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Reduced glutathione and anthocyans - Redox cycling and redox recycling in biological systems

I Introduction

Structure, form, in-formation and function are closely interrelated - indeed interdependent - at all biological levels. The flow of electrons between electron donors and electron acceptors common to all bioorganic molecules, a process which appears to be strictly determined in space and time by the electromagnetic field energies of the molecules involved, takes place in a milieu governed by finely but differently tuned redox potentials oscillating about characteristic mean levels.

Chemical reactions in general can be looked upon as complex processes in which electrons are "newly" distributed [3, 4]. The location, direction and underlying mechanisms of chemical reactions depend on the distribution of the electrons in the molecules involved in the reaction. As a rule the electrons in molecules are not shared equally; the electron cloud is polarized. Molecular groups with a predominantly negative charge align opposite those with a positive charge. These polarizations can be represented by static and/or dynamic models, the latter probably being a closer perception of a supposed reality [3, 4, 18, 34, 52, 81, 118].

In the static model of polarization the entire molecule is seen as analogous to the strength of the dipole moment. A substituent X is seen as causing a polar C-X bond and interacting with other bonds in the molecule [3, 4].

In the dynamic polarization model complex external fields are seen as interacting with the oscillations in space and time of the molecule itself, a situation allowing us to calculate only an approximate mean polarity as an assumed global molecular constant. Since the two polarity models give similar results, a certain degree of imprecision is obviously involved.

The following transfer mechanisms can be distinguished dialectically in the biological material of living systems (3, 4, 16, 18, 19, 24, 31, 34, 46, 55, 57, 58, 69, 87, 118, 123]:

1) **Induction effect:** Conduction by successive polarization of δ bonds

2) **Field effect (Coulomb effect):** Conduction through space according to the laws of electrostatics

3) **Conjugation effect (mesomeric effect):** Conduction by π-π overlap or overlap of free electron pairs by substituents with π systems

4) Orbital repulsion effects.

Of course, isoelectronic configurations of various molecules (e.g. fluorine and hydroxyl radicals) and the dynamic spin geometrics of nuclide components are a factor despite the BORN-OPPENHEIMER approximation. Where there is an equal number of neutrons and protons, molecular oxygen sports what is called a "double-magic" nuclide.

Redox reactions can be classified into nucleophilic (electron donors) and electrophilic (electron acceptors) reactants depending on their electron status and electron requirement [2, 3, 81] (table 1).

Nucleophilic groups		Electrophilic groups	
Negative ions Bonds with free electron pairs, olefinic double bonds, benzene and other aromatics	} Bases or Lewis bases	Positive ions Substances with incomplete electron shells, acetylenes, carbonyl groups, halogens	} Acids or Lewis acids

Table 1: Classification of some nucleophilic and electrophilic groups, taken from H.G.O. Becker [4]

Nucleophilic and electrophilic reactions always occur in tandem. L-cysteine, reduced glutathione (GSH) and other sulfhydryl (SH) groups are nucleophilic reactants in human and animal organisms. Nucleophilic groups readily react with free radicals, electrophilic molecules and/or reactive oxygen species (ROS), and also - as chelating agents - with toxic heavy metals. Many exogenous or endogenously generated cell toxic aldehydes react with SH groups at body temperature.

These common and preferentially occurring reactions deprive sulfhydryl (SH) groups of important supramolecular, molecular and submolecular regulatory tasks in enzyme and transport molecules, in protein folding, and in redox regulation.

A protein molecule composed of 300 amino acids, for example, with an assumed mean degree of conformational freedom of eight different space-filling states for each component, gives a total conformational capacity of 8^{300} (10^{270}) folding and/or structural motifs. Even for a small protein containing 100 amino acids with a dual state option for each amino acid the number of folding options would still amount to 10^{30} different conformational variations.

An important factor restricting the potential conformations in the three-dimensional arrangement of proteins is the intramolecular and intermolecular development of covalent disulfide cross-links composed of L-cysteine [18, 19].

Free, non-oxidized SH-groups also play an important role in the conformational changes of bioorganic molecules and the weak interactions resulting therefrom. Indeed, free SH-groups must be present in more than stoichiometric amounts since they interrelate via fluctuating thiol-disulfide exchange reactions in an attempt to develop the specific structure for a given biological task [18, 19, 52, 54, 57, 61, 81].

Information-controlled protein biosynthesis involves a high degree of SH group-disulfide dynamism determined by the redox potential of the respective compartment until the relevant SH and S-S situation for the folding of this protein at a defined amino acid sequence is reached; the final structure, i.e. the definitive formation of a disulfide group in a protein, comes at a very late step in the protein folding pathway, especially since a fairly large number of thermodynamically favorable (energetically favorable) molecular states are possible depending on the number of amino acids in a protein molecule [18, 19, 81].

Intermolecular disulfide bridges may form spontaneously in polypeptides which contain both SH and S-S groups. The oscillating interplay of thiol-disulfide and disulfide-thiol reactions is thought to be analogous to the complex conformational fluctuation pattern arising during the three-dimensional assembly of the atoms of a molecule about a median position [81]. This fluctuation is unlikely to be purely spatial, temporal, and mechanical in nature; it is more likely to be a wobble signal that can be tuned in to a specific range, a molecular oscillating system with short-range effects on intramolecular structure and function and on all weak bonds, and long-range effects on other molecules [18, 19, 81] (figure 1).

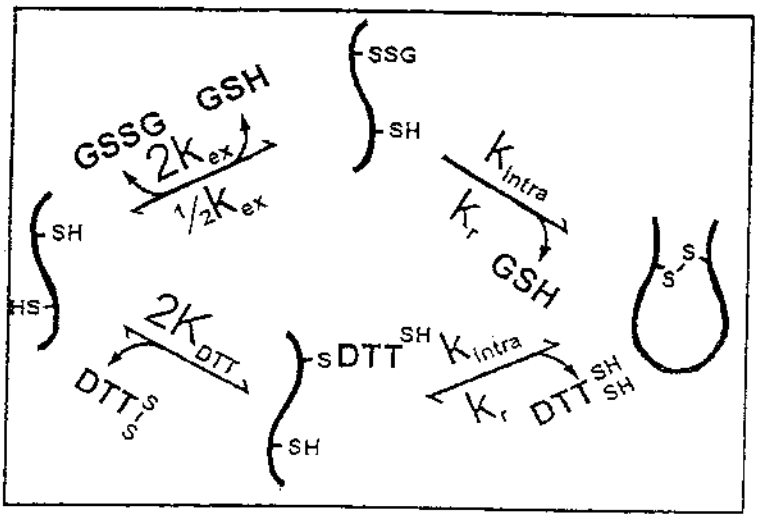


Figure 1: Protein-disulfide bridge formations and bond breaks via the thiol-disulfide interaction mechanism, both intermolecular (top) and intramolecular (bottom) with glutathione and dithiothreitol, taken from T. E. Creighton [18]

Interaction with the extremely sensitively tuned higher-order glutathione system responsible for coordinating the

GSH ⟷ mixed disulfides ⟷ G-S-S-G

non-equilibrium thermodynamic system in each compartment is of crucial importance to the upholding of these SH - S-S exchanges.

Protein thiols and disulfides play a central biochemical role in upholding the structure and function of all cell proteins [21, 29, 31, 54, 57, 62, 64, 81]. They stabilize the enzyme conformations essential to substrate bonding, thus and only thus making it possible for the manifold enzymatic catalyses in biological systems and compartments to take place. The calcium-dependent ATPases present in cell membranes, for instance, are sulfhydryl-dependent enzymes [21].

Elevated oxidation of thiol groups of the cytoskeleton protein actin, to give one example, results in cytoskeletal disruption and the formation of plasma membrane blebs [21]. To protect the function of all vital protein thiols, an extremely high concentration of reduced glutathione is maintained in each compartment by a non-equilibrium thermodynamic system. When a defined concentration of toxic, GSH-consuming xenobiotics is exceeded, the glutathione system becomes overstressed and breaks down, resulting in total degradation of all thiol proteins [21, 29-31, 54, 57, 62, 64, 81].

These pharmacokinetic/pharmacodyamic SH - S-S fluctuations pose an interesting quantum chemical problem with respect to chelate linkage to the topochemi-

cally diverse SH groups. A single metal chelation would suffice to inactivate or interfere with the fluctuations or, in other words, to knock the oscillations off balance [81].

It is interesting to note that oxidation, the energy-giving process driven by structural destruction and culminating in molecular oxygen and reactive oxygen species, is so close to sulfur and selenium on the periodic table of the elements (group 6), thereby highlighting the chemicophysical similarities and electron transfer relationships between the three elements O, S, and Se (figure 2).

These elements, although revealing something of their inorganic origins in the "higher" order of bioorganic molecules (SH groups, disulfide bridges, sulfur-containing amino acids, seleno-amino acids) by their susceptibility to oxidation, are in fact structure-stabilizing, structure-giving and structure-maintaining at the structural biochemical level [81].

(Further SH group problems in the intermediate metabolism in humans, their therapeutic potential and disruptions of the intracellular glutathione system are discussed in:
G. Ohlenschläger, Acetylglutathion - eine neue therapeutische Substanz in der Behandlung von Immunschwächen, Infektionskrankheiten und anderen devitalisierenden Erkrankungen, PRAXIS-telegramm special supplement No 3-4/93, July 94 (Ralf Reglin Verlag Köln)).

II Biochemical and quantum physical oscillations in biological systems

The metabolic pathways regulated and catalyzed by biochemical and enzymatic processes that underlie all living systems oscillate in space and time just as the prime molecule of life, DNA. Alternate inhibitions and activations, information flow and substrate flow are the chemical short-range and short-term regulators that interfere with the gene expression of certain enzymes. The enzyme concentrations at the site of catalysis, which depend on biosynthetic fluctuations, act as long-range and long-term regulators of metabolism by integrated chemical feedback control networks like chemical clocks or pulsators.

Chemical pacemakers are a prime example of dissipative self organization. The complex intermeshed and interconnected catalytic metabolic pathways in the human organism show that, under certain conditions, these pacemakers can be very reliable indeed, but in other conditions are apt to depart on a chaotic course [81].

Benno HESS and Mario MARKUS demonstrated this in their studies on glucose degradation (glycolysis) in the human organism. Although by no means all domains of metabolite concentration are affected during a chaotic process, similar

phase leaps in higher-order, feedback-controlled, oscillating regulatory systems such as the glutathione system may have serious repercussions for specific and general biochemical functions in many or indeed all cells.

The temporal and spatial structure of self-organizing non-linear systems results in a self-organized superordinate dynamism that coordinates the coupling of oscillators to superoscillators and the synchronization of morphogenetic processes at many levels. This is particularly true of the glutathione system [62, 81].

The symbiosis and autotransparency characterizing the autodynamism of self-organizing systems is arguably more important than symbiosis and self-transparency at the morphological level [81]; the phenomenon of self-organization at hierarchically interrelated levels is what sets the scene for autopoiesis and the evolution of a harmonic and intricate whole.

The initial effect of the chaotic disruption of the GSH ⟷ mixed disulfides ⟷ GSSG non-equilibrium thermodynamic system is the breakdown of the host of important regulatory and biomolecular functions dependent upon this system – enzyme molecules, transport molecules, structural proteins, the molecular mechanisms underlying gene regulation, etc. - followed by the breakdown of the system itself.

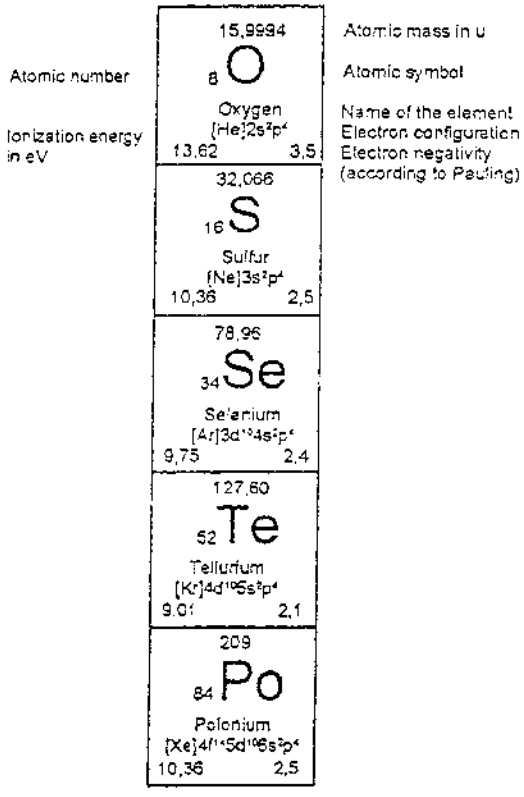


Figure 2: The elements of the sixth primary group of the periodic table of the elements with their chemicophysical properties

The maintenance of a defined negative redox potential in all cellular compartments is a fundamental physico-chemical precondition essential to the upholding of all molecular bioorganic functions. The maintenance of crucial SH groups and covalent disulfide bonds in the different compartments and biomolecules is the central task of the oscillating non-equilibrium thermodynamics of the glutathione system, which, along with its functions in connection with oxidative stress and detoxification of xenobiotics, is primarily responsible for the coordination and fine-tuning of the redox potential [62, 81].

The glutathione system constantly endeavors to defend the approx. 400:1 ratio in favor of reduced glutathione against all oxidative, electrophilic, catabolic, and destructive forces, with the aid of energy flow and entropy export. When this system of chaotic phase transitions breaks down, not only is there a breakdown of the metabolic pathway characterizing the system, but also of all the cellular protein functions regulated by SH groups and/or disulfide bridges.

The structure of biomolecules is not only determined during their development in the ribosomes and in protein biosynthesis by the folding up into a three-dimensional native conformation via disulfide bridges according to the most thermodynamically favorable route (from higher to lower energy states), as mentioned in section I. In fact, during the entire existence of thiol protein molecules, complex thiol-disulfide exchanges take place, which, together with the microfluctuations of weak interactions, also dependent upon the redox potential, guarantee the whole range of adaptive biomolecular oscillating interactions of a cell in cell stress.

Functional flexibility, the ability of biomolecules and molecular and subcellular regulatory mechanisms to adapt to changing circumstances, is dependent to a great degree on the fluctuations of weak interactions and, at catalytic or allosteric enzyme sites, on free SH groups [24, 55, 57, 62, 90-92, 123, 136].

III The glutathione system - gene regulation and transcription factors

Basal intracellular processes regarding cell function, cell viability, cell stress, cell aging, genetically programmed cell death (apoptosis), and, most of all, the regulation of stress-response gene families, are governed by the structure and function of a number of defined proteins [25, 80].

These cell stress response gene families are themselves regulated by transacting factors (transcription factors, nucleoproteins) such as NFκB, AP-1 (c-fos, c-jun),

the bZIP or C/EBP protein family, the heat shock factors (HSF) and other chaperonins, which in turn are stimulated and induced by bacterial lipopolysaccharides (LPS), free radicals and/or reactive oxygen species, phorbol esters (TPA), heavy metals, xenobiotics and short-wave photons (UV rays, x-rays, gamma rays and high frequency radiation).

The transcription factors and regulatory proteins NFκB and AP-1, acute phase proteins (APP), metallothioneins (MT), heat, salt and stress shock proteins, DNA repair enzymes and the extracellular matrix (Pischinger space) are the target molecules for cell stress coming from outside the cell and adapt their counterregulatory responses qualitatively and quantitatively as appropriate. Both factors, external phenomenological stressors and the highly integrated, often feedback-controlled response of biological mediators to maintain the inner milieu, are transported via signal transduction pathways (protein kinases interacting with phosphatases), flanked and additionally milieu-regulated by redox reactions involving thioredoxin, the glutathione system, glutathione reductase, glutathione peroxidases, glutathione S-transferases, redox factor 1 and NAD(P)H-NAD(P) kinetics.

NFκB is a multivalent transcription factor that quite rapidly regulates genes for inflammatory processes, immune responses and acute phase protein kinetics. Although NFκB was first found in pre-B, B and T lymphocytes, macrophages and hepatocytes, it is a ubiquitous transcription factor present in all cells [112, 115].

For the sake of simplicity the target genes for NFκB can be classified into four groups:

1) Immunomodulatory cytokines (TNF-α, IL-6, GM-CSF)

2) Immunoregulatory functions mediated by cell membrane receptors (MHC class 1 antigens, nonpolymorphic MHC subgroups, IL-2-" receptors)

3) Acute phase proteins (and others such as serum amyloid, angiotensinogen etc.)

4) HIV 1.

NFκB is made up of three subunits: p50 with DNA-binding functions and nuclear translation domains, p65, which is responsible for DNA binding, dimeriza-

tion, and constitutes the transcription activation character, and an inhibitory subunit, IκB, which can bind to p65.
The various functions of these NFκB substructures are regulated by the signal redox pathway already referred to, which, in cooperative association with the above-described superordinate redox regulation governed by the non-equilibrium glutathione system, plays a pivotal role in gene regulation. In fact, as far as the structure and function of the multivalent transcription factor NFκB are concerned, the actions of both intrinsic and extrinsic factors targeted at activating NFκB are dependent generally and implicitly on the redox status of the molecule (figure 3) [112, 115].

The cis-acting DNA-binding protein AP-1 was first found in the enhancer region of SV40, in human metallothioneins II_A ($hMII_A$), and in phorbol ester responsive genes. It is, however, known that molecules of the fos and jun gene families also bind to the AP-1 DNA sequence, and that these proteins belong to the bZIP transcription factor family. These proteins have a leucine zipper domain in common [112, 115].

Variable homo (jun-jun) - hetero (fos-jun) dimer complexes that interact with defined DNA motifs have been identified. Direct DNA contact is only possible via a nucleoprotein, redox factor 1. Gene regulations and redox potentials are very tightly coupled and require an intact glutathione system to function properly [57, 58, 62, 64, 81].

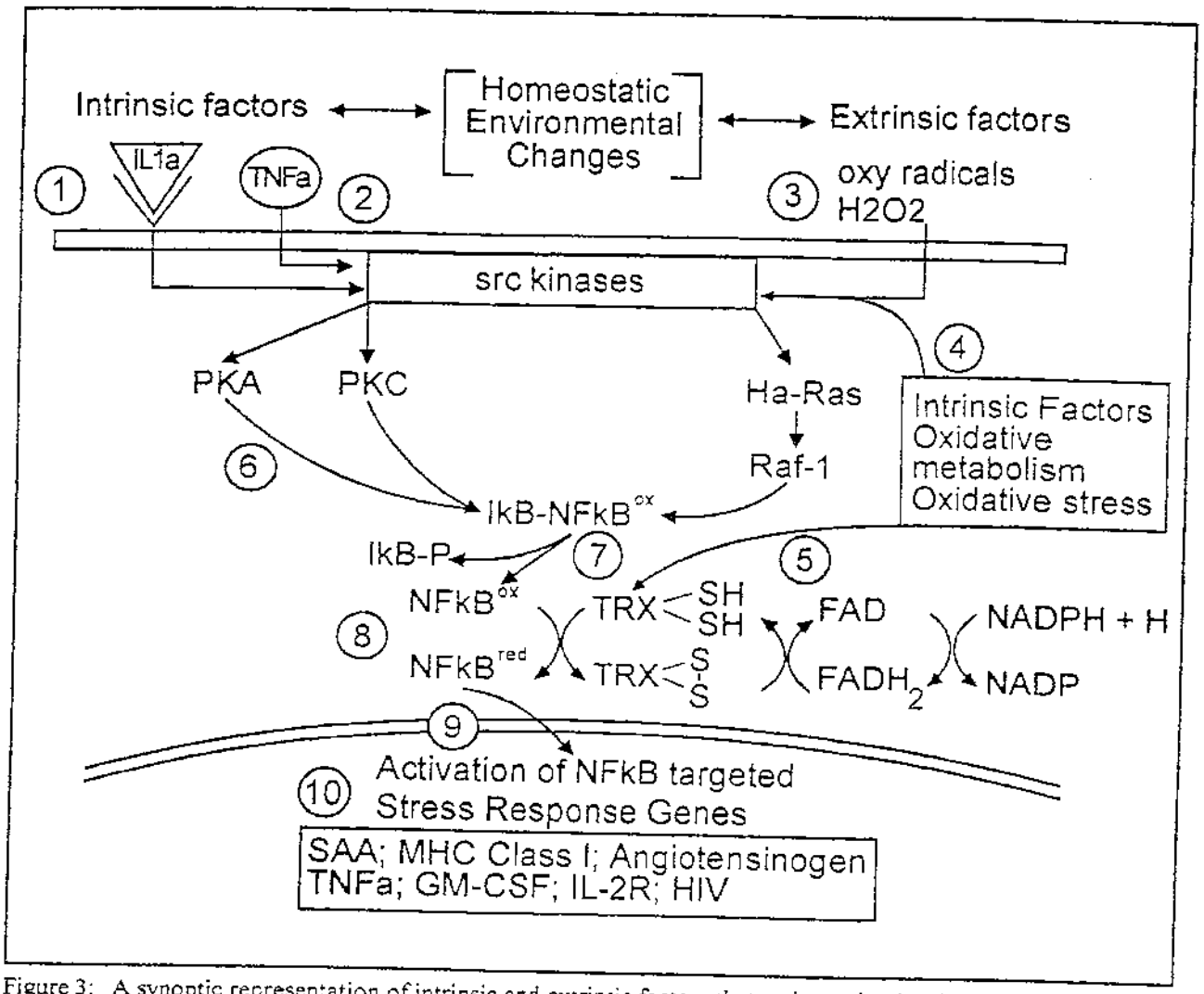


Figure 3: A synoptic representation of intrinsic and extrinsic factors that activate the signal transduction redox pathway and result in NF B induction (taken from J Papaconstantinou [112])

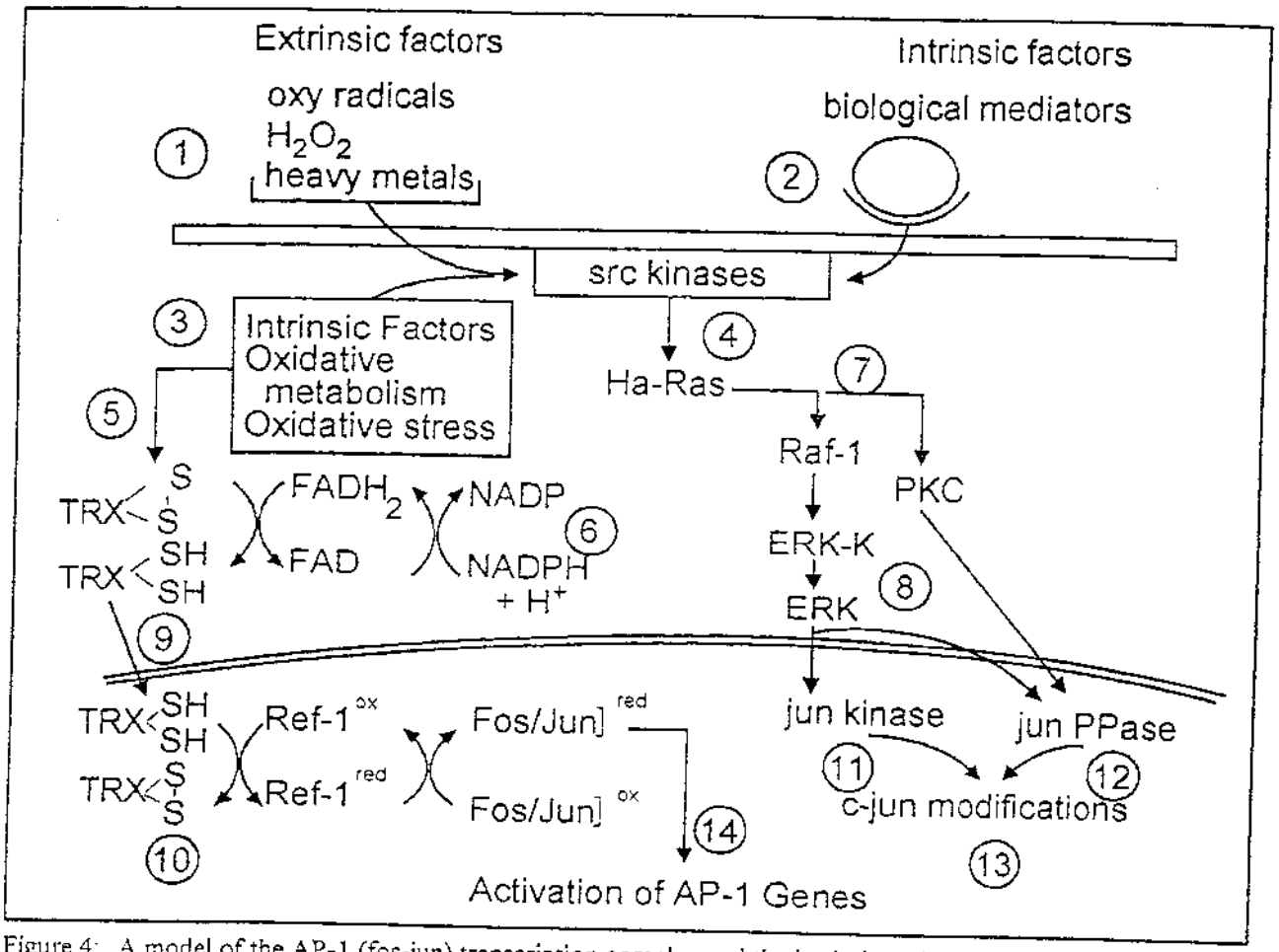


Figure 4: A model of the AP-1 (fos-jun) transcription complex and the intrinsic and extrinsic factors that induce this transactivator to activate stress response genes (taken from J Papaconstantinou [112])

The AP-1 (fos-jun dimer) proteins belong to a transcription factor superfamily that can be stimulated by the following stressors:

A) Intrinsic factors: polypeptide hormones, growth factors, cytokines, neurotransmitters, etc.

B) Extrinsic factors: short-wave photons (UV rays, x-rays, gamma radiation), xenobiotics such as alkylating agents, aromatic carbohydrates, halogenated carbohydrates, nitrosamines, fungal toxins, synthetics, etc.

UV exposure, to give one example, results in a rapid and dramatic change in the phosphorylation of serine residues in c-jun proteins that require initial activation by protein kinases. In the light of this AP-1 is a crucial DNA signal transmitter for the regulation of stress genes and their chaperonins in the response to many and varied external and internal stressors [57, 58, 62, 64, 81, 112, 115].

Src-tyrosine kinases → HaRas → Raf-1 → Raf-1 $(P)_n$ → stimulate protein kinases which in turn activate the C-jun domain and AP-1 activity and, by feedback, also activate the AP-1 response gene c-jun itself [112, 115] (figure 4).

It is now considered proven that oxidative stress is induced by phenomenologically very different stressors which activate both the signal transduction pathway through the cell to the nuclear genome with the aim of inducing cell proliferation as well as the stress genes [21, 25, 35, 36, 52-74, 78, 80-87, 90-94, 96-108, 112, 115, 131, 136].

Treatment with L-cysteine, 2-MPG (2-mercaptopropionylglycine), GSH, and acetyl-GSH with or without anthocyans modulates c-src activation. C-jun gene expression in turn modulates photochemically generated radicals, reactive oxygen species ($O_2^{\bullet}$, H_2O_2, $OH^{\bullet}$, $O_2(^1\Delta_g)$), radicals from lipid peroxidative processes ($L^{\bullet}$, $LO^{\bullet}$, $LOO^{\bullet}$), but also LOOH exogenous and/or endogenous aldehydes. Epoxides, which in turn alter the glutathione-regulated redox potential by reacting with the free SH group, a vicious circle that disrupts redox potential regulation and thus prevents physiological reactions with enzyme SH groups, metallothionein SH groups, and others [81].

Alterations of the redox potential immediately result in the activation of a number of tyrosine kinase systems and in the inhibition of tyrosine phosphatase reactions. Alterations in the glutathione-regulated redox potential and the resultant SH group oxidation at active and allosteric enzyme sites lead to "nonsensical" cell division, malignant transformation, or cell death.

These examples of the dependency on the redox potential of the molecules involved in gene regulation demonstrate the close relationship between the quality and effectivity of regulation and the redox status and are a proof of the implicit dependence of antioxidant and reducing enzymes themselves on the redox milieu supplied by thioredoxin and GSH.

The physiological NFκB reaction is closely associated with the reduction of L-cysteine (position 65) in p50 and redox factor 1 stimulates the DNA binding activity of the following transcription factors in vitro: Fos, Jun, AP-1, NFκB, Myb and CREB [112, 115]. The redox activity of Ref-1 is due to a "conserved" basic L-cysteine motif (KCR) [112]. Similar cysteine residues have been detected in the DNA binding domains of p65 NFκB (KICR) and Myb (KQCR) [112, 115].

The gene regulation activities of many of the repair or preventive mechanisms involved in the defense against oxidative stress and its devastating effects are integrated in a complex humoral, endocrine and neurosynaptic network. The basic functions and activities involved in this network are conducted at the level of discrete molecular groups and definable functional groups - especially SH and S-S groups - and are governed by a local microscopically oscillating redox potential ultimately defined by the glutathione system and which renders cellular functions and life itself possible in the first place [3, 4, 10, 11, 14, 16-21, 24-31, 35, 38, 40, 43-49, 51-110, 112, 115, 118, 119-121, 123, 128, 129, 134-136].

IV Biochemistry, pharmaceutical potential and pharmacology of the flavonoids and anthocyans

A C-15 carbon skeleton composed of three acetate units (C_6) and a phenylpropane unit (C_6-C_3) is typical for all flavonoids. Pharmaceutically and pharmacologically tried and tested flavonoids are derived either from diphenyl-1,3-propane or from 2-phenylbenzopyran [2, 127, 133] (figures 5, 6 and 7).

Flavonoids contain two differently substituted aromatic rings linked by a C_3 bridge [2]. Whereas ring A follows the substitution pattern of phloroglucin or resorcin, the B ring is mostly hydroxylated at position 4', 3', 4' or 3, 4', 5' [127] (figures 5, 6 and 7).

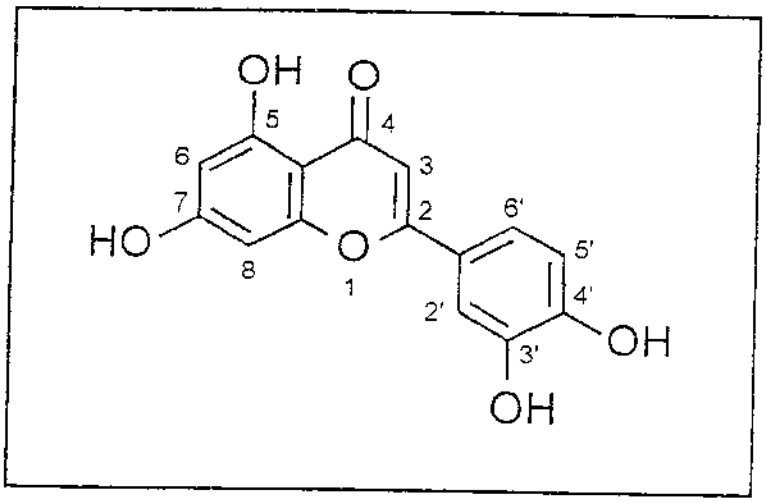


Figure 5: Structural formula of the flavonoids. They are O-heterocyclic with a skeletal structure composed of 15 carbon atoms divided into two aromatic rings and linked by a C_3 bridge. One benzene ring shows a substitution pattern like that of the shikimiates (cinnamic acid, p-coumaric acid, caffeic acid, ferulic acid). The second benzene ring reveals its acetogenin origins by its meta substituted O functions (phloroglucin or resorcin patterns). Taken from E. Steinegger and R. Hänsel [127]

Most flavonoids have a carbonyl group at position 4. They also contain phenolic OH groups that may be found uncombined, as glycosides and/or in methylated form [2, 127, 133].

Interesting synergistic effects, notably redox cycling relationships, exist between certain flavonoid derivatives, in particular anthocyanidins, flavadiols and catechins (figures 6 and 7), ascorbic acid and reduced glutathione (GSH). This interplay between different antioxidants is of central importance [15, 41, 81].

The antioxidant properties of the flavonoids and anthocyans are attributable to their phenolic OH groups, which is why the antioxidant potency increases with the number of OH groups in a molecule [6, 15] (figure 8).

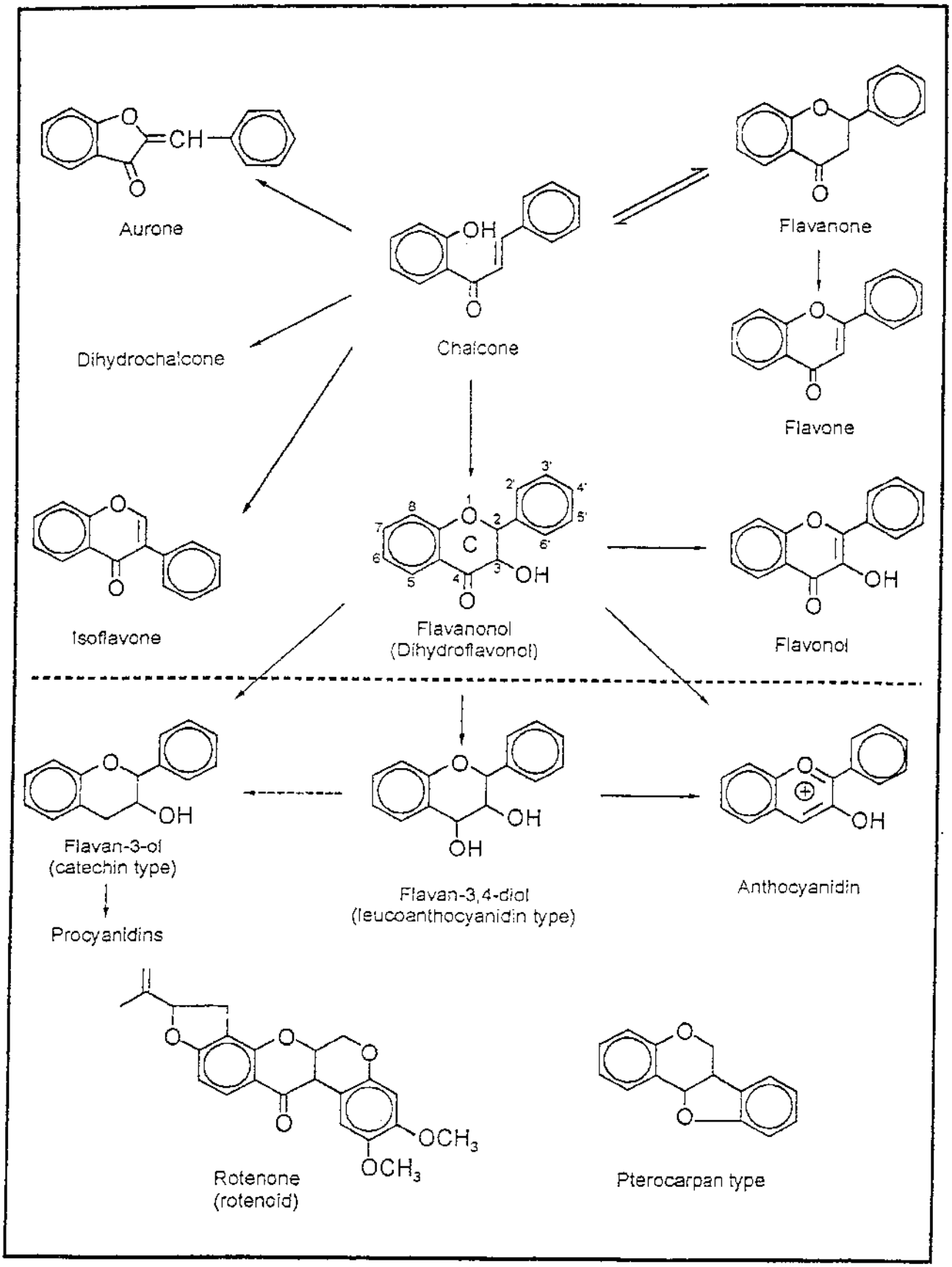
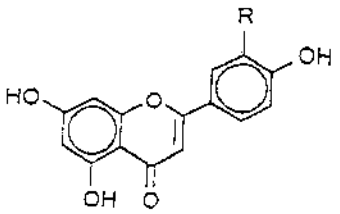


Figure 6: Metabolic pathways of the flavonoids, taken from H. Wagner [133]

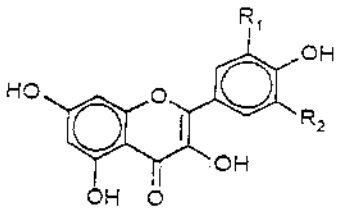
Flavone family

	R
Apigenin	H
Luteolin	OH
Apigenin-, luteolin-7-O-glucoside	
Apigenin-8-C-glucoside	= vitexin
Luteolin-8-C-glucoside	= orientin



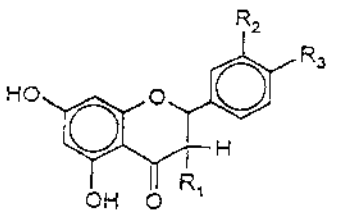
Flavonol family

	R_1	R_2
Quercetin	OH	H
Kaempferol	H	H
Myricetin	OH	OH
Isorhamnetin	OCH_3	H
Quercetin-3-O-galactoside	= hyperoside	
Quercetin-3-O-glucoside	= isoquercitrin	
Quercetin-3-O-rhamnoside	= quercitrin	
Quercetin-3-O-rutinosoid	= rutin	
Kaempferol-3-O-glucoside	= astragalin	



Flavanon(ol) family

	R_1	R_2	R_3
Naringenin	H	H	OH
Eriodictyol	H	OH	OH
Homoeriodictyol	H	OCH_3	OH
Hesperetin	H	OH	OCH_3
Taxifolin	OH	OH	OH
Naringenin-7-O-neohesperidoside	= naringin		
Hesperetin-7-O-rutinoside	= hesperidin		
Eriodictyol-7-O-rutinoside	= eriocitrin		
Hesperitin-7-O-neohesperidoside	= neohesperidin		



	R_1	R_2
Pelargonidin	H	H
Cyanidin	OH	H
Delphinidin	OH	OH
Peonidin	OCH_3	H
Malvidin	OCH_3	OCH_3

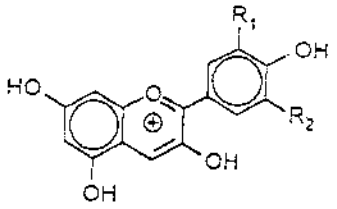


Figure 7: The many structures and substituents of the flavone, flavonol and flavanonole family, taken from H. Wagner [133]

Experiments with 10^{-3} molar solutions clearly show the antioxidant activities of flavones, flavonones, flavonols, flavanonols, catechins, anthocyanidins and their

glycosides with a 3-hydroxyl and 4-carbonyl group at the γ-pyrone ring and/or a 3,4-catechol structure at the B ring [15].

Studies on the reduction of dehydroascorbic acid to ascorbic acid by thiols in an anaerobic milieu showed that the flavan-3-ol configuration of factor C2 compounds such as L-epicatechin plays a major role in this reaction regardless of the existing redox potential [15].

Dihydrochalcones, aurones and, by a phenyl change, isoflavones, too, can form from chalcone (figure 6) [133]. Flavone is generated by the dehydration of flavanone. Flavanonol seems to be generated directly from chalcone by oxidation (figure 6). Dehydration of flavanonol results in flavonol which in turn produces flavan compounds by reduction. Flavan derivatives include the leucoanthocyanidins (flaval-3,4 diols), anthocyanidins and flavan-3-ols of the catechin type [133] (figure 6). The oligomeric proanthocyanidins are possibly generated from flavan-3,4 diols and/or flavan-3-ols by oxidative dimerization or acid-catalyzed condensation [2, 133].

Three features characterize naturally occurring flavonoids as a group despite their fairly broad chemical diversity:

1) Divergent degrees of oxidation of the C-ring (flav-on, flav-on-ol, flavanone, flavan-on-ol) [133]
2) The fairly large variations in the number and arrangement of the A and B ring OH, O, and C-alkyl substitutions
3) Differences in the quality, number and position of glucose residues [133].

The flavone family comprises C-glycosides as well as O-glycosides [133]. Mono- and disaccharides are the most common compounds. The most important disaccharides (biosides) are:

Rutinose	(6-O-rhamnosidoglucose)
Neohesperidose	(2-O-rhamnosidoglucose) and
Sophorose	(2-O-glucosidoglucose) [133].

Less common disaccharides are rubinobiose, primverose, sambubiose, gentiobiose and apiobiose [133] (table 2).

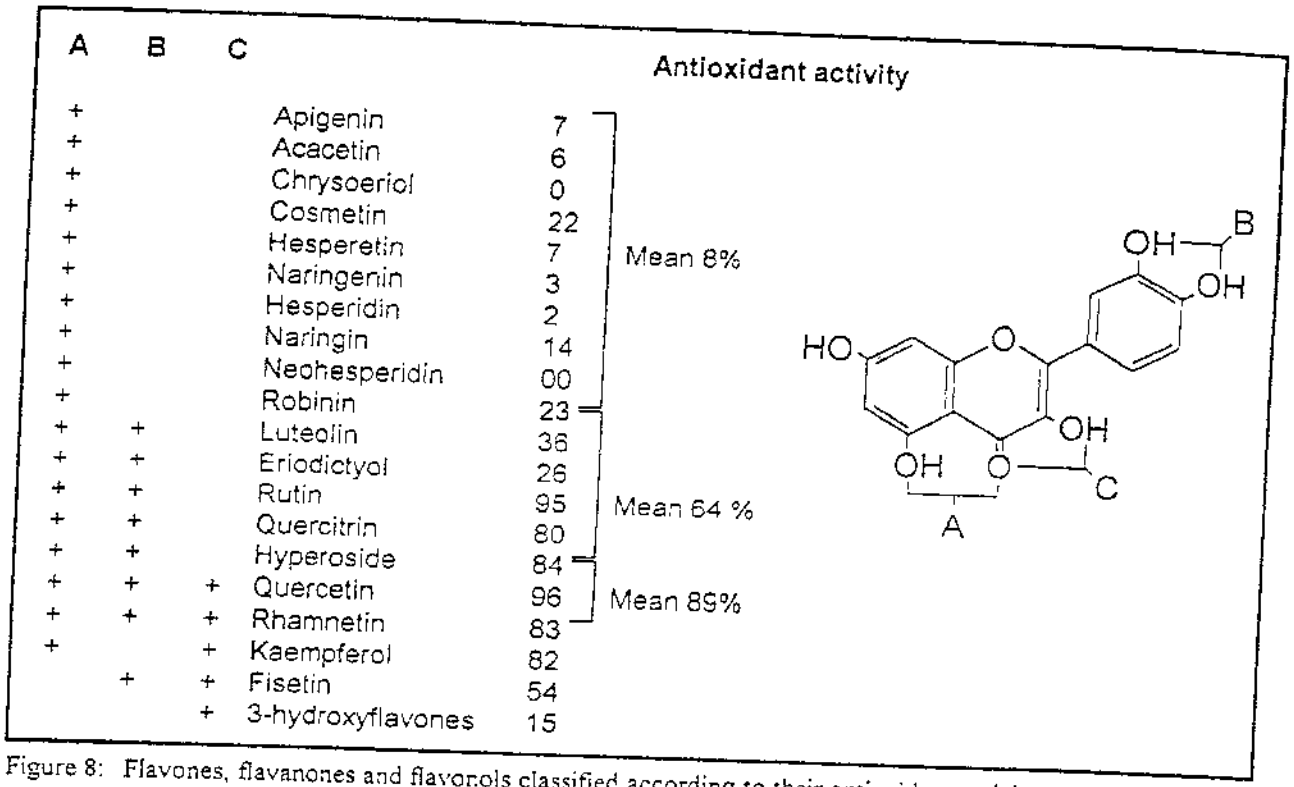


Figure 8: Flavones, flavanones and flavonols classified according to their antioxidant activity. The 3-hydroxy-4-carbonyl substituent pair at the gamma-pyrone ring and the 3'4' catechol substituent pair seem to be responsible for the antioxidant properties and the redox cycling behavior with vitamin C and GSH (taken from C.A. Clemetson [15])

Source	Anthocyans
Hibiscus flowers (Hibiscus sabdariffa)	Del-3-glc-xyl, Del-3-glc; Cy-3-glc-xyl, Cy-3-glc
Poppy flowers (Papaver rhoeas)	Cy-3-glc
Cornflowers (Centaurea cyanus)	Pg and Cy-3,5-di-glc; Pg-3-(caffeoylglucoside)-5-glc
Mallow flowers (Malva sylvestris)	Mv-3,5-di-glc
Peony flowers (Paeonia officinalis)	Peo-glycosides Cy-3-glc
Rose leaves (Rosa centifolia and/or Rosa gallica)	Cy-3,5-di-glc Pg-3,5-di-glc Pg-3-glc, Cy-3-glc
Hollyhock flowers (Alcea gallica)	Del-3-glc, Mv-3-glc
Bilberry fruits (Vaccinium myrtillus)	Glycosides of Pg, Cy and Pet; Del-3-glc, Del-3-gal, Mv-3-glc

Abbreviations: Cy = cyanidin, Del = delphinidin, Mv = malvidin, Peo = peonidin, Pet = petunidin, Pg = pelargonidin, gal = galactosid, glc = glucoside, xyl =xyloside, glc-xyl = sambucosid (sambubiosis = β - D - Xylp - (1 → 2) - D - Glcp.).

Table 2: Anthocyans in flowers and fruits, taken from E. Steinegger and R. Hänsel [127]

In flavonoles glycoside linkages are most common at C-3, C-7 and C-4'-OH, in flavones at C-7 and C-4', and in flavanones at C-7-OH [133].

Both **Anthocyans** and **Anthocyanidins** are not just found in flowers, but are also present in fruits, stems, bark and leaves (table 2). Pro-anthocyanidins are mainly found in fruits and polymeric catechins in roots and shrubs [133].

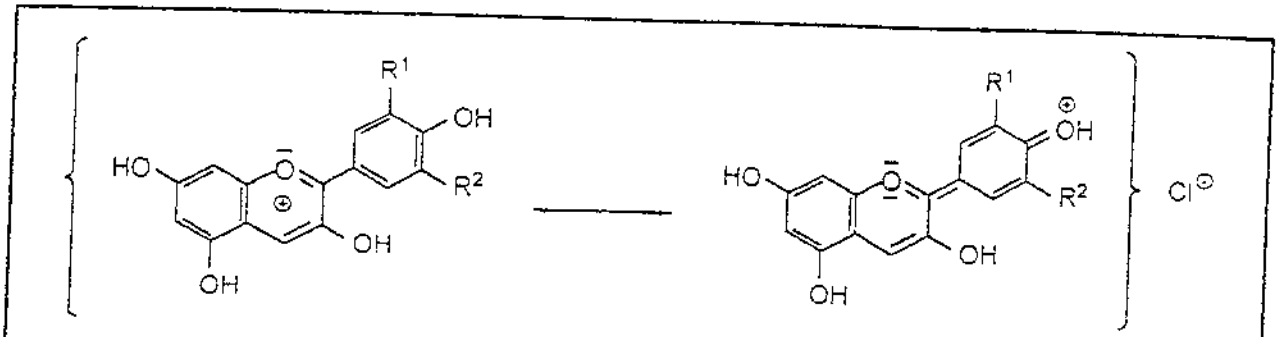
Anthocyans are glycosidic water-soluble 2-phenylchromenol derivatives that usually occur in higher-order plants (table 2). They are the red to violet and blue to black color principles (redox colors) in fruits, flowers and leaves [2, 127, 133].

Anthocyanidins may become photochemically active; as red-violet compounds their peak absorption is in the 520 nm and 544 nm range in the spectrum of visible light [127] (figure 9).

The colors of anthocyans depend on the pH of the solutions. With increasing pH a number of color changes take place, culminating in yellow chalcone phenolate anions due to ring opening in an alkaline milieu (pH > 8) [127]. Some color effects result from the occurrence of glycoside-metal chelate complexes with - for example - Fe(III) and/or Al(III) ions.

The aglycone components of the anthocyans are the anthocyanidins of the pelargonidin primary type with a 3,4',5,7-hydroxylation pattern corresponding to that of kaempferol in the flavonol group (figure 7). The introduction of further hydroxyl groups in the B ring gives cyanidin (3',4-dihydroxy type) and delphinidin (3',4',5'-trihydroxy type) (figure 7). Partially methylated derivatives also occur [127].

The pharmacological actions of flavones and flavonoids are summarized in table 3.



R^1	R^2	Common name	λ_{max} (nm)	(Color)
H	H	pelargonidin	520	(reddish orange)
H	OCH_3	peonidin	532	(reddish violet)
H	OH	cyanidin	535	(reddish violet)
OCH_3	OCH_3	malvidin	542	(violet red)
OH	OCH_3	petudinin	543	(violet red)
OH	OH	delphinidin	544	(bluish violet)

Figure 9: Absorption peaks of anthocyanidins in solution (in methanol: containing 0.01 % Hcl), taken from E. Steinegger and R. Hänsel [127].

V The bioavailability of orally ingested reduced glutathione and anthocyans

Questionable elements in the average modern diet (genetically engineered foods, flavorings and preservatives, hormones, halogenated carbohydrates, radioactivity and radioactive nuclides with different half-lives...) mean that the human gut must cope with an ever mounting barrage of primary and/or secondary toxic xenobiotics. Many of these foreign substances are primary or secondary radicals,

induce reactive oxygen species and trigger unpredictable radical chain reactions of varying duration in a number of very different biological compartments.

All these reactions impair or prevent the physiological antioxidant effects of certain enzymes, including reduced glutathione. The latter is a universal radical scavenger whether acting as a molecule in its own right or as a coenzyme in association with enzymes such as glutathione-dependent peroxidases and S-transferases. GSH protects SH groups against oxidation and metal chelation; it also protects the vital ubiquitous metallothioneins, a protein family built up of amino acids whose molecules contain up to one-third L-cysteine. Disastrous cell toxic aldehyde interactions are also prevented or inhibited to a greater or lesser extent by GSH [52-110].

Similar effects can be claimed for certain anthocyans [2, 6, 8, 15, 21, 23, 29, 30, 114, 127, 133].

The harmful reactions in human intestinal lumen provoked by different forms of diet and the chemical, radioactive and genetic manipulation of foods have reached such proportions that it is reasonable to doubt whether GSH and anthocyans are available to deeper compartments in the amounts required to maintain a physiological redox balance, given the colossal task they have to perform in coping with the dietary chemical onslaught.

In contrast to a persisting popular misconception, GSH is absorbed in the human small intestine by a number of transport mechanisms and penetrates with varying degrees of efficacy into the deeper biological compartments of the human organism. Of course, the intact GSH molecule and the absorption mechanisms have to compete against a number of radical reactions (hyperradicality) occurring in their topochemical proximity per unit time. However, there is a physiological influx of GSH from the small intestinal lumen via peripheral cells [44, 81].

Nonetheless, GSH can be oxidized, chelated, and thus inactivated to a greater or lesser degree by any number of biological and kinetic pathways. The harnessing of a mutually regenerating redox couple such as GSH and anthocyans would therefore be of considerable therapeutic benefit [81].

The human small intestine itself also possesses a lumenal mechanism for the re-reduction of G-S-S-G to GSH that employs glutathione reductase and NADPH as the reducing equivalent [41, 81].

The following absorption mechanisms of GSH in lumenal small intestinal cells have been postulated [18, 19, 44, 48, 81]:

- activated transport
- facilitated transport
- sodium-dependent transport
- diffusion
- endocytosis.

Flavones and flavonols (figure 5 - figure 9) are absorbed unchanged from the human gut only slowly and incompletely, which is why pharmacodynamically high doses must be administered to the first compartment if prophylaxis and therapy are the desired aims [2, 6, 81, 127, 133].

In addition to absorption through enterocytes, diffusion through the pores in the tight junctions between the enterocytes is also possible. The more or less readily absorbed microbial metabolites of the flavones, flavonols and anthocyans play a role in their systemic effectivity; some flavonoids have even been found to exert favorable effects in the bradytrophic tissues of the eye (cornea, lens, vitreous body) [2, 81, 127, 133].

The synergism between GSH, anthocyans and ascorbic acid already referred to (figures 10 and 11) is crucial to the protection of bradytrophic tissues against oxidative stress, especially in middle and old age when these tissues physiologically lack antioxidant enzymes (catalases, peroxidases) and as such are particularly susceptible.

VI Synergistic effects of reduced glutathione (GSH) and anthocyans in redox recycling with implications for combination therapy in a broad range of therapeutic areas

Although reduced glutathione (GSH), both as a coenzyme (selenium-dependent glutathione peroxidase - sel-dep G-SHPx) and as a cofactor (glutathione S-transferase), not to forget its role as non-enzymatic scavenger and electrophile substance, is able to detoxify electrophilic xenobiotics (primary or secondary radicals) and radicals generated in the cell metabolism by exposure to high-energy radiation, therapeutic use of GSH on its own, even if administered in sufficient amounts, may be insufficient to prevent depletion.

GSH depletion can be due to:

1) Congenital deficiency in one or more antioxidant enzymes (enzymopathy)

2) Poor rate of biosynthesis of antioxidant enzymes in various compartments associated with certain unfavorable lifetime phases or illness (enzymopenia)

3) Oxidative exhaustion of GSH reserves under certain circumstances (intoxication, inflammation, infection, competitive sports, deficiency in non-enzymatic or enzymatic scavengers, etc.) harboring the extremely unfavorable possibility of thiyl radical formation (G-S$^{\bullet}$), glutathione disulfide anion radicals (G-S-S-G$^{\bullet}$), or glutathione peroxysulphenyl radicals (G-SOO$^{\bullet}$) [52, 81].

4) De novo impairment of biosynthesis of endogenous reduced glutathione (GSH).

5) General causes (stressful life events, competitive sports, wasting disease, cachexia, old age, oxidative stress).

Each of these depletion scenarios alters the negative redox potential present in the biological compartments of living systems and perturbs the redox shuttle system

$$GSH \longleftrightarrow \text{mixed disulfides} \longleftrightarrow G\text{-}S\text{-}S\text{-}G,$$

resulting in enzyme imbalance (particularly in antioxidant enzymes and repair enzymes), disruption of cell metabolism, mutation, malignant transformation, and cell necrosis and/or apoptosis.

Lack of response to therapy with GSH on its own due to these pathobiochemical disturbances can be prevented by combining GSH with anthocyans (figure 11), as shown in extensive studies [15, 81].

Anthocyans, heterocyclic 2-phenylchromenol multiple ring systems with a number of different hydroxylation patterns, act as effective scavengers for the superoxide anion radical ($O_2^{\bullet}$), hydrogen peroxide (H_2O_2), the hydroxyl radical ($OH^{\bullet}$), alkoxyl radicals ($LO^{\bullet}$), peroxyl radicals ($LOO^{\bullet}$), numerous other radicals, and for singulet oxygen ($O_2(^1\Delta_g)$).

Anthocyans in their capacity as photobiological inhibitors (figure 9) are also able to intervene as regulators and detoxifiers in oxygen-mediated sensitizing photoreactions, thereby preventing cytotoxic and molecule-damaging radical-radical chain reactions of whatever origin [2, 81, 127, 133].

Anthocyans protect against cytotoxic and carcinogenic aldehydes (4-hydroxyhexenal; 4-hydroxyoctenal; 4-hydroxynonenal, propanal; butanal; pentanal; hexanal; 2,4-heptadienal, malondialdehyde etc.) [54]; in fact, they prevent their formation by lipid peroxidative chain reactions. They detoxify the acetyl aldehyde released during ethanol degradation and the formaldehyde generated during methanol degradation or present by other means [54, 81].

Combined therapeutic use of properly dosed anthocyans and GSH (redox recycling, redox shuttle) results in optimum synergies in the treatment of cellular disturbances and the restoration of disrupted regulatory pathways governing cell and enzyme function. Apart from the qualitative therapeutic aspects, the quantum yield in terms of scavenger functions is considerably enhanced with the GSH-anthocyan combination.

Thiyl radicals (G-S$^{\bullet}$), glutathione disulfide anion radicals (G-S-S-G$^{\bullet}$), and glutathione peroxysulphenyl radicals (G-SOO$^{\bullet}$) are either prevented from developing or detoxified by anthocyans [81].

Reduction of oxidized glutathione (G-S-S-G) and prevention and/or detoxification of glutathione radicals are not the only benefits. An additional bonus is the sustained regeneration of radical-detoxification functions necessary for scavenge purposes resulting from an oscillating reaction cycle operating between the two substances/substance classes (GSH and anthocyans). These additive benefits of this new combination launched for therapeutic use in humans and animals have been proven in vitro and in vivo (figure 11).

A regenerating redox cycle exists between GSH and anthocyans on the one hand and vitamins E and C on the other (figure 10). As shown by favorable responses to therapy proven and reproduced in experimental settings, this combination is superior to all hitherto known antioxidants of natural and synthetic origin [15, 81].

The mutual enhancement of effect is so optimal that GSH is subsequently able to fully resume its vital regulatory tasks at the genetic and enzymatic level and eventually at all levels.

radicals across hydrophilic and lipophilic compartments [15, 62, 81]. **GSH** can redox recycle with anthocyans, vitamin C and vitamin E (figure 11).

VI) **Anthocyans** and **GSH** provide antioxidant protection for hydrophilic compartments (intravascular space, interstitial space, intracellular space, intramitochondrial space, cerebrospinal fluid space) against radical reactions.

VII) **Anthocyans** and **GSH** protect bradytrophic tissue (cornea, optical lens, vitreous body, and cartilage, bone, and conjunctive tissue) against oxidative stress.

VIII) **Anthocyans** and **GSH** protect all SH and S-S groups of bioorganic molecules - particularly the SH groups of metallothioneins -, thereby resulting in optimization of the functions of these molecules, molecular bonds, and molecular cascades. By virtue of the redox recycling phenomenon, the two substances in combination protect each other and defend the metallothioneins that play such a key role in all cells (figure 11).

IX) **Anthocyans** and **GSH** defend quinoid polymer molecules of human melanin against free radical catastrophes of chemical and phototoxic aetiology and the associated chromosomal damage in melanocytes, and in so doing prevent the otherwise inevitable development of malignant melanoma.

X) **Anthocyans** and **GSH** in combination provide optimum antioxidant and free radical protection that is superior and superordinate to all other natural antioxidant defense systems.

The basic synergistic and pharmacological effects of the GSH-anthocyan combination indicate that it may have considerable potential in the prevention of malignant melanoma and cataracts and in the treatment of the broad range of diseases presented in table 4.

Throughout the physiological pH range and in all self-regulating redox potentials established via the non-equilibrium thermodynamic system GSH $\rightleftharpoons$ mixed disulfides $\rightleftharpoons$ G-S-S-G, a self-regenerating redox recycling relationship exists between reduced glutathione and anthocyans (as already shown for anthocyans and vitamin C [15]) in addition to all the other functions listed above (I - X). This redox recycling relationship helps to optimize the redox milieu and detoxify

radical reactions and radical chain reactions in an effective and sustained manner.

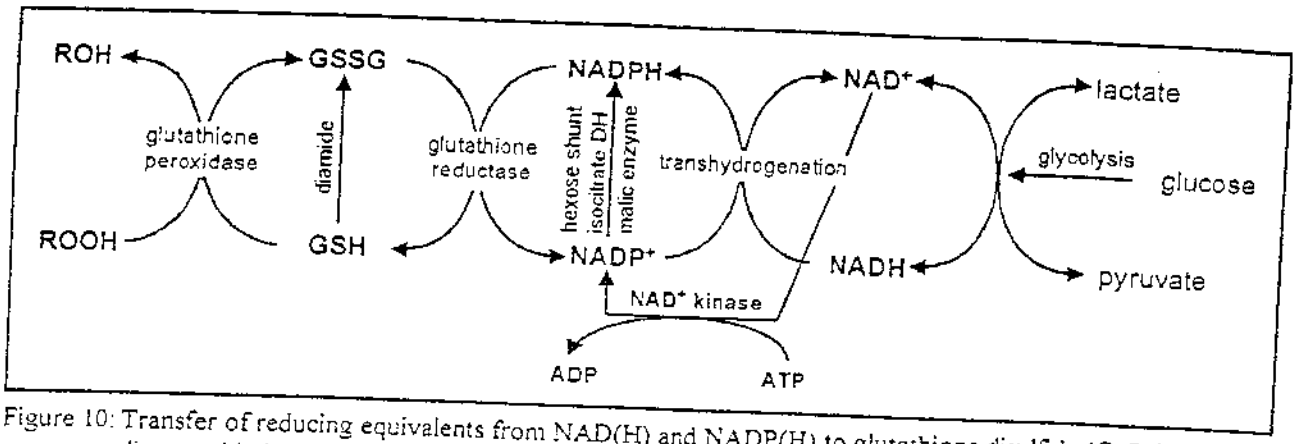


Figure 10: Transfer of reducing equivalents from NAD(H) and NADP(H) to glutathione disulfide (G-S-S-G) via direct oxidation or mediated by the enzyme peroxidase, taken from J.P. Kehrer and L.G. Lund [41]

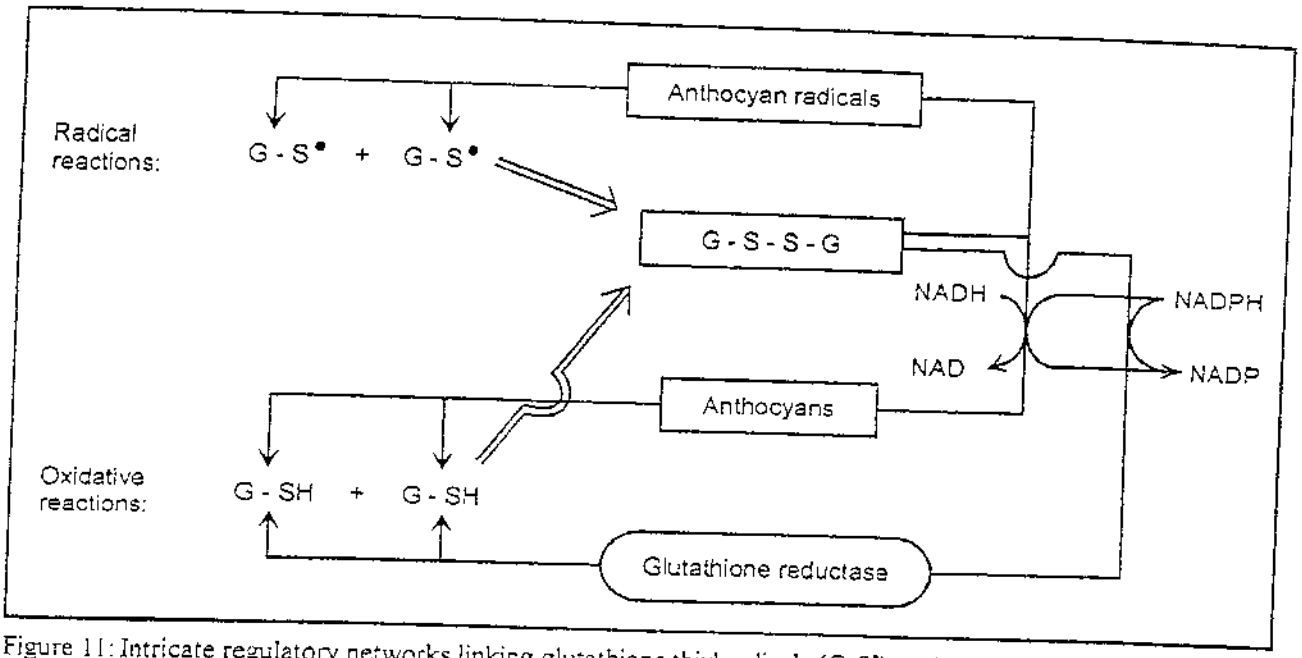


Figure 11: Intricate regulatory networks linking glutathione thiyl radicals (G-S•), reduced glutathione molecules (G-SH), anthocyans, anthocyan radicals, oxidized glutathione molecules (G-S-S-G), transhydrogenation coenzymes NADH/NAD and NADPH/NADP and the enzyme glutathione reductase

The glutathione-anthocyan combination constitutes a strong alliance against oxidative stress, its devastating effects at the molecular pathological level, and the diseases thereby caused; a broad defense system that works well in hydrophilic (polar) compartments, protein membrane structures, bradytrophic eye tissue, connective tissue and glial tissue - regions in which conventional liposoluble antioxidants are useless.

Hence, GSH in combination with anthocyans represents a valuable and vital addition to the ranks of antioxidants engaged in the prevention and treatment of

oxidative stress. What is more, the combination has a broad spectrum of action in the prevention of destructive radical reactions. Only this combination can maintain and optimize the host of enzyme-catalyzed metabolic reactions dependent on SH group processing.

Self-evidently, the protection of SH groups and/or the potential of inducing targeted thiol-disulfide exchanges (figure 2) afforded by the combined effects of GSH and anthocyans also offers considerable protection against mutations in nuclear and mitochondrial DNA and against many or all of the mechanisms involved in the multifactorial process of carcinogenesis.

The GSH-anthocyan combination optimizes and normalizes such key functions as enzyme repair, chaperonin effects, gene regulation, gene expression, mitosis, transcription, polymerase activity - only some of the potential areas of action for this fascinating hydrophilic redox couple.

Pharmacological effect	Plants, flavonoids
Antihemorrhagic and antisclerotic	Citrus bioflavonoids (hesperidin, eriocitrin), rutin and derivatives
Antiphlogistic and antiedematous	Rutin (oxyethylrutin = Venoruton®)
Coronary vasodilative and positively inotropic	Crataegus, arnica, ginkgo
Spasmolytic	Procyanidins Camomilla (apigenin glyc.) Liquiritia (isoliquiritigenin)
Antihepatotoxic	Silybum (silymarin)
Choleretic	Helichrysum
Estrogenic	Isoflavones (clover and broom species)
Diaphoretic	Tilia, spiraea, sambucus
Diuretic	Betula, ononis, solidago, viola, virgaurea, orthosiphon

Table 3: Overview of various known pharmacological effects of flavones and flavonoids, taken from H. Wagner [133]