.g., because it regulates their fluidity and, hence, their ability to react upon external stimuli by rearrangements, which activate enzymes and initiate transport processes (Bloch, 1983). However, the presence of an excessive cholesterol concentration leads to a stifling of the membranes and their physiological functions, with the ultimate result of the development of atherosclerosis and the occlusion of blood vessels (embolism). Several genetic errors predispose the patients for this condition (Brown & Goldstein, 1984). A common example is the absence or deficiency of LDL receptors, primarily of those on hepato-

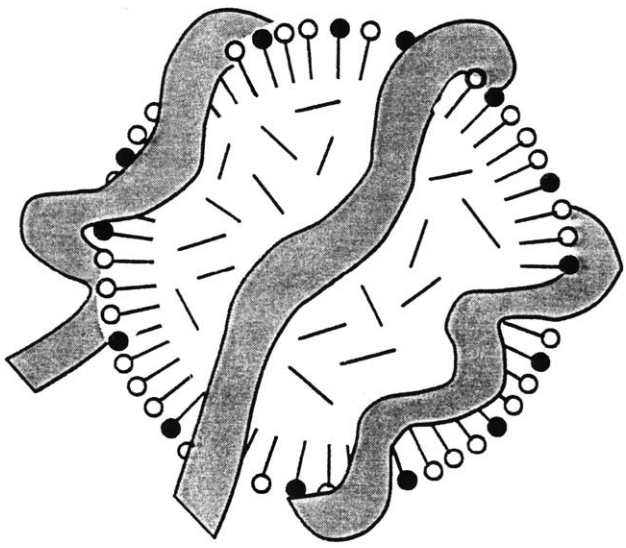


Fig. 111. Schematic model of an LDL. The lipids are wrapped in a protein (B-100, hatched), the conformation of which is yet unknown. A surface structure of this protein docks into a corresponding receptor protein on the surface, e.g., of hepatocytes. Genetic errors causing changes in the cognate protein structures can cause hypercholesterolemia, which leads to the progression: atherosclerosis → embolism → ischemic disease (myocardial infarction/apoplexy) → death. ·–, cholesterol (50% is esterified, primarily with palmitic acid); o-, phospholipids; —, triglycerides. Adapted from Buddecke (1994).

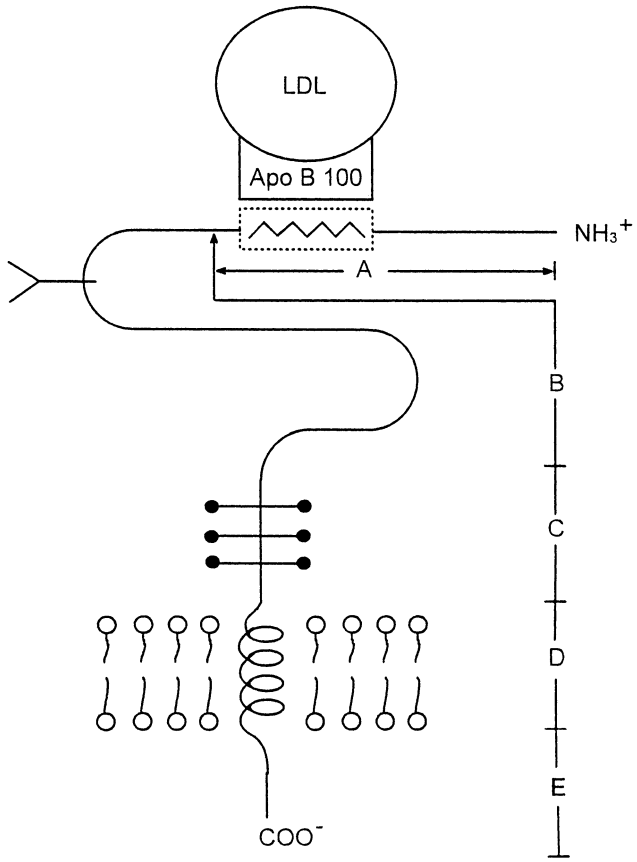


Fig. 112. Model of an LDL receptor in the plasma membrane of a hepatocyte, which consists of the segments: A, negatively charged, cysteine-rich, repeats of 40 amino acid long homologous units, 292 residues; B, N-glycosidic peptide of 350 amino acids; C, 58 amino-acid long, O-glycosidic peptide; D, transmembrane domain (22 amino acids); E, cytosolic, regulatory, C-terminal domain. The latter communicates with a coated pit to induce endocytosis. Apo, apolipoprotein. Adapted from Stryer (1995).

cytes. Due to their hydrophobicity, like other lipids, cholesterol and its ester are carried through the blood wrapped in proteins, which provide the necessary hydrophilicity. These lipoprotein particles are classified according to their lipid content and their density into chylomicrons, high-density lipoproteins, intermediate-density lipoproteins, LDLs, and very LDLs. These lipoproteins can exchange lipids via the liver and thus, interconvert. The forms of particular pathological significance are LDLs, which are positively related to the development of atherosclerosis, and high-density lipoproteins, which resists this pathogenesis. An LDL particle (schematically shown in Fig. 111) carries on the surface a cognant protein structure, which fits into a corresponding protein receptor on the surface of some cells, primarily hepatocytes and gonad cells.

A sketch of the LDL receptor is shown in Fig. 112. Its N-terminal cysteine-rich portion, which is strongly negatively charged due to the presence of many acidic residues, interacts with the positively charged cognate segment of Apo B-100 (Fig. 112). Thus, the acidic environment inside a lysosome severs the electrostatic linkage by protonating the carboxylate ions. Subsequently, the receptor returns to the cell surface in a receptosome, whereas the B-100 protein is digested into small peptides. The penultimate N-glycosylated segment fends off attacking enzymes with antennary carbohydrates. It is followed by an O-glycosylated segment, which keeps the receptor erected perpendicularly to the cell surface, and by a hydrophilic transmembrane segment. The intracellular C-terminal end controls the interaction of the receptor with coated pits, and participates in the endocytosis of the LDL receptor complex (Wilson et al., 1991).

The process of endocytosis has already been described (see Section 9.2). It results in the release of cholesterol into the cytoplasm, from which it is transferred to the Golgi apparatus for esterification and to peroxisomes for oxidation and conjugation to bile acids. The sources of cholesterol for this purpose are endogenous and dietary. Since cholesterol is an allosteric inhibitor of the normal version of a key enzyme in the cholesterol biosynthesis, HMG-CoA reductase, the endogenous production of cholesterol is lowered when the dietary intake of this substance is high. However, inborn errors exist that have abolished this effect and thus, cause hypercholesterolemia. There are several other sites in the apparatus for the uptake and metabolism of cholesterol that often suffer from changes caused by genetic errors in the structure of a pivotal protein. Most often, the LDL receptor suffers from a mutation. The frequency of homozygous inborn errors is $1{:}10^6$ and of heterozygous mutations 1:500.

17.17.1. Treatment of hypercholesterolemia

Patients suffering from homozygous familial hypercholesterolemia usually suffer their first myocardial infarction before they reach the age of 20, and it is often fatal. Such a threat demands radical protective action. In this case, liver transplantation may be deemed appropriate. The much more frequently occurring heterozygous form of the familial hypercholesterolemia takes a milder course, and may be treated conservatively. A desirable goal is a stronger expression of the gene encoding the LDL receptor to increase the number of such protein molecules in the plasma membrane of hepatocytes. The steroid regulatory element gene mentioned in Section 17.8 may be used for this purpose. It is activated by steroids, the intracellular concentration of which is raised by removal of bile acids from the intestine. The bile acids are bound to a cationic ion exchange resin, which is given orally. The resin, loaded with bile acids, leaves the body with the faeces, thus extracting steroids from the body. They are replaced by an increased uptake of dietary cholesterol from the blood into the hepatocytes, where it is oxidised to new bile acids. The result is a decrease in the concentration of cholesterol in the blood and, hence, a diminished rate of catabolism of the LDL receptors (see above). This mechanism can reduce the cholesterol level in the blood by 20% or more. An additional reduction of this level of the same magnitude can be achieved by competitive inhibition of the HMG-CoA analogue mevinolin (lovastatin) (Fig. 113).

A combination of the two approaches removes many patients from the limit of danger, which is believed to lie at around 250 mg/dL, but to depend upon the strength of the individual antioxidant defence. The reason is that the macrophages develop into foam cells. They preferentially engulf oxidised LDL, become sick from all that lipid, and strike the alarm by producing cytokines, e.g., ILs (primarily IL-1), IFN, and active oxygen species (see Section 17.12). The result is an immediate vascular contraction that may close some capillaries and by protracted stress, a tendency to atherosclerosis (Green et al., 1993).

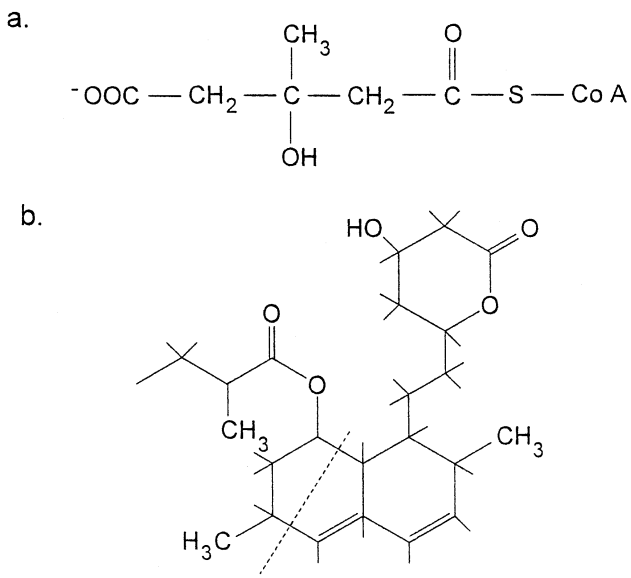


Fig. 113. **a**: 3-Hydroxy-3-methyl-glutaryl CoA. **b**: Mevinolin (lovastatin). Note the resemblance of the portion above the line to the true substrate (a).

17.17.2. Sites of flavonoid action in cholesterol metabolism

The activity of the key enzyme of cholesterol biosynthesis, HMG-CoA reductase, is regulated by phosphorylation/dephosphorylation reactions. It is inhibited by the action of a cAMP-dependent protein phosphokinase, which phosphorylates serine or threonine residues using ATP as substrate (Stryer, 1995). However, since the coenzyme cAMP is cleaved by the enzyme cAMP PDE, which is inhibited by flavonoids (Ferrell et al., 1980; Stein et al., 1999), the consequence is that the cAMP concentration increases and that phosphorylation of the HMG-CoA reductase is enhanced, but endogenous cholesterol production is diminished. In addition, the flavonoids can interact with the enzyme protein phosphatase, which liberates the aliphatic phosphoesters from HMG-CoA reductase, thus restoring the activity of this enzyme. The reason is that all known phosphatases are metalloenzymes, which carry a divalent heavy metal ion cofactor (Zn^{2+}) in their active site, where it is indispensable for catalytic action (Yamanaka et al., 1997). However, heavy metal ions form complexes with suitable ligands, and happen to have a strong affinity for flavonoids. The result is that the metal ion changes its position in its site. In this case, the rearrangement has a negative effect on catalytic activity (Hiermann & Kartnig, 1978). Thus, flavonoids inhibit HMG-CoA reductase by a dual mechanism. However, flavonoids probably also exert their influence on steroid metabolism at other pivotal points. The hydrophobicity of their aglycone suggests that they are carried into hepatocytes by lipoproteins, released from the lysosomes into the cytosol, and bound to the cytoplasmic steroid receptor, which carries them into the cell nucleus. There, the receptor part of the complex is likely to interact with the steroid regulatory element or the flavonoid may intercalate itself between the bases of this DNA segment. Any of these mechanisms may explain the known oestrogenic effect of some flavonoids, e.g., silymarin (Sonnenbichler et al., 1980; Mikziek, 1993; Mitcher et al., 1982; Sani et al., 1993; Cavallini et al., 1978; Plump et al., 1992), and the frequently observed lowering of the blood cholesterol level after the regular intake of the flavonoids (Igarashi & Ohmuma, 1995; Fremont et al., 1999).

The pregnancy-preventative activity of some flavonoids (Santti et al., 1998; Sani et al., 1993) found by mice opens the interesting possibility of an application in humans. Since the toxicity of most flavonoids is much lower than that of, e.g., morning-after pills, the prospect of developing a safe drug for women who have second thoughts the day after coitus seems promising (Grotz & Günther, 1971).

17.18. Stimulation of the immune system by flavonoids

The immunodeficiency diseases dramatically illustrate the immense importance of the immune system for the protection of our health, especially against infection. The inborn error, which inactivates the enzyme adenine deaminase, causes a generalised inefficiency of the cellular immune system in a way that is yet poorly understood. Infants of such heritage are very susceptible to infections, and experience a ceaseless series of influenza, pneumonia, etc., until they wear out and die within a few years, unless they are confined to a sterile tent or receive a complete adenine deaminase gene by transplantation. In fact, this operation was the first example of a successful therapeutic gene transplantation. Since the bureaucratic and technical effort required to carry out such an operation is still high, even in the United States, it is useful to bear in mind that flavonoids kill many infectants or bring about their destruction by activating endogenous defence systems that may still be available (see Section 17.2). In the case of HIV infections, we have to deal with a resourceful virus, which so far has resisted most attempts on its life that could be tolerated by the patient. Theoretically, flavonoids should have an excellent chance of preventing the proliferation of HIV since they inhibit reverse transcriptase, induce IFNs, and inactivate the enzyme that prepares the precursor of the protein building blocks of the viral capsid (Brinkworth et al., 1992). However, to the knowledge of the author, so far no one has attempted to eliminate HIV in this way, neither in vitro nor in vivo.

A clear reciprocal relationship exists between the proliferation of HIV and the decline of T_4-lymphocytes [see Section 17.13, as well as Nowak & McMichael (1995)]. Apparently, the HIV is preferentially engulfed by follicular dendritic cells, which are members of the macrophage family (Heath et al., 1995) residing in the spleen and the lymph nodes. These cells provoke, by mechanisms such as those outlined in Section 9, a hyperactivity of the immune system, which renders it more susceptible to the virus. Over a period of $\sim$10 years of incessant battle, the HIV patient is vulnerable to all kinds of opportunistic infections, which, within a relatively short time, leads to death. Some of these infectants are likely to be sensitive to flavonoids (Ching & Baguley, 1987; Hornung et al., 1988; Wiltrout et al., 1988; Dimov et al., 1991; Wang et al., 1998). It seems that flavonoids, which are much less harmful to patients than the antiviral drugs in current use, could improve the quality of life of those suffering from AIDS. They may even prolong the lives of such patients, but so far, no one has examined the effect of flavonoids on AIDS. In the case of a disease of similar gravity, cancer, flavone acetic acid has been applied with significant result, at least in vitro (Parkins et al., 1993; Ching et al., 1994; Deschner et al., 1993; Evelhoch et al., 1988). As mentioned in Section 17.8, the biological effect of the synthetic compound flavone acetic acid may differ from that of natural flavonoids, e.g., quercetin.

The stimulating effect of flavonoids can be rationalised as follows: PGs suppress T-lymphocytes by inhibiting T_H-cells or activating T_S-cells. (Goodwin & Webb, 1980; Webb & Jamieson, 1976; Barasoain et al., 1980). The flavonoids inhibit the PG COX, thus eliminating the suppression of the immune response.

17.19. The potential of flavonoids in the acquired immunodeficiency syndrome prophylaxis and therapy

17.19.1. Introduction

AIDS is one of the recent epidemic diseases that has been feared the most, because it is insidious, lethal, and incurable. Since the first case of this disease was not recognised until the 1960s, the principal users of flavonoids, the lay medical practitioners, had no experience with AIDS. They also lacked the insight into the nature of this disease that could justify an experimental treatment of AIDS with flavonoids. Hence, the indications of the possible usefulness of flavonoids as preventive, or even therapeutic, agents came from basic medical research, especially on viral infection and proliferation. In the 1970s, Montainer and Gallo isolated and identified a virus, which at first was named human T-lymphocyte-associated virus (HTLV)-III and later renamed HIV (Gallo & Montagnier, 1988). It belongs to the family of lente virus, which also includes herpes and hepatitis viruses. The members of this virus family propagate slowly. Therefore, the infection is difficult to recognize in its early stage. In the case of the prevailing strain that causes AIDS, HIV-I, the virus gains time to hide in a class of T-lymphocytes, which primarily are located in lymph nodes and the spleen. The target cells of HIV-I particles are T_H-lymphocytes. The latter are characterised by the presence in the plasma membrane of a K^+-ion channel named CD4, through which the virus particles enter the cell. This entry is facilitated by a neighbouring lymphokine receptor.

Since HIV particles reach their target cells within seconds after entry into the blood, immune surveillance hardly has a chance to take notice and to respond to the threat until much later when the host cells die and liberate scores of new virus particles. Hence, the disease usually has progressed far before anti-HIV antibodies can be detected in the blood. If the infection is left untreated, the reservoir of T_H-lymphocytes in the body will become depleted over a period that depends on the efficiency of the individual immune defence, but which usually lasts ~ 10 years. Then, the disease enters the active AIDS phase, in which opportunistic infections of many kinds, including bacteria, viruses, and carcinogens, spread unopposed by the destroyed immune system. Eventually, the patients die in misery from a coagulopathy induced by one of the secondary infectants.

AIDS has been one of the greatest challenges to modern medicine, and an unprecedented investment has been made in research into this disease in the past 30 years. Yet, despite all the efforts, AIDS remains incurable, although considerable advances have been made in the suppression of the viral load and, hence, in the quality of life of the patients. Besides, much new knowledge has been gained on the structure of viruses, on viral pathogenesis, and on prophylaxis.

Since HIV is a retrovirus, most attention initially was given to the development of specific inhibitors of the retrotranscriptase that converts the viral RNA genome to the corresponding DNA form, which can be incorporated into human chromosomes. Unfortunately, this process resembles vital cellular processes to such an extent that the effective inhibitors of this enzyme also were very toxic. Hence, they could only be given in low doses, and uninfected cells were simultaneously severely damaged. Interestingly, some of the flavonoids also specifically inhibited the retrotranscriptase in vitro, and they did so without undesirable side effects in cell culture. Since experimental animal AIDS models are rare and expensive, no intact HIV-infected animals have been treated with flavonoids so far.

Another promising point of attack on the life cycle of HIV is the preparation of the precursor of the virus capsid protein. This precursor protein must be freed of a terminal peptide by proteolytic cleavage before it can fold into a conformation that aggregates to a sphere enclosing the viral genome. If this process fails, the viral genes are exposed to endonucleases in the cytoplasm, which soon will destroy them. Specific inhibitors of this viral protease have been developed and used with some success, especially when they were used together with retrotranscriptase inhibitors. Flavonoids capable of inhibiting the viral protease without deleterious effects have been found, but they have not been tested on intact HIV-infected organisms for the same reason as the one mentioned above.

Since flavonoids have been applied successfully in the treatment of a variety of diseases, including such which are components of AIDS, the question has been raised several times whether patients suffering from AIDS could also benefit from these compounds, the toxicity of which is much less than that of common antiviral drugs. Previously, the answer to that question has been hesitant for a number of reasons. First, AIDS patients are often emotionally very sensitive due to the gravity of their illness, the very poor prognosis, and the low quality of life during the unpleasant and expensive, yet ineffective treatment. Second, this disease is often contracted by persons of the lower social strata and by persons suffering from psychiatric diseases that tempt them to seek any pleasure, which is still available, including narcotics, alcohol, tobacco, and sex. Third, the public and, not the least, the AIDS patients themselves are skeptical toward claims of the benefits of simple, inexpensive, and mild drugs after the failure of the intensive search extending over more than 10 years for antibodies capable of eliminating the HIV that is causing AIDS. Despite the lack of therapeutic methods capable of eradicating the disease, significant advances were gained with combinations of drugs, which reached different targets on the virus and the enzymes it encodes simultaneously. Thus, the viral titer could be suppressed for a period of ~ 10 years and the quality of life improved considerably during this therapy. Since flavonoids might contribute to such a development, e.g., by elimination of some of the opportunistic infections, which often become fatal, when the immune defence finally is destroyed by the virus, a brief discussion of the potential benefits of flavonoids to AIDS patients seems to be appropriate.

17.19.2. The origin of acquired immunodeficiency syndrome

Since some Old World primates can contract Simian immunodeficiency virus (SIV), which strongly resembles HIV in its base sequence, the prevailing opinion among epidemiologists and virologists is that HIV arose by a mutation in the SIV genome, which enabled the virus to cross the species barrier from monkey to humans. The first report of a new disease suspected to be viral appeared in the 1950s after a Missionary nurse in Central Africa had contracted a fatal, previously unknown infection, which may have been the result of a bite of a monkey. In retrospect, several similar incidents in that period were probably actually early cases of AIDS, although blood samples were rarely retained and reliable diagnostic tests were not developed until the 1970s. The improved infrastructure (roads, air service, etc.) and the increasing affluence broadened human contact and permitted the virus to spread quickly worldwide. HIV is disseminated with blood, lymph, and sperm, and it is harboured in the cells of these body fluids, where it is protected against most effectors of the immune system, as long as its products are not presented on the cell surface. Therefore, HIV must be destroyed by drugs that are capable of penetrating the cell. Individuals who are particularly susceptible to HIV infection, and subsequently to AIDS, are drug addicts sharing needles, recipients of transfusions with HIV-contaminated blood products that have not been adequately treated by heat, and persons engaging in promiscuous sex. However, normal human contact, sharing of gastronomic utensils, or the use of common towels, etc., is regarded as entirely safe procedures. Since AIDS patients need all available support and consolation, human contact with them should not be avoided.

Previously, fear, e.g., of the threats of the Cold War and traumatic experiences in Vietnam caused many depressions, leading to a life style favorable to an HIV infection. Later, these causative factors were supercede by others, e.g., the misery from natural disasters and failure to cope with the demands of a new political situation. Hence, the potential for a new outburst of the AIDS pandemic exists, although the rate of new infections in most parts of the world recently has slowed. It is apparently not too late to consider the use of flavonoids as prophylactic or therapeutic drugs.

17.19.3. The human immunodeficiency virus gene

HIV belongs to the lenti (slow) family of viruses. Other members of this class are SIV (mentioned in Section 17.19.2), herpes, hepatitis, and HTLV-I and -II. Among the diseases caused by these viruses are AIDS, leukaemia, and carcinoma, as well as liver and skin disorders. The slowness of the infection by this group of viruses explains the insidious nature of the disease. It often escapes diagnosis before it has disseminated to such an extent that it is difficult or impossible to eradicate. HIV is a retrovirus because it possesses an RNA genome. Hence, it cannot infect the chromosomes of the host cell until its genes have been transcribed to the DNA form by a retrotranscriptase. Like all RNA viruses, HIV contains the three genes *gal*, *pol*, and *env* (Fig. 114). The *gal* gene encodes a protein that regulates viral metabolism. More precisely, the gal protein, p17, forms the outer shell of the viral core and lines the inner surface of the viral lipid membrane. It directs viral assembly by retaining env proteins in the vicinity of the viral genome (Matthews et al., 1994). The *pol* gene encodes the polymerase that transcribes the RNA genome to DNA, and the *env* gene encodes a basic building block for the virus mantle. In addition, the HIV genome contains genes for insertion and transcription activation at its ends (long terminal repeats), as well as for surface proteins, e.g., gp120, for a protease performing limited proteolysis of mantle protein precursors and for further regulatory proteins (vif, vpr, tat, rev, and nef). The product of the viral gene *nef* binds to cell membrane protein phosphokinase, which, in turn, removes an inhibitor from the transcription activator NF-κB. The latter moves from the cytoplasm into the cell nucleus, where it stimulates viral replication and suppresses the immune response (Cohen, 1995). The *rev* protein binds to a viral mRNA, thus protecting the latter from nuclear sequestration (Brighty & Rosenberg, 1994). The glycoprotein gp120 closely resembles the MHC II proteins (see Section 9), which mark cells as belonging to the organism, i.e., not foreign. Hence, the patrolling T-lymphocytes can easily mistake a virus-infected cell as normal and endogenous (Maddox, 1991). One of the proteins encoded by the HIV genome is a superantigen, which renders the T-cell autoimmune, i.e., self-destructive. This mechanism contributes to the depletion of T_4-lymphocytes in HIV-infected patients (Marx, 1991; Brighty &

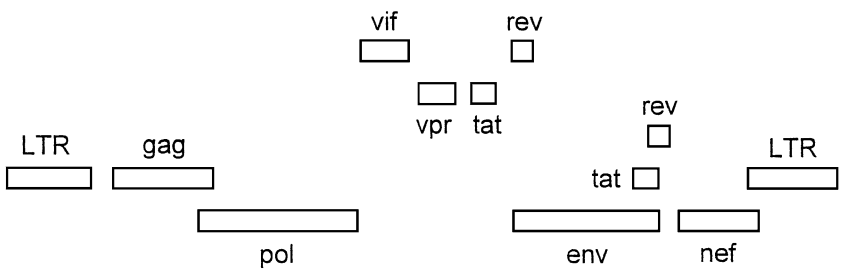


Fig. 114. Structure of the HIV-1 genome.

Table 7
The life cycle of HIV and its control

Steps	Potential or actual drugs
Attachment of virus to cell	Soluble CD4, anti-CD4 antibodies, CD4 peptides, dextrane sulphate
Uncoating of the virus	Hypericin
Reverse transcription	3′-Azido-3′-deoxythymidine, ddc, quercetin
Degradation by RNase H	Illincaquinone
Synthesis of second DNA strand	None
Migration to nucleus	None
Integration	None
Latency	None
Viral transcription	Ro 24-7429
Nuclear transport of RNA	Rev-responsive element decoys
Limited proteolysis of pre-gag	Protease inhibitors such as flavonoids
RNA stability	Antisense molecules
RNA packing, virus assembly	Myristic acid, antisense RNA, ribozyme
Virus release from the cell	IFN-γ
Maturation of the virus	Immunomodulators

Rosenberg, 1994). The viral genes are overlapping in a fashion typical of RNA, but not of DNA, viruses (Gallo et al., 1988; Cocchi et al., 1995). Thus, the genome is compact and difficult to decipher. Yet, in principle, its products offer possible targets for attack by specific antibodies. This approach has been attempted for several decades with limited success of short duration since the rate of mutation of the virus is high.

17.19.4. Possible targets of antiviral drugs

The classical strategy of combating infectious diseases is to inhibit a specific, but vital, part of the metabolism of the parasite using drugs or antibodies. Unfortunately, this method has yielded only minor results due to the high mutability of the viruses, to the high toxicity of the antiviral drugs, and to the scantiness of the virus-specific metabolism. Most of the enzymes and substrates for the integration of the viral genome into the chromosomes and for the viral anabolism are requisitioned from the host cell and, hence, excluded from the list of possible drug targets. Table 7 shows a list of viral functions, which theoretically could be inhibited, and examples of drugs that have been used in the anti-HIV therapy.

Despite the difficulties mentioned, the first attempts to foil viral integration were based on the use of faulty building blocks for the viral DNA, especially Acyclovir (acycloguanosine), 5-azacytidine, and arabinoside (Fig. 115). However, these substances were also accepted by the metabolism of the uninfected cells and proved to be highly toxic. Thus, the cure could be more dangerous to the patient than the viral disease from which he suffered. Therefore, it is interesting that some flavonoids, e.g., quercetin, inhibit the retrotranscriptase strongly in vitro (Bauer et al., 1981; Ishitsuka et al., 1982; Kaul et al., 1985; Simuth et al., 1986). An extension of such experiments to animal models seems to be timely.

Another promising target for a treatment with flavonoids is the protease, which is required to shorten and activate the precursor of the protein that subsequently aggregates to the viral mantle (Fig. 116). It is also possible to demonstrate in in vitro experiments that this enzyme is strongly inhibited by flavonoids. An important, still remaining question is whether or not such a substance is admitted into the cytoplasm of the infected cell and whether it escapes possible resistance mechanisms, e.g., such as those of certain cancer cells (Perez-Victoria et al., 1999; Mitrocotsa et al., 1999; Hooijberg et al., 1997). Several proteases seem to act in concert. Hence, their inhibition requires the simultaneous application of a spectrum of inhibitors of different specificity (Richman, 1995).

The recent discovery of the complicity of a chemokine receptor, CD28 or CCR5, of the plasma membrane of T_4-lymphocytes in the entry of HIV particles has raised the hopes of the development of an antiviral drug that is tolerable even to weak AIDS patients (Hill & Littman, 1996). The chemokines, which are docking at the receptor CD28, are RANTES and macrophage-inflammatory protein-1α and -1β. They are secreted by $CD8^+$ lymphocytes (Cocchi et al., 1995).

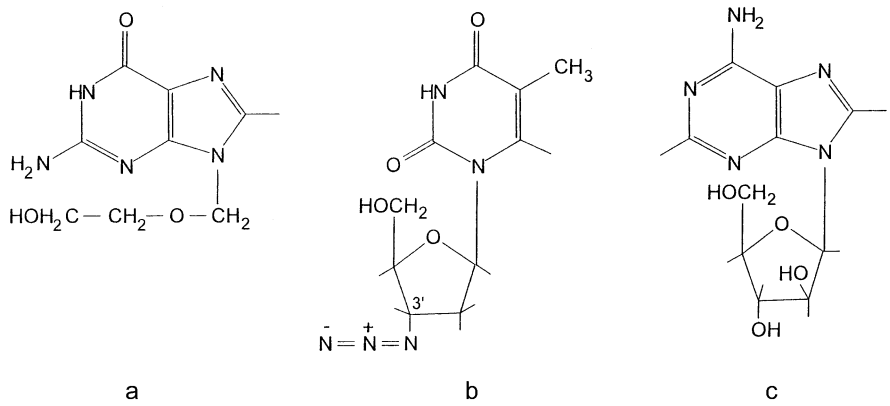


Fig. 115. Structure of some antiviral drugs. **a**: Acycloguanosine (acyclovir). **b**: 3′-Azido-3′-deoxythymidine. **c**: Arabinosyladenine.

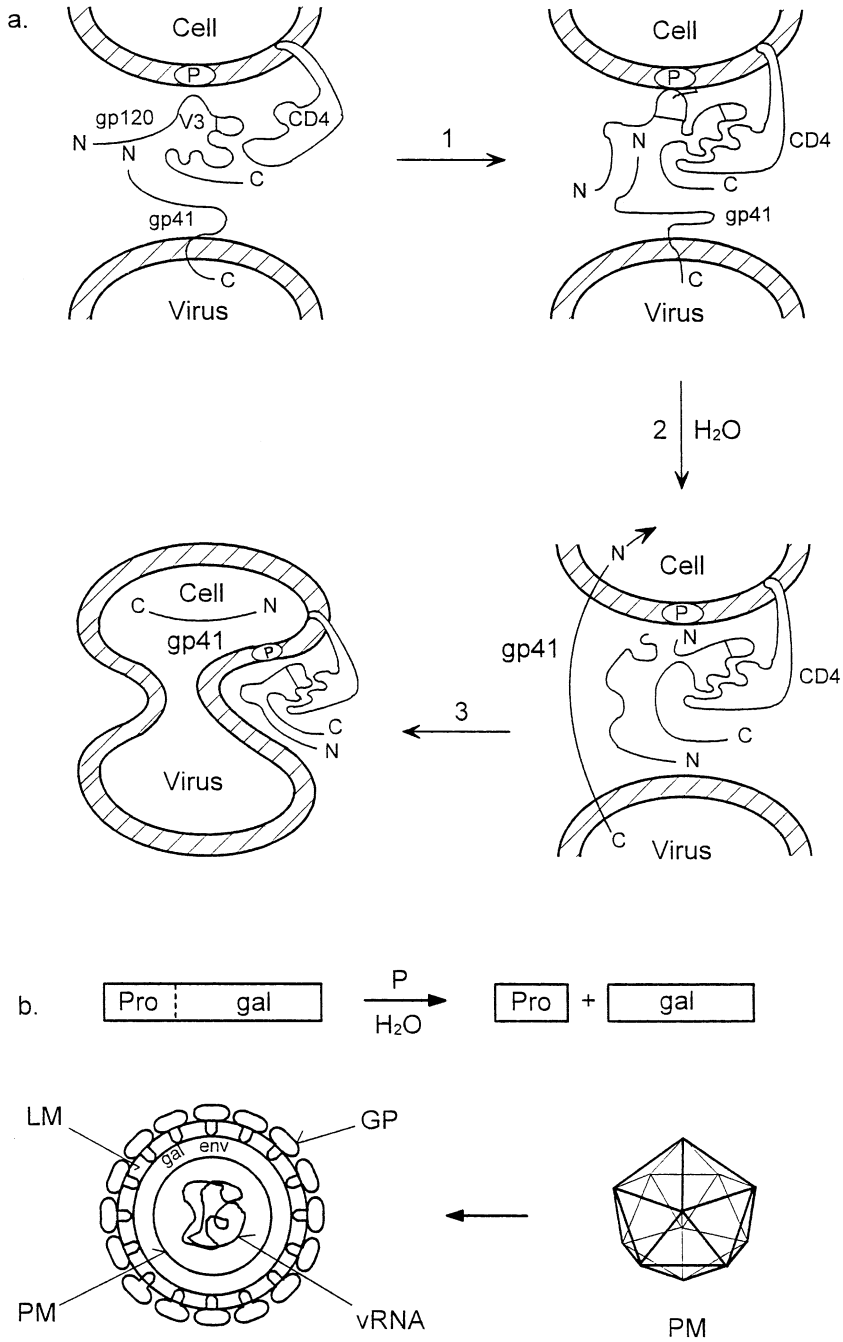


Fig. 116. Examples of the functions of viral proteases, some of which are sensitive to inhibition by flavonoids (Mucsi, 1984; van den Berghe et al., 1986; Vrijsen et al., 1988). **a**: Infection of a T-helper ($CD4^+$-T-Ly) lymphocyte by an HIV-1 virion. The point of entry is furnished by a protease (P) and the protein CD4, which is part of an ion channel that is specific for K^+ ions. The virus touches the cell surface with the glycoproteins gp41 and gp120. The loop, V_3, near the N-terminus of gp120 is juxtaposed to the protease. Step 1: The protease quickly binds to the V_3 loop and the CD4 protein binds the C-terminal segment of gp120. Step 2: The protease cleaves gp120 hydrolytically in the V_3 loop, thus liberating the N-terminal peptide of gp120 and clearing the way for the entry of gp41 into the cell. Step 3: gp41 induces the fusion of the cell with the virion to complete the infection. The cell surface protein CD28 (a chemokine receptor; see text) is also participating in the mechanism of the entry of the HIV virion into the cell, but it is omitted here for the sake of clarity. **b**: The precursor of the HIV mantle protein gal is proteolytically cleaved by a protease that can be inhibited by flavonoids. After removal of the pro-peptide, gal aggregates to an icosahedron, of which it forms the outer surface, whereas the env protein lines the inner surface. This protein mantle (PM) encloses the viral RNA and is surrounded by a lipid membrane (LM), which is garned with glycoproteins (GPs) on the outside. The lipid membrane seems to originate from the plasma membrane of the invaded cell.

17.20. The use of flavonoids in birth control (fertility control)

The world is threatened by a population explosion that can only be controlled medically, since restrictions imposed by the authorities, e.g., severe taxes on children, are insufficient to calm one of the basic human urges, the sexual urge. Therefore, the medical profession is called upon to develop safe methods of reversible, transient sterilisation. Although surgical techniques for this purpose exist, most of the individuals involved prefer the use of drugs. Effective contraceptive drugs and inhibitors of the implantation of fertilised eggs in the uterus have been used for several decades with a low rate of thrombotic accident, about 1:100,000. However, the alarming press reports of these few incidents have scared millions of women, with the result that these measures to counteract the baby boom are resisted.

However, there are other means of contraception. They have existed for centuries, but most of them are long forgotten. These methods were based on herbal concoctions prepared by lay practitioners of dubious, mostly empirical training, but some of these procedures worked quite well. The authorities, which needed more babies, especially male ones that would grow up to become soldiers, banned these witch brews and burned many of their protagonists at the stake. Interestingly, a recent article (Weniger et al., 1982) reports that the flavonoids in a common plant are inhibitors of the implantation of the fertilised mouse egg in the uterus. Such flavonoids may very well have been present in medieval preparations, which were used for population control in the villages. Since flavonoids neither give rise to coagulopathies nor cause neurological side effects such as headaches, except for a few transient allergic responses, the possible use of these compounds for birth control should be investigated. Some flavonoids also inhibit the hyaluronidase in sperm, which facilitates the entry of the spermatocyte into the oocyte (Li et al., 1997).

18. Interaction of flavonoids with other drugs

Drug interactions create increasing problems to the design and control of modern medical treatments, as new insights into pathogenesis lead to the introduction of ever more complex drugs. Thus, the mutual interaction between two drugs or between a drug and a food component, e.g., alcohol, can drastically displace the therapeutic window of one or both of the drugs and, thus, render them useless or dangerous. Although the flavonoids are considered as non-toxic components of common foods, they can influence the pharmacological potency of certain drugs significantly. One of the first examples of this complication was observed in a kidney dialysis ward, in which patients with a stable kidney transplant received drugs that improved blood circulation. Some of these patients consumed grapefruit juice with their meals, but soon after suffered the deleterious effects of the increased potency of their medication. This phenomenon was chosen as the topic of a medical dissertation at the University of Kiel in 1998, and the effect was confirmed in a controlled, prospective project.

This finding has been corroborated recently by Benet and colleagues (Soldner et al., 1999; Wacher et al., 1998; Kim, H. K. et al., 1999; Chan et al., 1998; Ameer & Weintraub, 1997). They found that a component of the juice, probably the flavonoid naringenin, which is present in significant concentration, activates phosphoglycoprotein in the epithelial cells of the intestine and suppresses the expression of the cytochrome P450 3A4 gene. The former protein enhances the uptake from the intestine of many drugs, including vinblastine, cyclosporine, digoxin, fexofenadine, and losartan, and the latter initiates the oxidative decomposition of the drugs. Since the drugs mentioned are used frequently in the therapy of cancer, hypertension, HIV, immune disorders, and other serious conditions, any interference with their bioavailability has to be taken into consideration by clinicians (Wiseman, 1999; Jeong et al., 1999; Di Pietro et al., 1999; Fukuda et al., 1997).

The mechanism of the enhancement of the activity of the drug for improving blood circulation was identified as the inhibition of a cytochrome P450-dependent monooxygenase, which initiates the destruction of the vascular agent by multiple hydroxylations of its aromatic nucleus. A subsequent oxidative step then cleaves the aromatic ring, which renders the product sufficiently water soluble for its excretion through the kidneys with the urine. The site of interaction of the flavonoid with the cytochrome P450 has not been determined yet, but that clarification should be a simple matter since this enzyme possesses several structural features, which are likely points of attack for flavonoids (Ramos & Sultatos, 1998):

(1) It contains several heavy metal ions, e.g., Fe^{2+} and Cu^{2+}, which can bind flavonoids if the metal ion is located in an accessible position near the surface of the protein (see Section 16).
(2) The enzyme needs co-substrates, e.g., tetrahydrofolic acid and NADPH, which bind to the enzyme at sites at which flavonoids by similar enzymes can compete (see Section 12).
(3) The catalytic mechanism of cytochrome P450 contains steps in which there is a transient formation of a free radical. Such radicals are efficiently scavenged by flavonoids (see Sections 3.6 and 10).

Although grapefruit juice contains several flavonoids, naringenin is qualitatively and quantitatively considered as the principal component. Hence, the influence of this compound in its pure form on blood pressure has been investigated. Other studies on different flavonoids, primarily quercetin, have confirmed the ability of some such substances to influence the efficiency of various kinds of medication. Since flavonoids are ubiquitous to food products from plants, the lesson to be learned from these

observations is that nutrition and medication must be considered together in future therapy plans.

19. Prospects of further applications of flavonoids

The use of flavonoids for the prevention and cure of diseases is already widespread. The conscious use of these substances is more common in the developing and in the emerging countries than in the industrialised part of the world. In the latter, the demands by the medical authorities on the proven efficiency and safety of new candidate drugs, for which authorisation is applied, are high and rigid. Hardly anybody opposes such strict rules, but the consequence is that only very few flavonoids are ready to pass the grade yet, because the required tests have not been completed. Besides, the wealth of information on flavonoids in the scientific literature, more than 1000 articles have appeared in reputable journals, has only rarely been reviewed from a medical point of view. Hence, the subject is absent in the curricula of all but a few medical and pharmaceutical schools, and most young researchers in medicine or biochemistry would not come upon the idea or want to risk their career on pioneering work in a virgin field, even if it is promising, when there are enough safe developmental projects from which to choose.

In this situation, most developed countries allow only the sale of products based on mixtures of flavonoids, e.g., labelled as propolis or its derivatives, as food supplements, instead of as drugs. These restrictions are not likely to be eased before much more work on the properties of individual flavonoids has been documented. This review supplies some of the necessary information and encourages work in the field of flavonoids. As stated in Section 4, a great variety of different flavonoids exists, and each of these compounds possesses its own physical, chemical, and physiological properties. Although the latter are overlapping to a certain extend for closely related species, each compound intended for medical purposes must be evaluated by a specified procedure that is time-consuming.

Another problem is logistic. The procurement of flavonoids requires their extraction from plants or bee products, e.g., propolis, or chemical synthesis. Since the physiological concentration of flavonoids in plants, as it is often the case for signal substances, is low and the extraction, therefore, is laborious, an attractive alternative is to use propolis, which contains a much higher flavonoid concentration—an average of 4% by weight. In other words, the bees have done a considerable part of the selection and collection work. A typical propolis sample contains about 25 different flavonoids in significant concentration. Therefore, the extract from propolis contains most of the biochemical properties commonly associated with flavonoids, e.g., the antioxidant effect and radical scavenging. More special physiological effects, such as oestrogenic or contraceptive actions, must be sought in extracts from selected plants. Neunaber (1995) has examined the flavonoid content of various European, Asian, and American varieties of propolis by HPLC. Neunaber also tested the antimicrobiological properties of some of the isolated flavonoids.

The production of drugs by organic synthesis is often preferred when it is feasible because the raw materials are simple and inexpensive; the manufacture can be controlled and automated; and the costs of cultivation, harvesting, refining, and transportation are small or eliminated. About 20 synthetic flavonoids of high purity are already available on the market. More probably could be added since the organic synthesis of some classes of flavonoids is quite simple (Yang, 1980). However, the current price of raw propolis on the market, where it is offered on the scale of several tons, is so low that the synthetic flavonoids can only compete if a pure substance is required, e.g., for experiments or for the treatment of certain diseases.

The possibility of extracting the flavonoids in propolis by a supercritical method has been tried using CO_2. The initial results were not encouraging because the product was an unattractive powder, which did not seem to be usable for further processing. However, it is possible that the problems can be circumvented by a change in the process variables or in the solvent. Supercritical water, which is astonishingly hydrophobic, is a possible alternative to CO_2. These methods are new and require expensive installations. Besides, the problem of separating the flavonoids remains. In the laboratory, it is easily achieved by HPLC or capillary chromatography (Stobiecki et al., 1988; Pietta et al., 1983; Ackermann, 1991), but the experience on the performance of such procedures on the industrial scale is scant.

In the near future, most applications of flavonoids probably would have to rely on relatively few synthetic substances and products based on propolis. If the expectations of successful treatment of a wide variety of diseases with flavonoids are fulfilled, then the market on these substances could soon be empty and the prices of flavonoids products would rise to a level comparable with that of other drugs. The bee keepers, among others, would welcome such a development since their economy suffers from rising cost of feed (sugar) and veterinary service (e.g., to treat Varoa infections) and falling prices of their traditional products, e.g., honey. Bees are indispensable for pollination of grain and other plants that provide food products for human consumption. An increased use of flavonoids (e.g., from propolis) for health purposes would indirectly support plant breeding and, hence, the society as a whole by facilitating the expansion of apiculture.

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