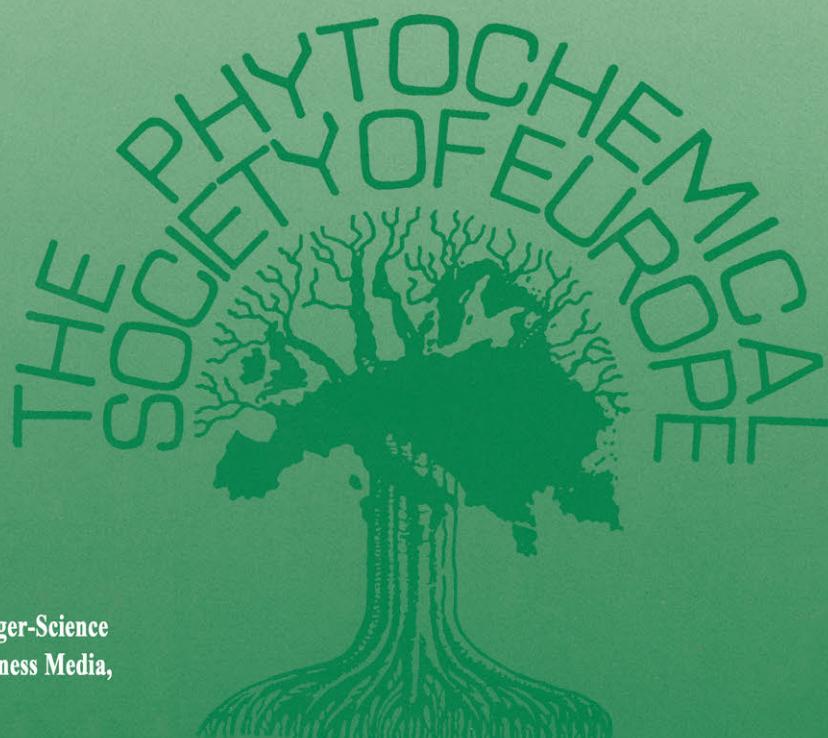


PROCEEDINGS OF THE
PHYTOCHEMICAL SOCIETY OF EUROPE

Polyphenols, Wine and Health

Edited by Catherine Chèze, Joseph Vercauteren
and Robert Verpoorte



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Polyphenols, Wine and Health

Proceedings of the Phytochemical Society of Europe,
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Edited by

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and

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Preface

Where is the truth, at the end of this century, in the relationship between Polyphenols, Wine and Health?

Humanity has drunk wine and eaten grapes for millenaries but a particularly important use was mostly restricted to the Mediterranean countries. Epidemiological studies have revealed some strong lines of evidence that this nutritional habit was good for health and mainly in protecting against cardiovascular and neurodegenerative diseases. During the last decade, not only the knowledge on the polyphenolic content of wine increased considerably but also were reported numerous scientific information attesting that the *in vitro* biological properties of some of these polyphenols were fundamental enough to support strongly the hypothesis that those molecules could have something to see with this protection.

This volume is the compilation of the nine speakers who have delivered a plenary lecture at the International Symposium of the Phytochemical Society of Europe and who accepted to present a deeper analysis of their own field in writing these texts. This Symposium was entitled '*Polyphenols, Wine and Health*' and held in Bordeaux, France, in April 1999. Three words for three days of discussions and fruitful exchanges, at the highest scientific level, by conjugating them by pairs: "*polyphenols and wine*", "*polyphenols and health*" and "*wine and health*". It was thus aiming at clarifying the *in vitro* arguments sustaining or refuting this idea and at making the point on their scientific value.

After the marvellous analytical survey of the literature, given by Edwin Haslam, going back to last centuries, related to the antioxidant and to the radical scavenging properties but also to the complexation with the proteins of polyphenols that could explain the mechanisms by which some diseases such as scurvy could be cured and world-wide disappeared, the debate was launched and kept always an incredibly interesting level.

No definitive conclusions were drawn but simply was observed the necessity to obtain further information, at a cellular and even at a molecular level, in order to make allowance for these very difficult questions.

It is a great honour for us to have the opportunity to thank very much the plenary lecturers for such concise and so clever presentations, along with Dr. Catherine Chèze, for her continuous and tireless help.

Many thanks also, are due to the Phytochemical Society of Europe, the University Victor Segalen Bordeaux 2, the Aquitaine Region, the Gironde Department and to the private sponsors without whom the organization would not have been that great.

Joseph Vercauteren

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Chapter 1

NATURAL POLYPHENOLS AS DRUGS AND MEDICINES: POTENTIAL MODES OF ACTION

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INTRODUCTION

Plus ça change, plus c'est la même chose.

Scurvy and vitamin C; vitamin P and bioflavonoids

Scurvy is an ancient disease of mankind which is characterised by a marked tendency to haemorrhage and by structural changes in cartilage, bone and teeth. Crews who manned the sailing ships which took part in the great voyages of discovery, to Africa, India and the New World, undertaken in the 15th and 16th centuries by the Portuguese navigators Bartolomeu Diaz, Vasco de Gama and the Genoese born Cristóbal Colón, were extraordinarily prone to develop the disease. The means of prevention of scurvy was succinctly stated (see Stryer)¹ by a Scottish physician, James Lind, in 1753: -

" Experience indeed sufficiently shows that as greens or fresh vegetables, with ripe fruits, are the best remedies for it, so they prove the best preservatives against it. "

Englishmen who sailed the high seas 40 years on, were given a plentiful supply of citrus fruit to combat scurvy, thus earning the nickname "limeys". The disease is caused by a deficiency in the diet of primates (and guinea pigs) of vitamin C (ascorbic acid). Isolation of a pure, water soluble, antiscorbutic substance from fresh fruit and vegetables was first achieved by the Hungarian Nobel Laureate Albert Szent-Györgyi in the 1920s and its structure was determined in 1933. It was named ascorbic acid once its striking biological activity had been demonstrated. The acidity of ascorbic acid is due to the ene-diol grouping (pK_1 4.2; pK_2 11.6) and its most distinctive property, its reversible red-ox action, is manifest in this same grouping (Figure 1).

Collagen is arguably the most abundant protein in the animal body. Its properties are diverse and the dense connective tissues of tendon, skin and ligament are just one of the supramolecular structures formed from the collagen molecule. In all the collagen types a major component is a triple helical structural domain composed of three polypeptide chains which each have an extensive (~1000 amino acids) and characteristic [Gly-X-Y] repeat sequence, where X and Y are frequently proline and γ -hydroxyproline respectively. Once in the extracellular fluid, collagen molecules aggregate spontaneously first into fibrils and ultimately into fibres; they do so moreover in a highly regular manner in which they are staggered axially with respect to one another, giving a liquid crystal-like supramolecular structure. γ -Hydroxyproline residues in collagen are not derived from exogenous γ -hydroxyproline but by direct conversion of certain proline residues in the assembled polypeptide chains, mediated by a di-oxygenase enzyme *prolyl hydroxylase* (Figure 1). The enzyme has a ferrous atom at its active site and the hydroxylation reaction requires a reducing agent, such as L-ascorbate, to keep the iron atom in the ferrous state.

If a solution of collagen is heated substantial changes occur in the tertiary structure of the protein, including the distinctive triple helix. The temperature at which half of the helical structure is lost is called the melting temperature. Collagen synthesised in the absence of L-ascorbic acid is insufficiently hydroxylated and has a lower melting temperature; it cannot form fibres and this leads to skin lesions and weakened blood vessels that are so characteristic of scurvy.

Vitamins, minerals and other nutritional supplements do not work in isolation; they are not drugs. There is a delicate system of checks and balances with other nutrients.

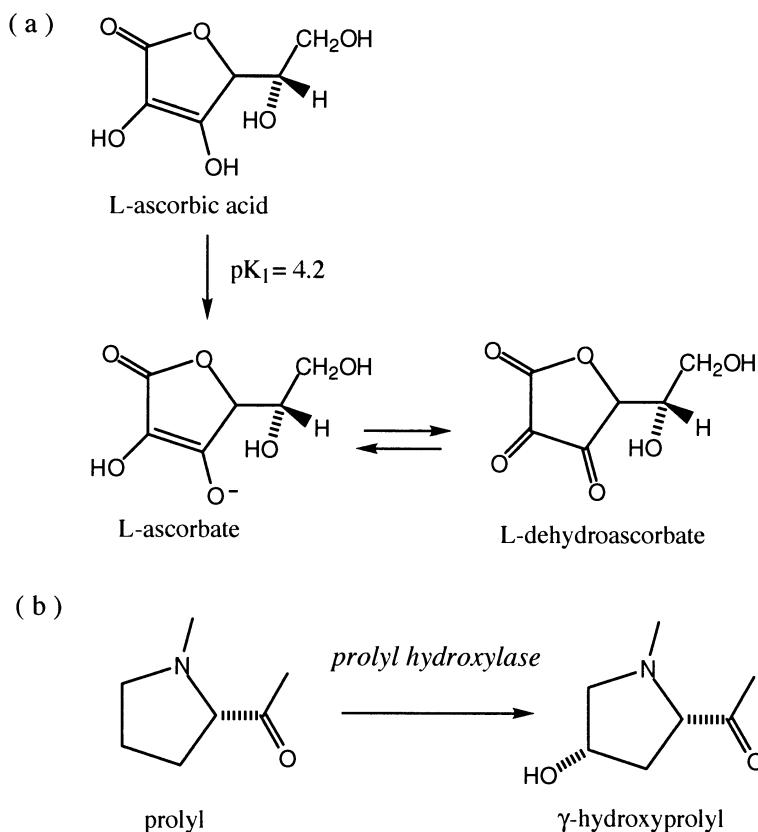


Figure 1. (a) - L-ascorbic acid, L-ascorbate, L-dehydroascorbate; (b) - conversion of L-prolyl residue in collagen to L- γ -hydroxyprolyl by the enzyme *prolyl hydroxylase*

In the case of fruit and vegetables these may include other phytochemicals. In 1936-37 Szent-Györgyi, Bentsath and St.Rusznyak²⁻⁴ showed that vitamin C, augmented with flavonoid preparations from paprika and citrus peel (described as "citrin"), could restore to full health scorbatic guinea pigs - *when vitamin C alone could not*. The flavonoids were designated the name **vitamin P** (P for permeability). On the basis of present evidence it seems reasonable to assume that flavonoids, such as hesperitin, hesperidin and eriodictyol in the fruit, were primarily responsible for these observations (Figure 2). Similarly whilst only vitamin C was curative for acute scurvy, flavonoids had a favourable effect on chronic, borderline vitamin C deficiency; an effect which has been termed - vitamin C sparing activity, (Singleton⁵). This property may well be thought to involve the 3,4-

dihydroxyphenyl ring (ring B) of a substrate such as eriodictyol acting as a surrogate red-ox functionality in place of ascorbic acid (Figure 2).

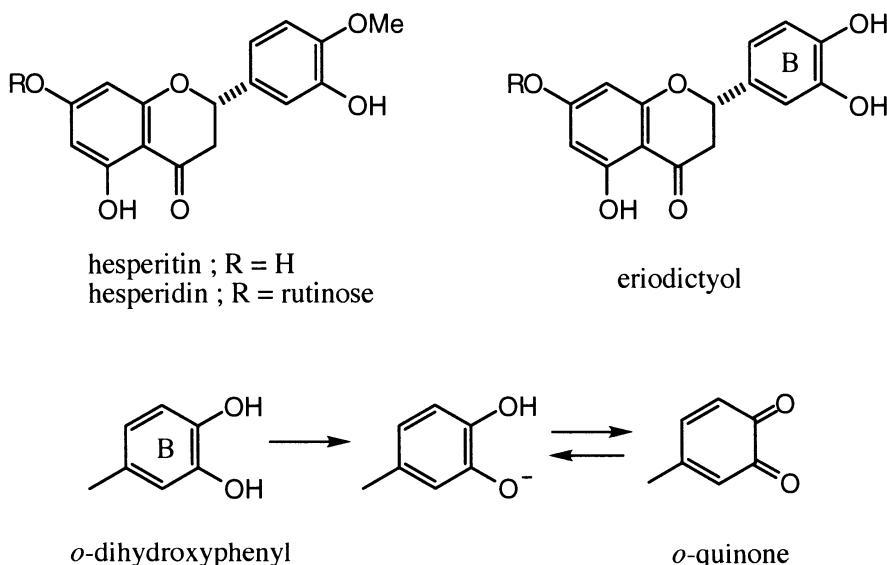


Figure 2. Bioflavonoids; red-ox functionality of ring B of substrates such as eriodictyol

Such was the interest these observations engendered that by 1960 over 1000 papers had been published on this and related topics. Several reviews document this period in detail (*e.g.* Hughes and Wilson⁶; Singleton⁵). However no clear view emerged and it is probably true to say that the history of flavonoids as dietary constituents has been, and continues to be, attended by similar uncertainties and hence controversy. In 1950 the American Society for Biological Chemists and the American Institute for Nutrition proscribed the use of the description vitamin P, on the basis that true vitamin activity had not been established. However its sporadic use persisted into the 1960s and 1970s in the European literature before being displaced by the umbrella terminology **bioflavonoid**, which has been employed to cover all phenolic substances which display "**bioflavonoid effects**". Nevertheless in 1968 the United States Food and Drug Administration (USFDA) withdrew approval for the use of bioflavonoids as drugs since it was considered there was no proven clinical efficacy for man.

Polyphenols (vegetable tannins) and herbal medicines

The use of herbs for therapeutic purposes has been associated with all cultures since the dawn of human history. They were invariably applied in an empirical way based on traditional knowledge passed down through succeeding generations. It is only in the past century that we have begun to learn something about the chemistry and pharmacology of the "active ingredients" (natural products) of these herbal remedies. These range from alkaloids to terpenoids, flavonoids, phenols, phenolic glycosides, polyphenols (vegetable tannins), glucosinolates and cyanogenic glycosides. Nevertheless in spite of the fact that plants or plant extractives have been used in this way for several millenia, only a relatively few are currently officially recognised in the United States as effective drugs. This contrasts remarkably with a country such as China where there are more than 500 extant herbal formulas of which about 200 are commonly used in Chinese medicine today. Moreover these traditional methods of treating illness are often integrated with, and augment, the techniques of Western medicine. Adaptations of this approach, using herbs for both health care and disease prevention, may be found in other eastern countries. Present trends in the West point to an increasing interest in the use of these so-called "natural remedies". However, lest valour get the better part of discretion, a word of caution from Varro E. Tyler, long-time Professor of Pharmacology and Dean of Pharmacy at Purdue University (Tyler⁷):

"Medicine and quackery have always been close, if not compatible partners. At times, they may appear to have separated, but sooner or later, in one place or another, they wind up reunited. The present area of greatest mutual attraction for them appears to be in the treatment of diseases by means of herbal remedies. More mis-information regarding the efficacy of herbs is currently being placed before consumers than at any previous time, including the turn of the century heyday of patent medicines".*

[* quack (Oxford English Dictionary) - Ignorant pretender to skill especially in medicine or surgery; one who offers wonderful remedies or devices, charlatan].

One method of classifying herbal remedies is in terms of their reputed therapeutic actions. Hoffman⁸ lists 45 of these. Typical examples are the following: - anti-haemorrhagic, anti-inflammatory, anti-microbial, astringent, cardiac tonic, diaphoretic and diuretic. Many traditional herbal remedies are based upon plants which are rich in polyphenolic (vegetable

tannin) metabolites; all are usually described as **astringent**. A variety of related conditions are ameliorated by their use; some examples are shown in Table 1. Besides the therapeutic effects most commonly associated with astringent herbal remedies, the ripe fruits of **hawthorn** (*Crataegus* sp.) also provide one of the best tonic remedies for the heart and circulatory system and are used in the treatment of high blood pressure, arteriosclerosis and angina. They act in a normalising way upon the heart, depending on the need, stimulating or depressing its activity. Hawthorn (fruit, leaves, flowers) is a rich source of the flavan-3-ol (-)-epicatechin and proanthocyanidins related to (-)-epicatechin, e.g. - epicatechin-(4 β -8)-epicatechin (procyanidin B-2), Figure 3. It is thought that these and related flavonoids constitute the major active principles⁷, but Duke⁹ also notes the presence of various cyanogenic glycosides in both shoots and seeds. [These latter metabolites, however, are not recorded in the comprehensive "Dictionary of Natural Products", the 1994, Chapman and Hall database]. Although little known in the United States there are numerous preparations containing hawthorn extracts marketed in Germany and elsewhere. For an herbal drug, potentially as useful as this one and in the context of present interests in this area, scientific studies are urgently required to substantiate these empirical observations.

Another herbal remedy still widely used and cultivated specifically for its therapeutic applications in European countries is **meadowsweet** or **queen of the meadow**; one of the three most sacred herbs of the Druids. It was from this plant that salicylic acid, a forerunner of aspirin, was first isolated (1838). Meadowsweet is one of the best digestive remedies available. It acts to protect and soothe the mucous membranes of the digestive tract, reducing excess acidity and easing nausea. It is used in the treatment of heartburn, hyperactivity, gastritis and peptic ulceration, and relieves the pain of rheumatism. Its gentle astringency is very useful in the treatment of diarrhoea in children. Two of its principal polyphenolic constituents are tellimagrandin II and rugosin-D^{10,11} based upon gallic acid and D-glucose; rugosin-D is formally derived by loss of two hydrogen atoms from the "monomer" tellimagrandin II (Figure 4).

Table 1. Some medicinal plants containing polyphenolic metabolites

1. **Tree Peony** (*Paeonia lactiflora*): outer skin of the root, used to cure disorders of the bloodstream, including high blood pressure. Principal polyphenolic metabolites - *gallotannins*.
2. **Bearberry** (*Arctostaphylos uva-ursi*): dried leaves; infusions have a soothing astringent effect which have value as a diuretic, in kidney disorders and ailments of the bladder and urinary tract. Principal polyphenolic metabolites - *gallotannins, arbutin, galloyl esters of arbutin*.
3. **Agrimony** (*Agrimonia* sp.): roots and dried aerial parts of the plant; used as an astringent on the digestive system, as a diuretic and as a haemostatic agent. Principal polyphenolic metabolites - *ellagitannins*.
4. **Geranii Herba** (*Geranium maculatum, G. thunbergii*): dried rhizome and leaves; used as an astringent, anti-haemorrhagic and anti-inflammatory agent. Principal polyphenolic metabolites - *ellagitannin*.
5. **Meadowsweet** (*Filipendula ulmaria*): aerial parts of the plant - leaves and flowers used as an infusion; employed as a mild astringent, anti-rheumatic anti-inflammatory agent, and as a diuretic. Principal polyphenolic metabolites - *ellagitannins*.
6. **Raspberry** (*Rubus idaeus*): leaves and fruit; mild astringent used in disorders of the digestive system, raspberry leaf tea traditionally used during pregnancy. Principal polyphenolic metabolites - *ellagitannins*.
7. **Hawthorn** (*Crataegus* sp.): leaves and berries; used as astringent for digestive system, diuretic, cardiac tonic in treatment of high blood pressure. Principal polyphenolic metabolites - *proanthocyanidins*.
8. **Rose Bay Willow-herb** (*Epilobium angustifolium*): leaves used as substitute tea; roots and leaves have demulcent, tonic and astringent properties and are used as an intestinal astringent. It has been employed as an antispasmodic in whooping cough and asthma and in ointments for the treatment of infantile cutaneous affections. Principal polyphenolic metabolites - *gallotannins, ellagitannins*.

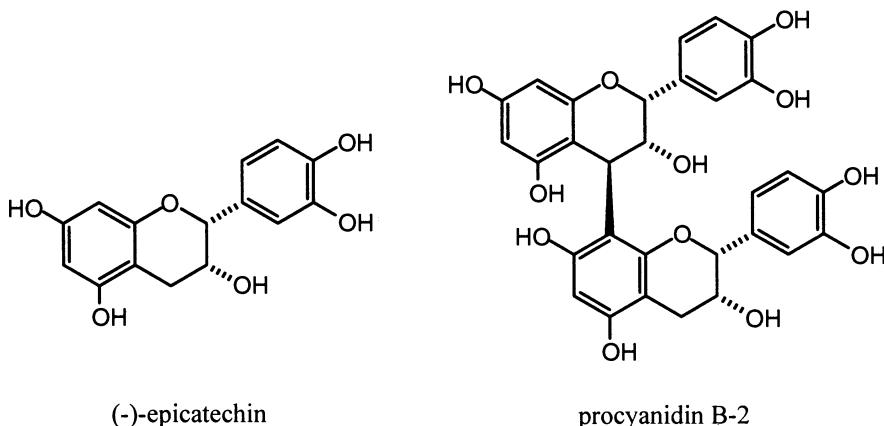


Figure 3. Flavan-3-ols of *Crataegus* species

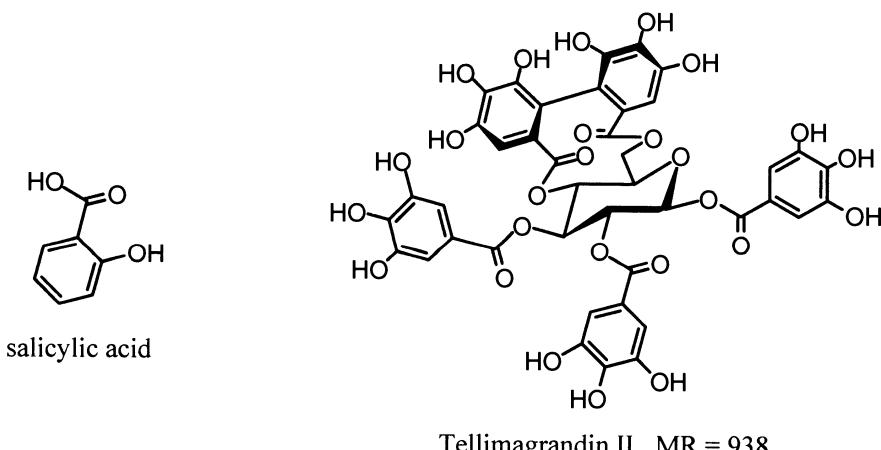


Figure 4. Phenolic constituents of meadowsweet (*Filipendula ulmaria*):
salicylic acid and tellimagrandin II

Finally note may perhaps be taken of the grape vine (*Vitis vinifera*). It is integral to the culture and cuisine of many European, particularly Mediterranean, countries. Although renowned for the production of wine from its berries, various parts of the grape vine (leaves, stem, fruit) have long established, if less well known, medicinal uses ranging from haemorrhage and menstrual problems to those of hypertension and high blood cholesterol levels¹². The fruit of the vine, particularly the skin and seeds, are rich sources of a complex mixture of condensed proanthocyanidins, similar to those found in hawthorn¹³ (Figure 3).

Contemporary developments

In 1962 Rachel Carson published her strong but cogent polemic "*Silent Spring*" awakening attention to the serious problems which arose from the indiscriminate use of pesticides and the widespread and prodigal application to crops of man-made fertilisers. Thereby she planted the seeds of environmentalism in the public domain. The results have been far reaching and continue to grow. At one extreme there are those siren voices which beckon a long past rustic Arcadia, but for a greater number it has led to a critical examination of current life-styles; an incipient "back-to-nature" movement has stirred. They search for reasonable alternatives, whether through diet or treatment, to certain aspects of present day medical treatment. In turn this has given rise to a renaissance of scientific interest in herbal medicines and to the benefits which a balanced "healthy" diet - less fat meat, more fibre intake, more fresh vegetables and fruit, etc - can confer. Over the past decade public enthusiasm for these developments has, in many instances, outstripped precise scientific evidence in support of them, a point amply illustrated by the following headlines and quotes from the London daily press in recent years relating to the putative therapeutic role of polyphenols as antioxidants: -

"Refreshing oriental secret of long life: The tea that Britain used to drink may help fend off heart disease and some cancers."

The Times, March, 1995

"Antioxidants: the key to a long life?"

The Times, January, 1996

"Meet me at the t-bar. Studies have shown that green tea, like wheatgrass and other fresh juices, is packed with minerals and antioxidants, which can help protect against heart disease and cancer."

The Financial Times, January, 1998

« Antioxidants, whether found in tea, malt whisky or red wine, protect the body from harmful substances called free radicals. These occur throughout the environment and are naturally produced in the body where they can damage its cells. The antioxidants have been likened by tea manufacturers to fire extinguishers. They are able to quench any 'fires' 'lit in our tissues by an excessive quantity of free radicals before the cells have been damaged. Taking antioxidants is a damage limitation exercise that reduces the incidence of both heart disease and cancer.

The sources of antioxidants are myriad. Many vitamins, but in particular vitamin E and vitamin C, have powerful antioxidant roles, as do trace elements such as selenium, and of course dark green leafy vegetables and peppers, carrots, etc. Black and green tea contain, just as do red wine and old malt whisky, polyphenols - flavonoids - which offer protection against the ravages of the free radicals and interrupt the slow change reaction of damage which could lead to cardiovascular disease and malignancy. «

Dr. Thomas Stuttaford, The Times, January, 1998

The success of the Rachel Carson crusade was based upon a number of factors, but most importantly it was the incontrovertible scientific facts, underpinning the arguments and the polemic, which ultimately changed the perceptions of public and politicians. Likewise, the case for the presumed link between polyphenols and human health must be based on fact and not supposition. Whilst a body of circumstantial epidemiological evidence supporting the protective effects of dietary polyphenols in respect of certain degenerative diseases has been garnered in the past decade the position remains uncertain. In the context of antioxidant therapy *via* the absorption of polyphenols from red wine, the following quotations¹⁴⁻¹⁶ from three recent papers in *The Lancet* illustrate the horns of the present dilemma: -

"Our results provide direct evidence that regular and long-term consumption of red wine, but not ethanol, inhibited LDL oxidation in vivo. It is suggested that red wine intake may reduce atherosclerosis and morbidity and mortality from coronary heart disease. In this context our study provides a plausible explanation for the "French Paradox".

"In our hands daily consumption of flavanoid-rich red wine did not influence the oxidizability of LDL. Our findings do not accord with the proposed beneficial effect of red wine consumption on LDL oxidation.".

"Contrary to recent reports, red wine is no more cardioprotective than white wine or beer. The data "robustly support" an inverse relation between alcohol consumption and CAD risk and each major beverage type seems equally protective".

The title of a recent paper in the same journal¹⁷ encapsulates this same uncertainty - *"Anti-oxidant therapy for ischaemic heart disease: where do we stand? "* Not surprisingly the urgent need for further detailed studies in this and related areas has been emphasised by, amongst others, Hertog¹⁸ and by

Suschetter *et al.*¹⁹. The goal, in this instance, must be to satisfy the requirements of the current definition of dietary antioxidant such as is used by the Food and Nutrition Board of the U.S. National Academy of Sciences: -"*A dietary antioxidant is a substance in foods that significantly decreases the adverse effects of reactive oxygen species, reactive nitrogen species or both on normal physiological functions in humans*".

Countries within Europe differ in the way in which health claims on foodstuffs are treated. In Britain, if such a claim is made, then it becomes part of the regulatory process of becoming a medicine. In the case of plant polyphenols then an absolute requirement would be to prove that these compounds reach the putative sites of action in the human body and that they fulfil, at these sites, the requirements as laid out in definitions such as that shown above.

BIOLOGICAL AND PHARMACOLOGICAL STUDIES

The increasing interest in the use of "natural remedies" and of appropriate diets to control illness and disease has been paralleled, over the past twenty years, by scientific studies whose aim has been to pin-point the origins of the particular biological activities observed. Notable studies in this area have been made by several groups worldwide, (Vlietinck *et al.*^{20,21}; Hamada *et al.*²²⁻²⁴; Hattori *et al.*²⁵; Kashiwada *et al.*²⁶; Kakiuchi *et al.*²⁷; Kinsella *et al.*^{28,29}; Nakashima *et al.*³⁰; Nishizawa *et al.*^{31,32}; Okuda *et al.*³³⁻³⁷; Perchellet and Perchellet³⁸; Sakagami *et al.*³⁹; Scalbert⁴⁰). *In vitro* testing has identified a wide range of potentially significant biological activities which are exhibited by natural polyphenols and a selection of these is shown in table 2. Although these studies have revealed important differences in pharmacological activity between individual polyphenols and between classes of different polyphenol, *overall they suggest selectivity rather than high specificity towards particular biological targets*. It should also be pointed out that, as yet, it is not clear in humans how, or if, many of these complex polyphenolic substrates are absorbed from the gut and this lack of precise knowledge on the fate of these compounds in the human body remains a major weakness in this area.

Table 2. Some Biological and Pharmacological Actions of Polyphenols²⁰⁻⁴⁰.

- (i) - Bactericidal action.
- (ii) - Molluscicidal action.
- (iii) - Anthelminthic action.
- (iv) - Antihepatotoxic action.
- (v) - Stimulation of phagocytic cell iodination.
- (vi) - Inhibition of Human Immunodeficiency Viral Replication (HIV).
- (vii) - Inhibition of Human Simplex Virus (HSV).
- (viii) - Inhibition of glucosyl transferases of *Streptococcus mutans* (dental caries).
- (ix) - Inhibition of ascorbate auto-oxidation (green tea).
- (x) - Inhibition of lipoxygenase dependent peroxidation; "French Paradox".
- (xi) - Host-mediated antitumour activity: cytotoxic effects, inhibition of tumour promotion, inhibition of ornithine decarboxylase (ODC) response.
- (xii) - Inhibition of xanthine oxidase and monoamine oxidase.

Thus far it has not been found possible to discuss, with any precision, the relationships between polyphenolic structure and "biological activity". Nevertheless, as the generality of effects displayed by many members of the polyphenolic class of compound show, phenols and polyphenols constitute the important active principals of particular diets, many medicinal plants and medicinal plant preparations. It seems reasonable to suggest therefore that these properties derive, at least in part, from the various physical and chemical properties which are associated with the possession of one or more phenolic nuclei within the same molecule. On the basis of this proposition it is therefore suggested that phenols and polyphenols exert certain of their roles in the amelioration and medical treatment of diseased states by virtue of three distinctive *general characteristics*⁴¹ which derive in essence from the properties of the simple phenolic nucleus itself, namely: -

- (i) - **their complexation with metal ions (iron, manganese, vanadium, copper, aluminium, calcium, etc.),**
- (ii) - **their antioxidant and radical scavenging activities, and**
- (iii) - **their ability to complex with other molecules including macromolecules such as proteins and polysaccharides.**

Metal Ion Complexation

The property of molecules, such as natural polyphenols, with catechol and pyrogallol nuclei of forming strong complexes with metal ions such as iron, vanadium, manganese, aluminium, calcium *etc.*, is not only a distinctive one but also an important one. In view of the importance of these metals to living systems it is logical to presume that species which form strong complexes with them, such as polyphenols, may well modify their biological activities. Insofar as the transition metals (vanadium, iron, manganese, copper and cobalt) themselves are concerned they have properties which clearly distinguish them from other metals found in living systems (*e.g.*, sodium, potassium and magnesium) namely (Frausto da Silva and Williams⁴²): -

- (i)- they are good Lewis acids, can act as π -electron donors and are red-ox active,
- (ii)- biological systems have evolved in such a way that, whilst much of the particular element may remain free, a considerable fraction is bound up with particular organic ligands, *e.g.* Fe - haem, and
- (iii)- functional properties, such as red-ox potentials, are very sensitive to ligand co-ordination.

Among the most closely studied, and important, of the inorganic elements necessary for life is iron. It is common to all life and is the most abundant transition metal found in the biosphere; its involvement in biological systems is however complicated. It is involved not only in red-ox catalysis but has numerous other functions - *e.g.*, storage and transport of oxygen, electron transfer, hydroxylation reactions, utilisation of hydrogen peroxide, superoxide dismutation, *etc.*, (Halliwell and Gutteridge⁴³).

The average adult male contains ~ 4.5 g of iron, absorbs some 1-2 mg per day from the diet and excretes about the same quantity when in iron balance. Plasma iron turnover accounts for some 35 mg per day and slight disturbances of iron metabolism readily leads to iron overload or iron deficiency. About 2/3 rd of body iron is located in haemoglobin, with lesser amounts in myoglobin, various enzymes, the transport protein transferrin and a small transit pool of iron chelates (nature uncertain). Otherwise iron that is not required is stored in ferritin which consists of a protein shell surrounding an iron core which holds up to 4500 iron ions per molecule of protein. In humans iron is of particular medical concern because of its involvement in

various red-ox reactions, its effect on infectious organisms and the diseases of iron overload and iron deficiency. Antimicrobial activity through iron depletion is, for example well documented.

Because iron in its most common form is not readily available, (the solubility of ferric hydroxide is 10^{-38}), many micro-organisms produce siderophores - low molecular mass chelating agents that bind and solubilize iron. The one which binds iron the most strongly under physiological conditions is the siderophore enterochelin (**ent**), (Figure 5) formed by *Escherichia coli*. The siderophore employs the three dihydroxybenzoyl rings to give a charged octahedral triscatecholate Δ -*cis* complex. The formation constant with ferric iron and the macrocycle of 10^{49} M^{-1} is the highest reported for a siderophore⁴⁴.

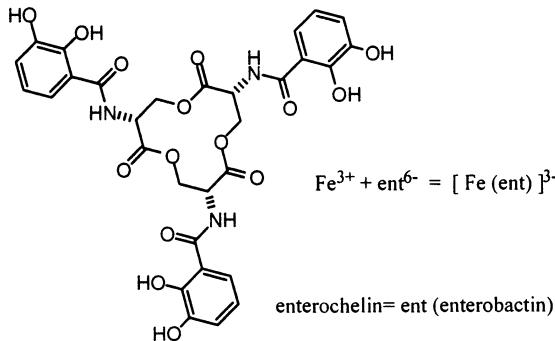


Figure 5. Enterochelin

Contemporaneously the development of new classes of selective, powerful, orally active iron chelators is an urgent need for the treatment of iron overload. The mechanism of capture and binding of the transition metal ion by enterochelin is clearly very similar, in principle, to that deployed by natural polyphenols in their complexation of such ions. There seems, *a priori*, therefore good reason to think that natural polyphenols have the potential, (should they possess the ability to penetrate to particular sites in the human body), to modulate physiological reactions involving iron and other transition metals.

Antioxidant activity

Increasing attention is being given to the role of free radicals and other oxidants in the mechanism of action of many toxins and, in recent years,

their involvement in the pathophysiology of major chronic diseases. Thus reactive oxygen species have been implicated in various human diseases including the processes of ageing, cancer, multiple sclerosis, Parkinson's disease, autoimmune disease, senile dementia, inflammation and arthritis and atherosclerosis. Many chronic diseases are also exacerbated by imbalances or perturbations in fatty acid and lipid metabolism. It is thought, for example, that excess dietary fatty acids are conducive to atherosclerosis and coronary arterial disease and uncontrolled lipid oxidation (enzymic or non-enzymic) is associated with arthritis, cancer and atherogenesis.

Whilst there is little doubt that cellular pro-oxidant states are implicated in many of these diseases it is not yet clear that they are the causative agents. Thus Ames has argued that a deficiency of micronutrients that protect against oxidative damage to DNA is a major contributor to human cancer; on the other hand others have stated⁴³ that increased oxidant formation is usually a *consequence* of disease.

Cellular pro-oxidant states

In cellular pro-oxidant states the intracellular concentration of activated forms of oxygen (reactive oxygen species) is increased, presumably because cells either overproduce these reactive substances or are deficient in their ability to destroy them⁴⁵. The ground state of the diatomic oxygen molecule (O_2) is a radical with two unpaired electrons (having parallel spins) in a π^* antibonding orbital (Figure 6).

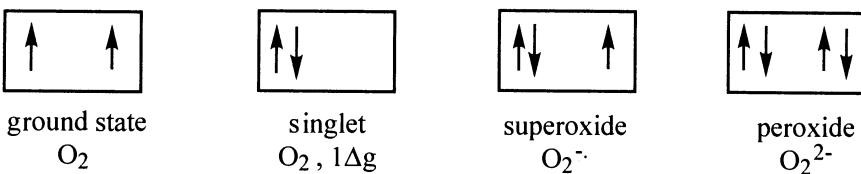


Figure 6. Occupancy of π^* 2p orbitals in various oxygen species

If oxygen is to oxidise another atom or molecule by accepting a pair of electrons from it, then both 'new' electrons must be of parallel spin to pair with the electrons in the π^* orbital. Most biomolecules are covalently bonded non-radicals and the electrons forming covalent bonds have opposite spins and occupy the same molecular orbital. As a result the reaction of ground state oxygen with biomolecules is spin restricted.

The reactivity of oxygen can be enhanced in a number of ways⁴³ (Figure 6) and in cellular pro-oxidant states the intracellular concentration of reactive oxygen species [ROS: *singlet oxygen, superoxide radical, hydroperoxyl radical, hydrogen peroxide, hydroxyl radical, lipid hydroperoxides, lipid peroxy radical etc...*] is enhanced - the cells either overproduce these reactive species or excessive production of these species is beyond the capacity of the cell to destroy them; the normal balance in the cell of antioxidant and pro-oxidant properties is destroyed.

In vivo the major route to the *hydroxyl radical* HO· is the metal-ion dependent breakdown of hydrogen peroxide. The highly reactive hydroxyl radical HO·, once formed *in vivo*, is very likely to react at or very close to its site of formation. It can for example initiate the process of lipid peroxidation by abstraction of a hydrogen atom from an unsaturated aliphatic lipid side-chain eventually giving rise by an autocatalytic chain reaction to a *lipid hydroperoxide* (Figure 7).

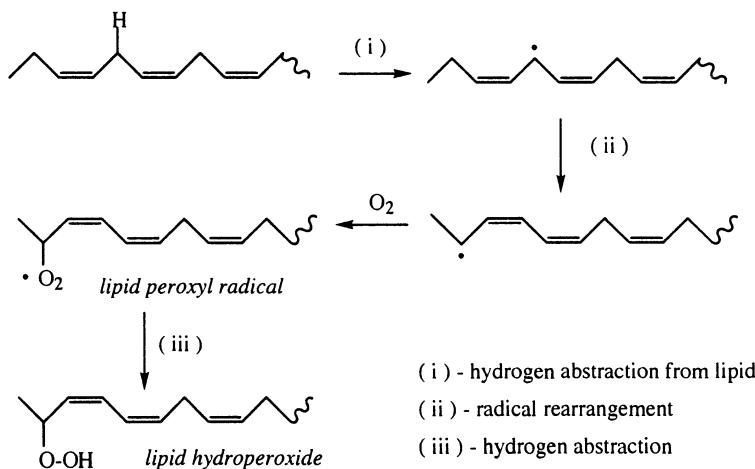


Figure 7. Lipid peroxidation

The decomposition of these lipid hydroperoxides, in the presence of transition metal ions, yields *alkoxyl* and *peroxy radical*s which may abstract additional hydrogen atoms and contribute to chain propagation. Compared with other oxygen-based radicals lipid peroxy radicals are more stable and capable of diffusing to cellular loci distant from their point of generation before reacting with say DNA or other macromolecules.

Oxidative stress represents a disturbance of the pro-oxidant/anti-oxidant balance of the body towards the former state and may arise from environmental or other external sources or by the endogenous production of free radicals accompanying diseased states. Cells and tissues normally possess antioxidant defence mechanisms to ensure the removal of reactive oxygen species - those that are controlled endogenously (*e.g.*, superoxide dismutase) and those [*e.g.*, antioxidants - α -tocopherol (vitamin E), ascorbic acid (vitamin C), β -carotene] that are provided by dietary and other means. If mild oxidative stress occurs, tissues respond by raising the level of the normal anti-oxidant defences. However severe oxidative stress causes cell injury and death.

Vitamins E and C are known and important antioxidants. Vitamin E is the general name given to a group of lipid soluble compounds of which α -tocopherol is the most familiar. It is found in lipoproteins and membranes and acts to block the chain reaction of lipid peroxidation by scavenging the intermediate peroxy radical which are generated (Figure 8).

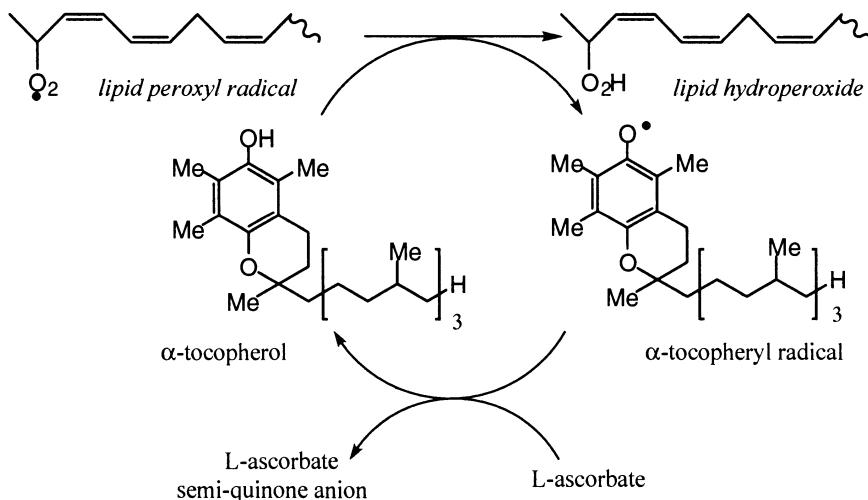


Figure 8. Lipid peroxidation - chain reaction blocking action of α -tocopherol

The highly sterically hindered α -tocopheryl radical is much less reactive in attacking other fatty acid side-chains and may be converted back to the parent phenol by ascorbic acid thus breaking the chain reaction. The

water soluble vitamin C (ascorbic acid) itself has many physiological roles; antioxidant activity (such as the re-cycling of vitamin E in membranes and lipoproteins) is only one of them.

Phenols and Polyphenols as Antioxidant Nutrients

Present interest in the possibility that many major human degenerative diseases may involve, in their aetiology, free radical processes, has its origins in researches which go back to the 1920's, (*vide supra*). Much of our present knowledge comes from epidemiological studies which, although they do not establish cause and effect relationships, do nevertheless present an emerging view that the incidence of certain degenerative diseases, such as cardiovascular disease and some forms of cancer, appears to be much lower in populations with a larger intake of antioxidant nutrients.

Thus it has been suggested that the following levels of intake (mg. per day) in the diet are likely to provide blood levels of the nutrients consonant with a low risk of degenerative disease⁴⁶⁻⁴⁷: -

- Vitamin E (40 - 60 mg./ day),
- Vitamin C (150 mg. / day),
- β-Carotene (9 - 12 mg. / day).

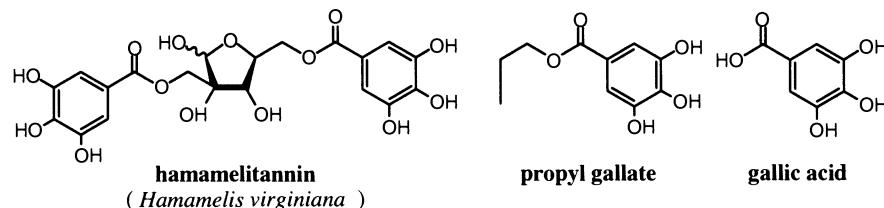
Concomitant with these developments there has, notwithstanding, been a surge in the production of proprietary medicines, offered for sale by major pharmaceutical companies, which are labelled as containing vital antioxidants and often accompanied by comments such as: - "Our new antioxidant will help you stay healthy".

However it is as well to keep these ideas in perspective. Thus until recently β-carotene, a typical carotenoid pigment found in plants and which can act as an antioxidant, ranked high on the list of "natural" remedies against degenerative disease. Trials have now cast doubts on these findings⁴⁸⁻¹⁷.

Phenolic compounds are widely employed as food additives to control non-enzymic lipid peroxidation and thereby maintain the nutritional qualities and the shelf life of foodstuffs. The use of the methyl, propyl and lauryl esters of gallic acid (Scheme 1), in this respect as food anti-oxidants is based upon their lipid solubility and the familiar ease of oxidation of the pyrogallol nucleus and hence their propensity to scavenge free radicals. The efficiency of gallic acid and some galloyl esters, compared to that of ascorbic acid, in scavenging some oxygen free radicals, which are known to be important in cellular pro-oxidant states, is shown in Tables 3 and 4.

Table 3. Radical scavenging activities of hamamelitannin, gallic acid and propyl gallate: IC₅₀ values (μM) - corresponding to 50% inactivation of active oxygen species, values determined by ESR spin trapping. Data from ⁴⁹.

Compound	Superoxide anion, $\text{O}_2^{\cdot-}$	Hydroxyl radical, HO^{\cdot}	Singlet Oxygen
Hamamelitannin	1.31	5.46	45.5
Gallic acid	1.01	78.0	69.8
Propyl gallate	1.41	86.5	66.7
Ascorbic acid	23.3	18.8	120.4



Scheme 1: examples of gallic acid esters

Table 4. Rate constants ($\text{M}^{-1} \text{ sec}^{-1} \times 10^5$) for the reaction of some strongly oxidising radicals with various flavonoids and natural phenols

Compound	$\text{O}_2^{\cdot-}$	RO_2^{\cdot}	HO^{\cdot}
Gallic acid	-	4.5	-
Propyl gallate	-	170	-
Ascorbic acid	-	1,300	-
Fisetin	0.13	4,100	-
Kaempferol	0.024	-	46,000
Quercetin	0.47	390	43,000
Eriodictyol	-	-	31,000
Hesperitin	0.059	-	58,000
(+)-Catechin	0.18	61	66,000
(-)Epicatechin	-	73	64,000

Table compiled from data derived from various sources⁵⁰⁻⁵⁴. Rates of radical reaction are directly comparable for each reactive oxygen species but are not directly comparable between reactive oxygen species.

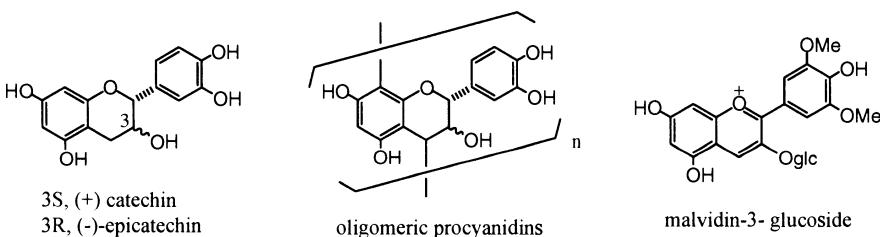
Polyphenols are ubiquitous as plant secondary metabolites¹⁰. They are present in fresh fruit, vegetables, and beverages such as red wines, teas and cocoa. As such they provide a potential source of therapeutically significant anti-oxidants in the diet. Plant phenols and polyphenols are known to inhibit lipid peroxidation and lipoxygenases *in vitro*³³⁻³⁷, and information has been accumulated over the past few years demonstrating their ability to scavenge radicals such as hydroxyl, superoxide and peroxy, which are known to be important in cellular pro-oxidant states. Although Halliwell⁵⁵ has urged a note of caution, pointing out that phenols and polyphenols are "possibly important as anti-oxidants" this fact has nevertheless generated intense speculation. Consumption of polyphenol rich items in the diet it is suggested leads to an increase in plasma anti-oxidant potential, which in turn might have an important role to play in the modulation of exposure to cellular oxidative stress. Various lines of evidence have lent support to this contention.

Hertog and his colleagues in the Netherlands⁵⁶⁻⁵⁸ have carried out a number of within-population cohort studies looking at the relationship between coronary heart disease (CHD) mortality and the intake of flavonoids, such as quercetin, in the diet. So far the epidemiological evidence points to a protective effect of antioxidant flavonols in cardiovascular disease, but it is not conclusive. Thus a protective role for flavonols in cardiovascular disease was found in 3 out of 5 prospective cohort studies, in addition to one cross-cultural study. However one prospective cohort study showed no association and one a weakly positive association between flavonol intake and coronary heart disease⁵⁹.

Epidemiological observations in two other areas have supported the benefits which may accrue from the intake of foods and beverages rich in polyphenols. Examination of WHO data shows marked differences in mortality from coronary heart disease amongst citizens in various countries - especially between French and U.S.A and U.K. populations. Subjects who have similar intakes of saturated fatty acids, similar risk factors and comparable plasma cholesterol levels show a much lower incidence of death from coronary heart disease in France. The regular consumption of red wines was one of the few dietary factors that seemed to correlate with reduced CHD mortality, although interestingly regular fruit consumption also has a good correlation with reduced CHD mortality.

Moderate alcohol consumption itself reduces CHD mortality and several possible mechanisms are now recognised - such as the ability of alcohol to alter blood lipid levels by lowering total cholesterol and raising

high density lipoprotein levels⁶⁰. The statistical data clearly show that *wine alcohol* consumption is much more strongly correlated with CHD mortality than total alcohol consumption and this has led to the view that the alcohol content of red wines may not be the sole explanation for this protection against coronary atherosclerosis. Red wine also contains significant quantities of phenolic compounds - thus a full-bodied young red wine contains up to 4.0 g/litre of phenolics - principally flavan-3-ols, oligomeric procyanidins and anthocyanin pigments (Scheme 2)¹³ and attention has been directed, principally, but not exclusively, at the role these particular constituents and their anti-oxidant properties may possess in rationalising the epidemiological data and with it the so-called "French Paradox"^{28,29}. Although they acknowledged the need for more detailed work on this problem these authors suggested that if potent anti-oxidant phenolic components of red wine are routinely ingested by the regular consumption of red wine they may collectively reduce oxidation of LDL and thereby contribute to the amelioration of atherosclerosis and morbidity and mortality from coronary heart disease. Other workers have presented data, which supports these findings, (e.g., Kondo *et al.*¹⁴).

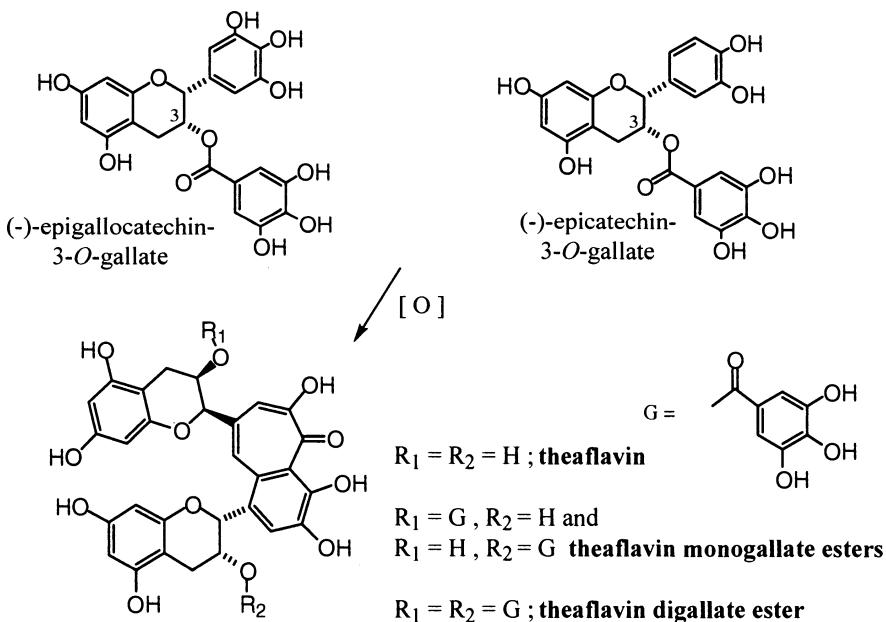


Scheme 2: main polyphenolics compounds from red wine

However it should be very clearly pointed out as a word of caution, if not scepticism, that not all workers in this field share these enthusiasms for the view that the phenolic constituents of red wines may act as anti-oxidants for LDL and so exert an anti-atherogenic effect. Thus de Rijke and her colleagues¹⁵ concluded, in contrast to the findings adumbrated above, that the daily intake of flavonoid-rich red wine **did not** influence the oxidizability of LDL. Halliwell similarly suggested⁶¹ that the enthusiasm for reports that phenolic constituents of red wine may act as anti-oxidants of LDL "*should be tempered by some scepticism*". More detailed biological

observations are clearly required to substantiate the epidemiological evidence at this stage.

Epidemiological evidence, principally from Japan and China, likewise strongly suggests that the habitual consumption of green tea as a beverage may protect against both cancer and the development of CHD. Attention has been similarly focused, with the presumption that polyphenols may ameliorate conditions of oxidative stress, upon the major polyphenolic component of the green tea flush (*Camellia sinensis*), namely (-)-epigallocatechin-3-*O*-gallate. Although the amounts may vary, dependent upon cultural and climatic conditions, flavanols usually constitute up to 20 - 30% of the dry matter of the fresh green tea leaf, of these (-)-epigallocatechin (~2.5%), (-)-epigallocatechin-3-*O*-gallate (~10%) and (-)-epicatechin-3-*O*-gallate (~2.75%) overwhelmingly predominate (Scheme 3).



Scheme 3: main polyphenolics compounds from green and black tea leaf

During fermentation to black teas approximately 15% of the phenolic flavan-3-ol substrates may remain unchanged some 10% are accounted for by the formation of the various theaflavins. Total **theaflavin** concentrations in black teas do not normally exceed 2% and can often be as low as 0.3%.

Serafini and his group⁶² showed that black tea had an *in vitro* anti-oxidant activity approximately one-third of the value reported by Maxwell for red wine⁶³ and that consumption of polyphenol-rich common items of the diet (such as black and green tea) is associated with an increase in plasma antioxidant potential. A preliminary report⁶⁴ indicates that in a series of tests in rats with orally administered (-)-epigallocatechin-3-*O*-gallate, some was adsorbed in the small intestine, but most reached the large intestine where decomposition occurred. At this stage therefore the position in regard to this problem is very similar to that described for red wines - precise "quantitative" experimental evidence in relation to the fate, after ingestion, of these phenolic compounds in humans is still awaited. Until such time the validity of the claims made, in relation to their possible effects in the amelioration of certain diseased states, for these dietary polyphenols should be treated with similar reserve.

Plant phenols and polyphenols are known³³⁻³⁷ to inhibit lipid peroxidation and lipoxygenases *in vitro* and information has been accumulated over the past few years demonstrating their ability to scavenge radicals such as hydroxyl, superoxide and peroxy, which are known to be important in cellular pro-oxidant states. However whilst the propensity of natural plant phenols and polyphenols to act *in vitro* with reactive oxygen species in the manner predicted for natural antioxidants is clear, it should nevertheless also be pointed out that they may additionally (like ascorbate) in some circumstances show pro-oxidant characteristics⁵². Thus it has been demonstrated that the food antioxidant propyl gallate and plant phenols can in fact also react with ferrous ions in the presence of hydrogen peroxide to produce reactive oxygen species which can subsequently damage other biological molecules.

Physiological antioxidant protection has been invoked as one of the principal mechanisms to combat free radical induced, mediated and promoted disorders and diseases. Attention has, until recently, been focused on the antioxidant vitamins and pro-vitamins - ascorbic acid, α -tocopherol and β -carotene. During the past decade the ability of natural phenolic compounds - flavonoids, stilbenes, and derivatives of the hydroxycinnamic acids and gallic acid - to act as antioxidants has also been extensively studied *in vitro*. However, *in vivo* rather less knowledge has been acquired concerning their availability, from the diet, at sites of oxidative challenge. The general consensus appears to be that, in aqueous media, polyphenols act

as electron donors and as hydrogen donors in non-polar systems. Numerous studies^{50,51,65-68} point to the fact that the major sites of antioxidant activity in flavonoids are the catechol or pyrogallol-type rings B [e.g. (-)-epicatechin; (-)-epigallocatechin; quercetin] or the pyrogallol gallate ester groups [e.g. (-)-epigallocatechin-3-O-gallate]. The reduction potential difference between the A and B ring phenoxyls, in a molecule such as (-)-catechin is such that even if the A ring phenoxy radical were to be generated it would normally irreversibly oxidise the phenolic B ring either intra- or inter-molecularly (Figure 9). Jovanovic and his colleagues have suggested^{67,68} that the conjugation between the A and B ring phenoxy-radicals of flavonoids is very inefficient (< 2.5%).

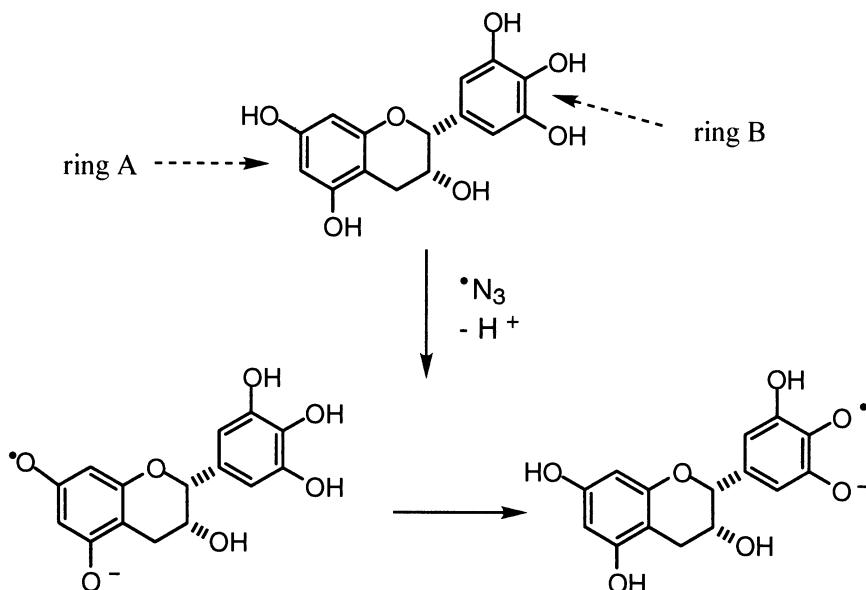


Figure 9. One-electron oxidation of (-)-epigallocatechin by N_3^{\cdot} (azide radicals) at pH 7.0⁶⁷

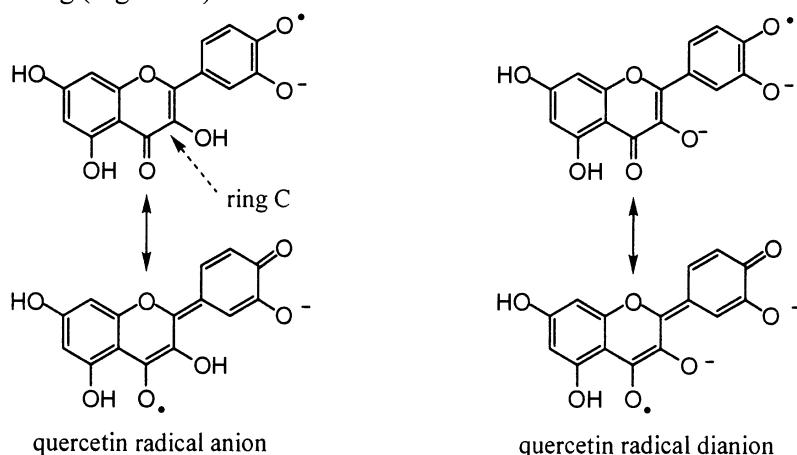
The efficacy of these molecules to act as anti-oxidants in biological systems depends on their ability to intercept and inactivate potentially damaging "foreign" free radicals or to repair damaged biomolecules or bioradicals. Measurement of the reduction potentials of phenoxy radicals is essential for an assessment of their antioxidant capabilities⁶⁸. Several such values (E_7/V ; pH 7.0) are reported in Table 5 for various flavonoids and model phenols.⁶⁸

Table 5. Reduction potentials of phenoxy and flavonoid radicals^{66,68}

Substrate	E_7 / V
Hesperidin	0.72
Rutin	0.60
(+)-catechin	0.57
(-)epigallocatechin	0.43
Quercetin	0.33
Catechol	0.53
Methyl gallate	0.56
3,5-dihydroxy-anisole	0.84

Steenken and Neta⁶⁶ report $E_7 = 0.30$ V at pH 7.0 for ascorbate.

Phenoxy radicals derived from ring A models (2,4-dihydroxyacetophenone, 3,5-dihydroxyanisole) have the highest reduction potentials ($E_7 = 0.89$ V; $E_7 = 0.84$ V, respectively), whilst those derived from the B-ring models (catechol $E_7 = 0.53$ V; 4-methylcatechol $E_7 = 0.52$ V; methyl gallate $E_7 = 0.56$ V) were much lower. The reduction potentials derived from various flavonoids were very similar to those of the models (Table 5). Quercetin radicals possessed the lowest reduction potential of all the flavonoid radicals investigated and this was attributed to the efficient coupling between the B ring radical and the carbonyl group in the C ring (Figure 10).

Figure 10. The quercetin radical anion and quercetin radical dianion⁶⁸

Based upon these results the natural polyphenols are inferior electron donors when compared to vitamin C. Even quercetin, which, of the various flavonoids investigated, is **unlikely** to be able to "repair" vitamin C radicals under physiologically relevant conditions (pH 7 - 9, etc.), reaction (a) (Figure 11). Likewise hesperidin which was the principal flavonoid constituent of Szent-Györgyi's vitamin P (*vide supra*). However the gallicatechins (and their 3-*O*-gallate esters) and quercetin **may** be able to reconstitute vitamin E (assuming the same reduction potential as Trolox C radicals, E₇ 0.48 V) under physiological conditions, reaction (b) (Figure 11).

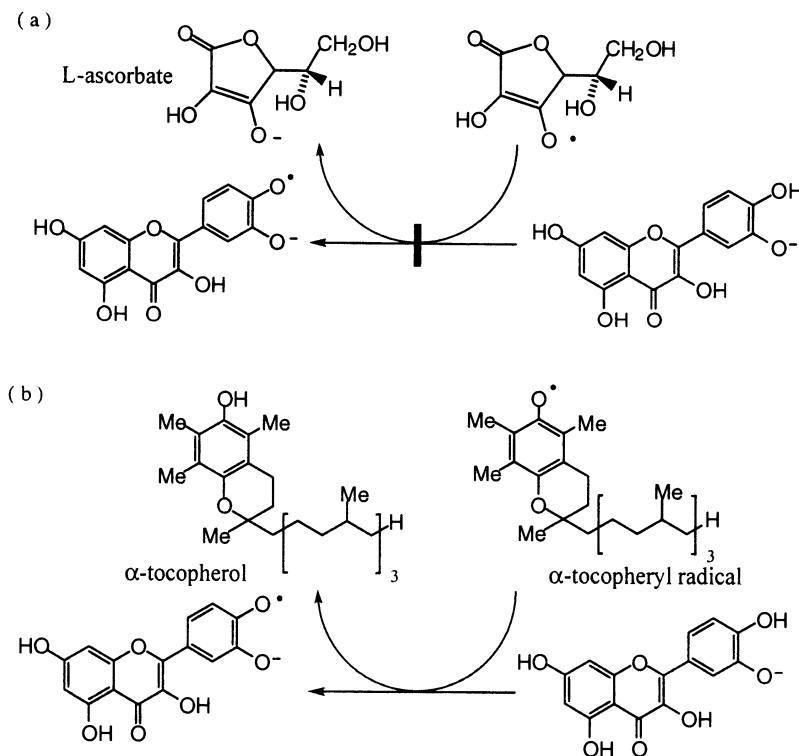


Figure 11. Flavonoid antioxidant activity: reconstitution of vitamin C (improbable) and α -tocopherol (possible) by quercetin?

Nevertheless the reduction potentials of these natural polyphenols are lower than those of important biologically damaging radicals such as O_2^- ,

HO_2^- and alkyl peroxyxl, ($E_7 \sim 1.06$). As a consequence the phenolic flavan-3-ols, flavones and flavonols may play an important role in the physiological defence against such radicals.

Polyphenol Complexation

Although the uses of herbal remedies, rich in polyphenols, as medicinal agents may be summarised under several broad headings, e.g. Tables 1 and 2, many of their actions appear to devolve, either directly or indirectly, on their ability to complex with proteins and polysaccharides. They thus aid the healing of wounds, burns and inflammations. In doing so they act to produce an impervious layer [polyphenol-protein and/or polysaccharide complex] under which the natural healing processes can occur. Presumably in such instances part of their action is facilitated by their complexation with the collagen of the skin, a reaction which underlies the age-old manufacture of leather from animal skins and hides. Thereby they harden and render the tissue impervious to abrasion and water, and thus to additional infection. Under this surface the normal healing processes may then take place. Decoctions of a number of polyphenol-rich plants were also frequently prescribed to stop internal bleeding, nosebleeds and to heal all internal wounds generally.

The widespread use of polyphenol containing plants and plant extracts in old herbal remedies for the treatment of disorders of the digestive system and the intestinal tract, Table 1, probably arises from the fact that gut secretions are hindered thus protecting the underlying mucosa from toxins and other irritants in the bowels. Likewise the healing action of many herbs and their ability to increase vitality has been variously ascribed to their ability to act to *purify* or to *cleanse* the bloodstream⁸. Many of these extracts contain polyphenols of one form or another and it is tempting to suggest that the idea of a blood cleanser, whilst hinting descriptively at much but saying little of substance in medical terms, may derive from the ability of such remedies to enter the bloodstream and preferentially complex with and remove toxins and other harmful materials of a proteinaceous character.

In strictly more scientific terms this propensity to bind to proteins also presumably accounts for the fact that polyphenols inhibit virtually

every enzyme that they are tested with *in vitro*^{69,70}. Assessment of the medical significance of polyphenol inhibition of particular enzymes (whose actions influence the course of development of illnesses and disease), determined *in vitro*, is therefore dependent on the, as yet, unanswered questions relating to the absorption and penetration of ingested polyphenols to the desired site(s) of action *in vivo*. Where the enzymes are extracellular then these problems do not arise. Thus the mutans group of streptococci, *Streptococcus mutans* and *Streptococcus sobrinus*, are principal cariogenic organisms and their major ecological niche is the tooth surface and dental plaque. Cariogenicity is considered to be strongly associated with the ability of these organisms to synthesise extracellular water-insoluble glucans by using glucosyltransferases (GTases). At least two major classes of GTase inhibitors, present in common foods and beverages, are known, namely certain mono- and oligosaccharides and polyphenolic compounds such as those found in betel nuts (*Areca catechu*) and tea leaves (*Camellia sinensis*). Hattori and his colleagues²⁵ have examined the effects of tea polyphenols on the glucosyltransferase (GTase) from *Streptococcus mutans* and they showed that whilst (-)-epicatechin, (-)-epigallocatechin and their 3-*O*-gallate esters, and their various diastereoisomers, showed modest inhibitory action against the enzyme, theaflavin and its mono- and bis-galloyl esters were potent inhibitors at concentrations of some 1-10 mM. Hamada and his colleagues^{22,23,24} have shown that ellagic acid and various extracts of partially fermented tea (Oolong tea) inhibit glucan synthesis from sucrose by the GTases from *Streptococcus mutans* and *Streptococcus sobrinus*. These workers suggested that the active components of the Oolong tea were the polymeric fractions that were "structurally different from those found in green and black tea", although they did not specifically characterise and identify them. Administration of polyphenolic extracts of Oolong tea into diets and drinking water led to a highly significant reduction in dental caries development and plaque accumulation in experimental animals infected with mutans streptococci. It was suggested that such extracts (like betel nuts) might well be very useful for controlling dental caries in humans, presumably *via* their inhibition of the glucosyltransferases.

Polyphenols have also been shown to have a broad antiviral spectrum *in vitro*, but to date their corresponding properties *in vivo* have not been well established³⁹. In a systematic study of the antiviral activity of a very wide range of natural products Vlietinck and his colleagues²⁰ concluded that polyphenols act principally by binding to the virus and/or the protein of the

host cell membrane and thus arrest absorption of the virus. They concluded that in consequence polyphenols are probably only viricidal in nature. Sakagami and his colleagues³⁹ have put forward a number of possible mechanisms whereby polyphenols may exert their anti-viral action. They suggested similarly that the major part of the anti-viral activity due to polyphenols probably derives from their direct inactivation of the virus and/or from inhibition of the virus binding to the cells. They also noted that although polyphenols are known to inhibit viral replication enzymes (such as reverse transcriptase for HIV and RNA polymerase for influenza virus) and other enzymes [*e.g.* poly(ADP-ribose) glycohydrolase], these effects seem to be non-specific.

This affinity of natural phenols and polyphenols for proteins also extends to very simple peptides. Thus gallic acid and its esters suppress the contractile response elicited from the smooth muscle of the guinea pig ileum by the action of the peptide hormone, bradykinin. The suppressive effect of members of a homologous series of gallate esters increases almost directly with the chain length of the ester group⁷¹. In the case of the long-chain esters the effect is only partially reversible. The mechanism is not clear but it is perhaps worth noting that the binding of phenols/polyphenols to bradykinin and other simple peptides is a reaction strongly influenced by hydrophobic effects.⁷².

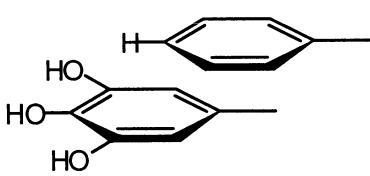
Scientific studies of the reversible association of polyphenols with proteins have a history going back almost 200 years to Sir Humphry Davy in 1803. Various quantitative investigations, completed over the past 25 years, have given considerable insights into the area; "structure-activity" relationships have been delineated and mechanisms proposed. **Molecular size, the number and disposition of phenolic nuclei, conformational flexibility and water solubility** are the dominant features in the determination of the strength of binding of a particular polyphenol to the protein. Good water solubility depresses the effectiveness of the polyphenol in protein complexation, whilst increasing molecular size and conformational flexibility enhance association with the protein. High-resolution NMR studies confirm, what has always generally been assumed, namely that the aromatic nuclei of polyphenols provide the principal sites for association with proteins.

In terms of its findings in relation to polyphenol-protein interactions there is little doubt that the paper by Butler and Hagerman⁷³ probably has been the most influential, particularly in the way in which it has directed subsequent work in this area. The Purdue workers used a competitive binding assay and showed that the condensed proanthocyanidin polymer (tannin) from *Sorghum bicolor*, at pH 4.9, had a relative affinity for various proteins and synthetic polyamides which ranged over four orders of magnitude - indicating that this polyphenol interacts quite selectively with proteins and protein-like polymers. Proteins were most efficiently precipitated at or near their isoelectric points. Tightly coiled globular proteins were not so readily precipitated as those, which had a random coil conformation, and, in particular, those which were rich in the amino acid proline (proline-rich proteins - PRPs). They also showed that the relative affinities of polypeptides and proteins for the polymeric proanthocyanidin were influenced by their size. The low affinities of small peptides and low molecular weight oligomers of proline for the tannin and the non-linearity of the increase in affinity which was observed with higher oligomers of proline implied, they suggested, that the complexation involved the deployment of multiple binding sites on both substrates.

In their seminal work Butler and Hagerman⁷³ besides using various oligomers of polyproline, utilised calf skin gelatine and a proline-rich protein from the rat parotid gland as examples of PRPs. Luck *et al.*^{74,75} later demonstrated the same high affinity of polyphenols for another group of PRPs - the milk proteins, α_1 -S, β and κ - caseins. These amphipathic molecules are, under appropriate conditions, able to form micelles that, it was suggested, have the ability to solubilize the added polyphenols within the micellar structure.

This affinity of natural polyphenols for proteins also extends to simple peptides, which are proline-rich and/or hydrophobic in character. In their studies Hatano and Hemingway⁷⁶ used intermolecular nOes to look at the association of (+)-catechin and the procyanidin catechin-(4 α -8)-catechin (*via* their phenolic nuclei) with oligopeptides ranging from Pro-Gly, Pro-Val and Pro-Phe to Gly-Pro-Gly-Gly and bradykinin (**Arg-Pro-Pro-Gly-Phe--Ser-Pro-Phe-Arg**). They concluded that strong selectivity for prolyl residues was not apparent; rather complexation was directed to the conformationally accessible hydrophobic regions of the peptide. Haslam and his colleagues^{41,72} made a detailed NMR study of the interaction of a series of galloyl esters with the bioactive peptide bradykinin and determined association constants for these interactions. Bradykinin is a proline rich

nonapeptide and with two phenyl residues is hydrophobic in character; its strong association with and precipitation by natural polyphenols is therefore not unexpected. A rapid decrease in the affinity of the polyphenol for the peptide was observed with decreasing galloyl content and this mirrors exactly the data obtained earlier with larger protein molecules, *i.e.* a strong dependence upon molecular size, the number and arrangement of phenolic groups and water solubility of the polyphenol. The most significant proton chemical shift changes in the bradykinin were associated with each of the three proline residues and the two phenylalanine groups. Strong intermolecular nOEs were observed between the 2-H galloyl protons and the protons of these groups suggesting that these amino acid side chains were participating preferentially in the complexation with polyphenols. π - π stacking was put forward as the mode of interaction with the phenylalanyl residues in the peptide, (Figure 12a). However it was suggested that there is probably not a specific mode of binding between the polyphenol and peptide substrates. Rather the driving force was visualised as the relatively unselective association of the aromatic nuclei of the polyphenol with prolyl and phenylalanyl groups on the nonapeptide, which *together* form a relatively hydrophobic area on the surface of the peptide. The role of the side chain of the amino acid arginine (**Arg**) in these complexations remains unclear. On the basis of observations in related fields it was suggested that the π -electrons of the 'electron rich' phenolic nuclei act as hydrogen bond acceptors forming quasi-hydrogen bonds to the hydrogen bond donor ($-\text{NHC}(\text{NH})\text{-NH}_2$) of the arginyl side-chain, (Figure 12b).

(a) Phenylalanine residues - ' π - π ' stacking

(b) Arginyl residues - hydrogen bonding

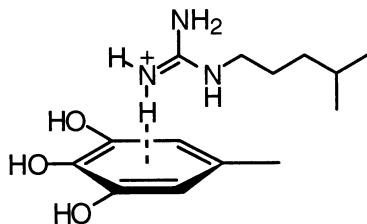


Figure 12. Possible non-covalent interactions of phenolic rings with amino-acid residues in the bioactive nonapeptide bradykinin

Similar comprehensive studies of the association of polyphenols with proline rich peptides, based upon the mouse salivary proline-rich protein

MP5, strongly support these observations^{77,78,79}. The processes are hydrophobically driven (water structure breaking) ones and the principal binding sites on the two peptides are the apolar methylene and methine groups on the prolyl residues themselves. The interaction was visualised as a hydrophobically driven association between a galloyl ring and the exposed hydrophobic surface of the pyrrolidine ring, preferentially that face containing the α -proton. Hydrogen bonding of one, or two, phenolic groups to the tertiary amide carbonyl group on the adjacent peptide linkage was presumed to be a secondary interaction helping to stabilise the complex, (Figure 13). Thus proline-rich peptides and proteins which have an open, random-coil type of conformation have a high affinity for polyphenols not only as a result of their extended structures, but also by virtue of the prolyl groups themselves which, in a figurative sense, provide "sticky patches" on the protein for the phenolic nuclei of the polyphenolic substrate.

Proline residues - hydrophobic stacking

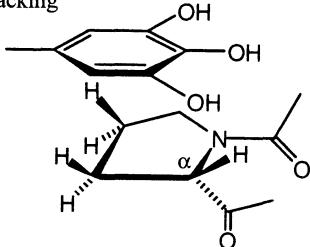


Figure 13. Figurative visualisation of the non-covalent hydrophobic interaction between the pyrrolidine ring of a prolyl residue and a phenolic ring

According to Jencks⁸⁰, "Hydrophobic effects" are probably the single most important factor providing the driving force for non-covalent intermolecular interactions in aqueous media. They may broadly be defined as an interaction of the molecules with each other, which is stronger than the interaction of the separate molecules each with water. No mechanism is implied by this definition. Water is a notoriously poor solvent for apolar compounds, such as hydrocarbons and the noble gases, at moderate temperatures and pressures. This reluctance to dissolve in water has been popularly attributed to the hydrophobicity of these substances - their *fear of water*. For an apolar compound to dissolve in water, it must intrude into a liquid that is characterised by an extended network of hydrogen bonds and has a high cohesive energy. Many rationalisations have focused upon the large losses of entropy which accompany the dissolution of

non-polar solutes, such as a noble gas or a hydrocarbon, in water. Frank and Evans⁸¹ first sought to rationalise the unusual thermodynamic properties of non-polar solutes in water by postulating a particular ordering of water molecules (structure making) around the solute. They described the process as "iceberg" formation: -

"When a rare-gas or a non-polar molecule dissolves in water at room temperature it modifies the water structure in the direction of greater "crystallinity" - the water, so to speak, builds a microscopic iceberg around it."

There is ample experimental evidence that relatively non-polar molecules have a favourable net free-energy of interaction with each other in aqueous media and that these "**hydrophobic effects**" (for want of a better term) are probably also the most important single factor, the most significant driving force for non-covalent intermolecular interactions in aqueous media.

The study of the molecular basis of polyphenol - protein interactions is not only of intrinsic importance to the wider questions of molecular recognition but it is crucially important in relation to the increasing 'therapeutic' use of plant materials, containing polyphenols, either as sources of potential anti-oxidants or, as herbal medicines. In such cases the palate, with its battery of associated proline-rich proteins (PRPs), may indeed be seen as a potential barrier which such medicines must first surmount before they may be absorbed into the plasma for these proteins are pre-eminent in their ability to bind polyphenols. Saliva is produced by the major salivary glands which empty their secretions into the oral cavity. The macromolecules in saliva consist almost exclusively of proteins (~ 1.0-3.5 mg/ml) and amino acid analyses of human salivary proteins have demonstrated the presence of an unusually large amount of proline (~ 16 - 33% of total amino acids). From the work in several laboratories^{82,83,84} it is now clear that saliva contains a unique group of proline-rich proteins or PRPs. They can be sub-divided into acidic, basic and glycosylated proteins, which have a repetitive primary structure particularly rich in the amino acids proline (P), asparagine (N) and glutamine (Q), and glycine (G). In human parotid saliva these account for 17%, 23% and 30% respectively of the total protein. Typical examples of such proteins are the acidic PRP - PIF-s (Figure 14), which may be likened to a detergent molecule with a charged hydrophilic "head" and a proline rich hydrophobic "tail", and the basic PRP IB-7 (Figure 15) which is amphipathic, but in which the hydrophilic side-chains (lysine and arginine) are distributed throughout the sequence.

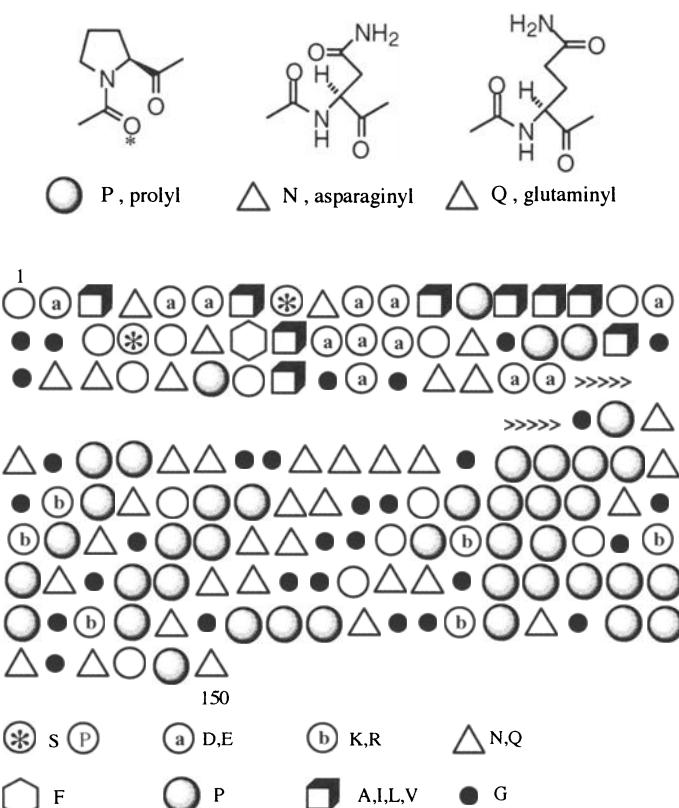


Figure 14. Diagrammatic representation of the amino acid sequence of the human acidic PRP: phosphoprotein PIF-s. The break shown in the amino acid sequence between position 51 and 52 [>>>>] illustrates the amphipathic nature of the molecule, with a strongly acidic N-terminus (~50 amino acids) and the proline-rich C-terminus (~100 amino acids).

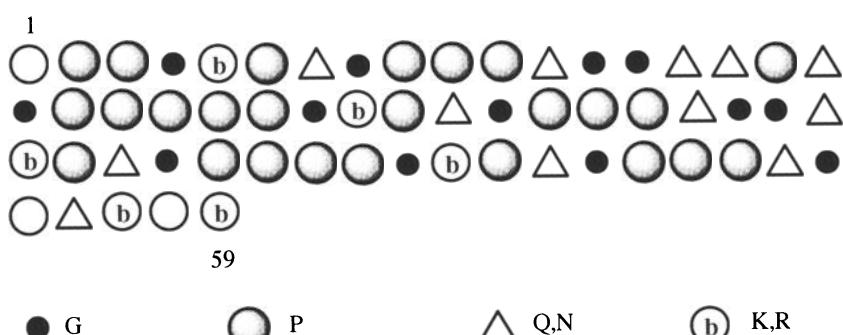


Figure 15. Diagrammatic representation of the amino acid sequence of the human basic PRP: IB7.

Basic PRPs complex with and precipitate polyphenols and recent observations suggest that both acidic PRPs and glycosylated PRPs are fully capable of forming *soluble* complexes with tannins but ones which are not so readily precipitated by virtue of the strongly solubilizing groups present elsewhere on the peptides. Complexation is presumed to occur selectively in the relatively hydrophobic proline-rich regions of the proteins. Consumption, as part of the diet, of fruits, fruit juices and wines, gives rise interactions of this type, and leads to an astringent response in the palate.

Astringency results from a loss of lubrication in the mouth. It is normally recognised as a feeling of extreme dryness and constriction, roughness or puckeriness of the palate which takes a significant time to develop. It is diffuse and not confined to a particular region of the palate. The word astringent is derived from the Latin *ad* (to) and *stringere* (bind); thus astringency is properly defined as a binding reaction, a sensation of touch. Indeed astringents in medicine are recognised as substances that bind to and precipitate proteins. Typical astringents include the salts of multivalent cations (Al, Cr, Zn, Pb, Ca, B), dehydrating agents such as alcohol and dimethyl ketone, mineral acids and natural polyphenols (vegetable tannins). A mucous membrane covers all the exposed surfaces of the mouth which are moistened by the secretions from the salivary glands. According to Bate-Smith (1973) the primary process whereby astringency develops is *via* precipitation of proteins and mucopolysaccharides in the mucous secretions. Accepting this view (which is still broadly assumed) then an understanding of the mechanism of the astringent response therefore focuses attention automatically upon (i) the molecular basis of the action of salivary proteins which give rise to lubrication in the mouth and (ii) polyphenols and their interactions with salivary proteins which result in the loss of lubrication. The data, on the comparative astringency of some natural polyphenols and shown in Table 6, were measured by an experienced taster from the food industry (Clapperton, Williamson and Haslam, unpublished data). Increasing aliquots of each polyphenol were taken and a "quasi-binding" curve established for each sample. From these curves the minimum threshold concentration for the perception of astringency in the palate was determined.

Even the most cursory examination of the data in Table 6 indicates a broad correlation of the astringent response of a particular polyphenol with

the picture presented earlier of the relationship between polyphenolic character and capacity to bind to protein, (e.g. **molecular size**, the **number and disposition of phenolic nuclei**, **conformational flexibility** and **water solubility**)

	940	0.009
Rugosin D	1874	0.005
Castalagin / vescalagin	936	0.4
(+)-catechin	290	0.3
(-)-epicatechin	290	0.9
(+)-catechin-3-O-gallate	458	0.1
(-)-epigallocatechin-3-O-gallate	474	0.4
Procyanidin B-2	578	0.05
Chlorogenic acid	353	0.4

Even the most cursory examination of the data in Table 6 indicates a broad correlation of the astringent response of a particular polyphenol with the picture presented earlier of the relationship between polyphenolic character and capacity to bind to protein, (e.g. **molecular size**, the **number and disposition of phenolic nuclei**, **conformational flexibility** and **water solubility**). Such a correlation strongly suggests that, in the case of polyphenols, it is (as originally suggested by Bate-Smith) their binding to salivary proteins which fundamentally underlies the development of astringency in the mouth.

Twenty five years later it is apposite to ask therefore :-

"What physical picture now emerges of the nature of the astringent response ?".

Since astringency results from the loss of lubrication in the mouth it is pertinent to consider first how lubrication is itself achieved in the oral cavity. It has long been held that lubrication is associated with saliva which derives its inherently viscous nature from the presence (~3.5 mg/mL) of thread-like molecules such as mucopolysaccharides and the PRPs (Figure 16a). It is presumed that such molecules adopt extended random coil conformations in solution and, probably by non-covalent interactions (e.g. hydrogen bonding),

form a tribological layer over the exposed surfaces of the palate, (Figure 16a). The strongly hydrophilic carbohydrate groups attached to the glycosylated PRPs (Figure 16c) may be envisaged to contribute not only to this overall structural process but also, by virtue of their approximate orthogonal relationship to the longitudinal peptide chains (similar to the hairs on the root of a plant), to place additional limits on the lateral compressibility of the PRPs as flow across the surfaces of the palate occurs. Their presence in the tribological layer would thus be expected to enhance lubrication in the palate.

Viscosity is a measure of the rate of energy dissipation in flow and for a macromolecule depends critically upon its shape and asymmetry, and its hydrodynamically effective volume. This last quantity must necessarily include not only the volume of the anhydrous material but also the solvent that is closely associated with the macromolecule. One view (developed in association with Professor A.J. Ryan, University of Sheffield) of the origins of astringency takes note of that fact. It is suggested that astringency derives directly from a collapse of the tribological layer under the influence of the astringent principles, to a release of bound solvent (water molecules) into the surrounding medium, and hence to a loss of lubrication. In the case of natural polyphenols this collapse would be engineered by intra- and inter-molecular non-covalent cross-linking of the salivary PRPs (Figure 14b), by mechanisms such as those described earlier. Although precipitation of polyphenol-protein complexes may occur ultimately this is not a necessary condition for the generation of the astringent response. The critical feature is the collapse of the tribological layer in the palate.

The principles which underlie molecular recognition phenomena may be analysed not only in terms of the composition, structure and conformation of the substrates taking part in the complexation reactions but also in terms of three idealised concepts. "*Die-mould*" (jigsaw) matching is essentially static with an exact fit of donor and acceptor molecules together. "*Key-lock*" matching is time dependent, since the key (donor) invariably has to be manoeuvred into the lock (acceptor) to achieve the correct fit. Finally "*hand-in-glove*" matching of donor and acceptor molecules is both *time dependent and dynamic*. Both donor and acceptor molecules are mobile and flexible and may assume a variety of subtly different shapes as complexation proceeds. In such situations it is important that both substrates are able to

sample a variety of different relative orientations with respect to each other such that ultimately the maximum number of strong contacts are made between donor and acceptor species. Such associative reactions frequently exhibit strong cooperative effects. Present evidence, summarised above, suggests that where polyphenol-protein complexation is at its most effective then it is largely of the "*hand-in-glove*" type where polyphenol and protein both possess considerable conformational mobility and structures which permit the possibility of bringing about multi-dentate complexation. If this view is accepted then this would also predict a delayed, time dependent, astringent response in the palate.

Finally brief comment should be made on the role of low molecular mass phenolic compounds, such as (-)-epicatechin, (+)-catechin, (-)-epigallocatechin-3-*O*-gallate and chlorogenic acid, in the development of astringency in the palate. The evidence from taste panels shows that the threshold values for the perception of these compounds as astringents in the palate is relatively *high*, c.f. Table 6. Work suggests nevertheless that where the concentration of these compounds in liquor is *high* then the liquor is generally perceived as mildly astringent. *A priori* it seems unlikely that the explanation is as suggested for polyphenols in Figure 16, namely direct cross-linking of the PRPs. The binding of simple phenols to proteins is relatively weak and the principal (if not sole) binding site(s) appears to be the *o*-dihydroxy, *o*-trihydroxy "B" phenolic nuclei. The participation of such substrates in direct cross-linking reactions seems, for steric reasons, also to be doubtful. Accordingly any explanation must center upon their relatively high concentrations in the liquor(s) and the hypothesis that under such conditions the equilibrium between phenolic compound and PRPs is driven towards the intermolecular complex. Coating of the surface (either wholly or partially) of the PRPs would lead to a relatively hydrophobic phenolic layer. In turn this would lead to aggregation and to the release of solvent (water) to the bulk medium.

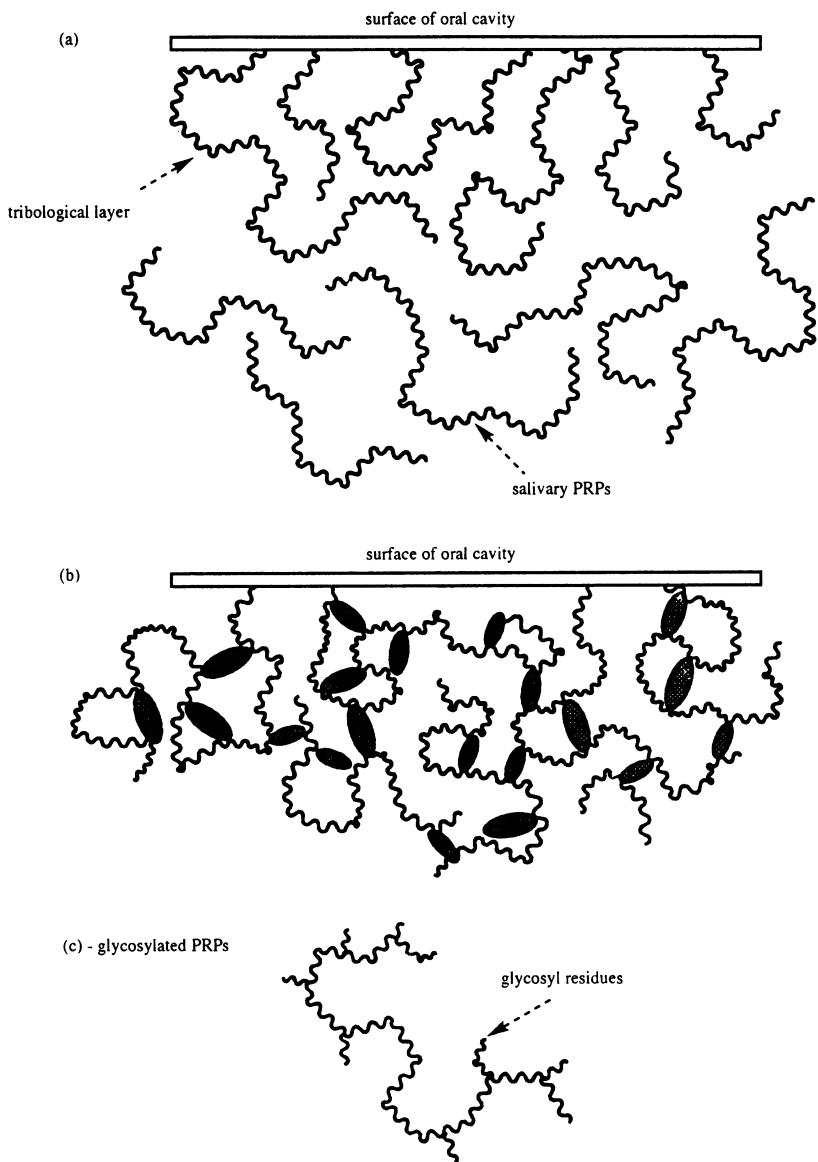


Figure 16. Polyphenol:PRPs interactions; lubrication and astringency
(a) - PRP molecules in solution: random coil conformations giving rise to a tribological layer in the palate
(b) - collapse of the tribological layer and release of water giving rise to astringency
(c) - glycosylated PRPs

The suggestion therefore that the role of these proline-rich proteins is as a "first line of defence" against the detrimental effects of polyphenols in the diet of herbivores is a natural and attractive one. Based on his own and other's work, Bate-Smith⁸⁵ first succinctly stated the presumed role of polyphenols in plant defence : -

"From the biological point of view the importance of tannins in plants lies in their effectiveness as repellents to predators, whether animal or microbial".

In either case the relevant property is **astringency**, rendering the tissues unpalatable by precipitating proteins or by immobilising enzymes, impeding invasion of the host by the parasite (see also recent discussion by McArthur *et al.*⁸⁶). If one accepts this proposition then, equally, it is important to consider to what extent the palate, with its battery of associated PRPs, may indeed be seen as a potential barrier, in humans, which plant materials (natural therapeutic medicines) must first surmount before they may be absorbed into the plasma. In particular what proportion of the ingested polyphenols in any consumed food or beverage passes eventually from the oral cavity into the digestive system in the free form and what proportion in the form of complexes with salivary PRPs? It is also therefore important to seek to discover the thermodynamic stability of these complexes and the extent to which the reactions by which they are formed are, *in vivo*, reversible. In this context it is interesting to note that the *in vitro* behaviour of different PRPs in re-solubilising PRP-polyphenol complexes is not the same⁷⁴. Thus addition of aliquots of solutions of gelatine (' partially unwound ' collagen) to solutions of β -1,2,3,4,6-pent-*O*-galloyl-D-glucose leads, in the first instance, to **precipitation** of the polyphenol as a complex with gelatine. As further gelatine is added there follows a process of **re-solubilisation** and the precipitated complex re-dissolves (Figure 17). This pattern is typical of the interaction of many proteins with polyphenols. However when a proline-rich protein from human saliva [or synthetic peptides such as poly-L-proline or poly (pro-gly-pro)] is used in place of the gelatine the precipitation phase is **not** followed by re-solubilisation and the polyphenol-PRP complex remains precipitated (Figure 17).

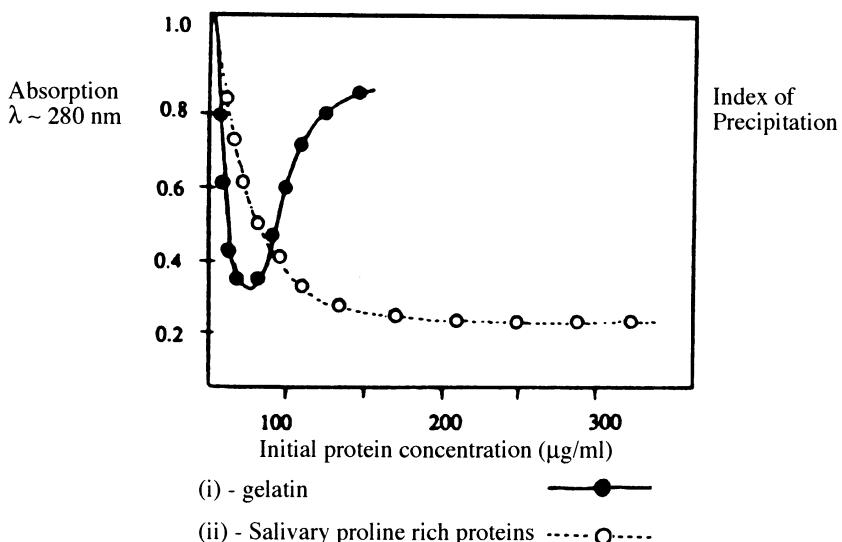


Figure 17. Comparison of the precipitation of β -1,2,3,4,6-penta-*O*-galloyl-D-glucose by gelatine (precipitation and re-solubilisation) and salivary proline-rich proteins (precipitation but no re-solubilisation) in glass distilled water. Plot of absorption at $\lambda \sim 280$ nm (phenol concentration) versus protein concentration

EPILOGUE

Insofar as the possible modes of action of natural polyphenols present in foodstuffs and beverages, and as constituents of herbal medicines, is concerned there is clear circumstantial *in vitro* evidence that they have the potential to act in at least three general areas (*i.e.* transition metal ion complexation, as antioxidants and by complexation with peptides, proteins and polysaccharides). The crucial questions of what happens *in vivo* in the human body however remain, at the very best, only partially answered and understood. Until such time the evidence for their remedial effects remains based largely on epidemiological data as opposed to scientific observation and evaluation.

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Chapter 2

GASTROINTESTINAL EFFECTS OF COMPLEX POLYPHENOLS FROM RED WINE AND TEA IN EXPERIMENTAL ANIMAL MODELS

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INTRODUCTION

DNA damage has recently attracted much attention because of its supposed relationship with processes such as ageing and carcinogenesis¹.

Dietary polyphenols and tannins from tea (TCP) and from wine (WCP) may reduce oxidative cell damage, therefore interfering with basic pathological processes. TCP have been shown in fact to reduce the formation of 8-OH-2'-deoxyguanosine (8OHdG), a marker of oxidative DNA damage, in rodents administered 4-methylnitrosamino-1,3-pyridyl-1-butanone². In mice, the administration of (-)-epigallocatechin gallate, prior to the application of 12-O-tetradecanoyl-phorbol-13-acetate, has been shown to decrease leukocyte infiltration, H₂O₂ formation and oxidation of DNA bases³. The consumption of a 2 % green tea infusion has protective effects

against oxidative damage to liver DNA and against hepatotoxicity in rats treated with 2-nitropropane (2NP)⁴. The formation of 8OHdG in liver after dosing with 2NP is one of the mechanisms underlying its carcinogenicity^{5,6}.

It has been assumed for a long time that red wine phenolics have antioxidant and oxygen radical scavenging properties⁷⁻⁹. The higher molecular weight compounds among these phenolics are usually described as tannins¹⁰. The major phenolics in green tea are monomeric flavanols ((–)-epicatechin, (–)-epigallocatechin and the corresponding gallic esters). In contrast, red wine phenolics consist mainly of polymeric material, including genuine grape tannins (proanthocyanidins) and complex polyphenols formed by reactions during winemaking and ageing. Monomeric flavanols ((+)-catechin, (–)-epicatechin and (–)-epicatechin gallate), flavonol glycosides, anthocyanins, and cinnamoyltartaric acids are also present. Black tea contains flavanol glycosides and novel flavanol-derived thearubigins, theaflavins and theacitrins^{11,12}. Health-related properties of these molecules, such as free-radical scavenging abilities¹³⁻¹⁵ or enzyme inhibition,¹⁶⁻¹⁹ increase with chain length and extent of galloylation, suggesting that complex phenols may be more active than monomeric phenols on some cellular processes.

Although monomeric flavanols and flavonols²⁰⁻²² are absorbed after oral administration, there is as yet no evidence regarding the bioavailability and biological effects in man of unmetabolised condensed tannins and related complex polyphenols from wine (WCP) or tea (TCP). In contrast, there are some data indicating that these substances may become available after metabolism by the gut microflora²³⁻²⁵.

On this basis, we wanted to verify whether WCP or TCP protect against oxidative DNA damage and chemically induced colon carcinogenesis. Colon cancer is one of the main causes of cancer in the western world²⁶ and it is generally agreed that the composition of the diet plays a fundamental role in the induction of this type of cancer²⁷. Among the many putative cancer-preventing components in foods, polyphenols have been selected for priority of attention for two reasons. Firstly, epidemiological studies suggest that populations exposed to polyphenols in beverages, such as tea, have a lower incidence of colon cancer²⁸ and, secondly tea has protective effects against experimental colon carcinogenesis^{28,29}. Dietary factors can in fact vary cell proliferation³⁰, a high cell proliferation being a factor of risk

for carcinogenesis; they can also alter the genotoxic effect of carcinogens on the mucosa.

Accordingly, we investigated the effect of WCP and TCP on F344 rats, studying the following parameters: colon mucosa proliferation, induction of aberrant crypt foci (ACF) and the number of nuclear aberrations (NA) induced by colon carcinogens.

MATERIALS AND METHODS

Animals

Male Fischer 344 rats (180–200 g) were purchased from Nossan (Correzzana, Milan, Italy). Rats were fed a high-fat diet with a composition based on the AIN76 diet, modified to contain a high amount of fat (230 g/kg corn oil w/w), a low level of cellulose (20 g/kg w/w), and a low level of calcium (1.3 g/kg w/w), to mimic the diet typical of western human populations at high risk for colon cancer. Dietary components were purchased from Piccioni (Gessate, Milan, Italy).

Chemicals

DMH, AOM and 2NP were obtained from Sigma, Milan, Italy; 8OHdG was synthesised³¹. IQ was obtained from Toronto Research Chemicals, Toronto, Canada.

Preparations of complex polyphenol and tannin fractions from red wine (WCP)

WCP were prepared in the laboratory of Dr Véronique Cheynier (INRA, Montpellier, France). As a starting material red wine (Cabernet Sauvignon, Arzen, France, harvested in 1994) was used. A ‘wine phenolic powder’ containing proanthocyanidins (condensed tannins) and derived tannins, but free from low mass phenols, was obtained by using the following procedure. Wine was first de-alcoholised under vacuum, filtered to remove tartaric precipitates and deposited onto a Relite SP411 column. After washing with water to remove sugars, organic acids and part of the phenolic

acids, the wine 'phenolic pool' was recovered with 90% ethanol, concentrated under vacuum and atomised. Batches of the phenolic powder thus obtained (380 g) were dissolved in water and chromatographed on a Toyopearl TSK HW-50 (F) column. The low molecular mass phenols were eluted with a mixture of ethanol : water : trifluoroacetic acid (55:45:0.005 v:v:v), and the polymeric fraction eluted with 60% acetone in water. The acetone fractions containing the wine polymeric tannins were pooled, concentrated under vacuum and atomised. The amount of total polymeric material, including non-proanthocyanidin derived tannins, was estimated from the absorbance at 280 nm.

The tannin powder contained trace amounts of flavonol aglycones (6 mg/g or about 15% of the initial flavonol content of wine) but no free anthocyanins, flavanol monomers, cinnamic acids, nor polysaccharides. Although quantitatively significant losses occurred in the last purification step, the proanthocyanidin profile of the tannin powder used in biological experiments, as determined by thiolysis, was similar to that of the wine. Genuine proanthocyanidins represent approximately half of the material present in the tannin powder defined in this paper as WCP, the other half presumably consisting of 'derived tannins'. These derived tannins, characteristic of matured red wine but largely absent from grape juice, have not been fully characterised, although some novel structures have been determined³²⁻⁴¹. In these experiments we used a dosage of WCP (57 mg/kg) which was calculated to be about ten times higher than the dosage of a man weighing about 70 kg and drinking 0.5 litres of red wine per day.

Preparation of complex polyphenol and tannin fractions from black tea (TCP)

The theafulvin (TFu) fraction was prepared according to a modification of the method of Bailey *et al.* (Ref. 42) using a cellulose column. The theafulvin was isolated from a Lattakari Assam black tea (Importers Ltd, Guildford, Surrey, UK) brewed for 10 minutes and decaffeinated with chloroform. Theaflavins and flavonol glycosides were removed by partitioning against ethyl acetate until the organic phase was colourless. The aqueous phase so obtained was treated with an equal volume of methanol, the mixture stirred, and the flocculent precipitate removed by centrifugation.

The brown solution was applied to the cellulose column. The column was washed with 1.0 litre methanol, followed by acetone until the eluate was colourless and the washings discarded. The material adsorbed to the column was eluted with 50 % (v/v) aqueous acetone, the acetone removed on a rotary evaporator under reduced pressure at room temperature and the aqueous solution freeze-dried to produce the TCP powder. This material is thought to have masses up to approximately 2,000 daltons⁴³ and is virtually free from flavanols, flavanol gallates and flavonol glycosides which would be characteristic of green tea.

Experiments on oxidative damage

Level of 8OHdG in DNA

Male F344 rats were treated for 14 d by gavage with WCP; controls received a gavage of water alone. After 14 d one group of rats was given an injection i.p. of 2NP and the controls an injection of vehicle alone. Animals were sacrificed 15 h following 2NP, and their livers were excised and frozen at -80 °C until analysis.

Rats were also treated with DMH, a specific intestinal carcinogen. These animals were administered WCP (57 mg/kg/d) by gavage for 10 d. Controls received water alone. After 10 d, DMH (20 mg/kg) or saline was administered by gavage. Rats were sacrificed 24 h after DMH administration, their colon was excised, rinsed with saline, and frozen at -80 °C until analysis. The colons were then thawed, the mucosa layer removed by scraping and processed for DNA analysis.

The isolation and hydrolysis of DNA in liver or colon mucosa was performed using a published method³¹. The analysis of 8OHdG and 2-deoxyguanosine (2dG) was performed with an LC/9A Shimadzu HPLC system using a UV and an electrochemical (Coulochem) detector in series. For chromatographic separation we used a C18 reverse-phase column (Supelco, 5 mm, i.d. 0.46 × 25 cm); the eluting solution was H₂O : CH₃OH (92:8 v/v) with 50 mM KH₂PO₄ pH 5.5 at a flow rate of 1 ml/min. The detection limit for 8OHdG was 20 pg. The 8OHdG levels were expressed as molar ratio 8OHdG/ 2dG × 10⁻⁶.

Measurement of DNA damage with the comet assay

The single cell gel electrophoresis assay is a method to allow detection of DNA single strand breaks at the single cell level. Acutely isolated cells are embedded in a thin layer of agarose gel on a microscope slide, the cytoplasm is eliminated by a lysis step to obtain naked nuclei, and the slides are submitted to electrophoresis in alkaline buffer. Non-damaged nuclei maintain a round morphology after electrophoresis, whereas the presence of single strand breaks allow the DNA to partly migrate out of the nucleus, giving rise to a comet-like appearance.

A modification of this method that allows the detection of oxidised bases in DNA has recently been described. It involves the use of bacterial DNA repair enzymes such as endonuclease III (ENDO III) and formamidopyrimidine-glycosylase (fpg) to introduce additional nicks at sites of oxidised pyrimidines or purines respectively. This specific oxidative damage is expressed as the difference between DNA damage detected with enzyme digestion and that detected without digestion.

After staining the slides with ethidium bromide, the cells have been analysed on the fluorescence microscope and classified according to the length of their comet into five categories (from 0 to 4 with increasing damage).

Colon mucosa cells were isolated from rat treated with polyphenols or water by oral gavage 10 d before sacrifice.

Experiments on carcinogenesis

Colon mucosa proliferation

Rats were treated s.c. with two injections (1 week apart) of azoxymethane AOM (15 mg/kg) or saline. Control rats were fed the reference diet for 90 d whereas treated rats were given a high fat diet supplemented with WCP (57 mg/kg). At the end of this period, cell proliferation in the colonic mucosa was assessed in mucosal biopsies obtained after sacrifice by measuring ^{3}H -thymidine incorporation *in vitro* by autoradiography, according to a well-described procedure⁴⁴.

Experiments on initiation

Fisher 344 male rats were fed the control diet ($n=15$) and treated rats ($n=16$) were fed the same diet supplemented with WCP (57 mg/kg/d). After 10 d the animals of both groups were treated s.c. with a single dose of AOM (20 mg/kg). The number and dimensions of ACF in both groups were evaluated 30 d after the administration of the carcinogen.

Induction of nuclear aberrations (NA) by colon carcinogens

F344 male rats were treated by gavage with saline or with WCP for 10 d and were given the indicated dose of carcinogen 24 h before sacrifice. All carcinogens were administered by gavage and dissolved in water except 2-amino-3-methylimidazo(4,5-f)quinoline (IQ) which was solubilised with 40% ethanol. NA were scored in coded samples by conventional microscopy.

RESULTS

We first studied the effect of thearubigin (TR) and theafulvin (TFu) on oxidative damage induced by DMH on colon mucosa (Figure 1).

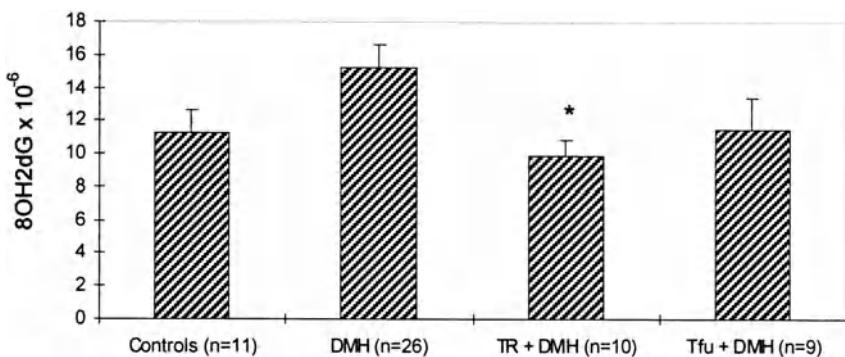


Figure 1. Effect of thearubigin (TR) (administered for 10 d at 40 mg/kg p.o. before DMH (20 mg/kg, p.o.) on oxidative DNA damage. Data are expressed as means of the ratio $8\text{OH}2\text{dG}/2\text{dG} \times 10^{-6} \pm \text{SD}$

It is apparent that TR exerted a significant protection against oxidative damage in the colon mucosa induced by DMH. This action, on the contrary, was not observed with WCP (Figure 2).

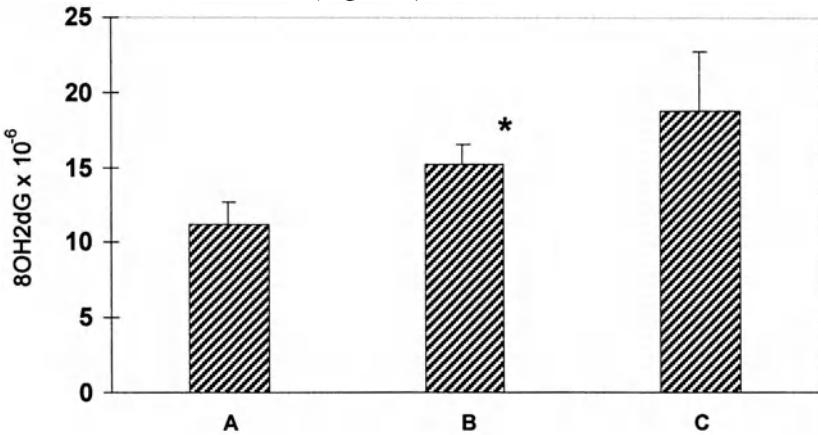


Figure 2. Effect of WCP (57 mg/kg for 14 d) on oxidative DNA damage induced by DMH.
Data are expressed as means of the ratio $8\text{OH}2\text{dG}/2\text{dG} \times 10^{-6} \pm \text{SD}$

WCP administered for 14 d was, however, able to reduce significantly the increased oxidative damage induced in the liver by the administration of 2NP (Figure 3).

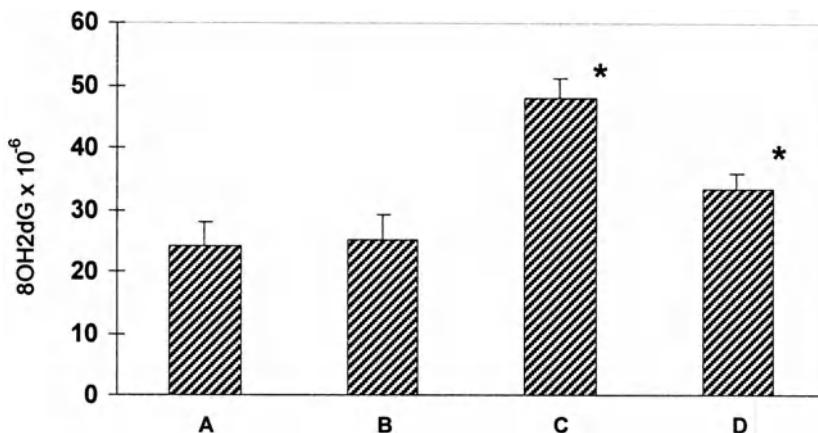


Figure 3. Variation in the oxidative damage in the liver of control rats (A), rats administered WCP for 14 d (B), 2NP (C) and WCP before 2NP.
Data are expressed as means of the ratio $8\text{OH}2\text{dG}/2\text{dG} \times 10^{-6} \pm \text{SD}$.

We also measured oxidative damage in the colon by means of the comet assay after digestion of DNA with endonuclease III, a specific restriction enzyme that cleaves oxidised pyrimidine bases. DNA damage is estimated by comparing the difference in the value obtained before (basal damage) and after treatment with ENDO III. The results are shown in Table 1 and 2.

Table 1. Basal oxidative damage in the colon mucosa of control rats and rats fed with WCP for 10 d (57 mg/kg) as measured with the comet assay after addition of endonuclease III. Data are expressed as mean Arbitrary Units (AU) ± SE

Treatment	ENDO III DNA Damage (AU)
Water (<i>n</i> =9)	53.4 ± 12.2
WCP (57 mg/kg × 10 d) (<i>n</i> =8)	20.1 ± 7.6*

* *p*<0.05

It is apparent that wine polyphenols reduced basal oxidative damage. The same trend was observed with TR, but the results were only borderline significant, due to high variability (*p*=0.08). More experiments are in progress to check this effect.

Table 2. Basal oxidative damage in the colon mucosa of rats fed with TR for 10 d (40 mg/kg/d) as measured with the comet assay after addition of endonuclease III. Data are mean Arbitrary Units (AU) ± SE

Treatment	ENDO III DNA Damage (AU)
Water	105.7 ± 31
TR (40 mg/kg/d)	87 ± 28

We then studied WCP on the colon carcinogenesis process. We started by determining the effect of WCP on colon mucosa proliferation. The results are shown in Table 3. After 90 d feeding, a relatively long period for a rodent, WCP did not modify the labelling index in the colonic crypts, a parameter connected with a variation of colon cancer risk. We only observed a small reduction in the number of cells/crypt after WCP, an effect of unknown biological significance.

Table 3. Proliferative activity in the colon mucosa of AOM- and saline-treated rats treated with WCP. Data are means \pm SE

Groups	No. of labelled cells/crypt	Labelling index (LI)
Controls (<i>n</i> =13)	6.02 \pm 0.73	7.81 \pm 0.97
WPC (57 mg/kg) (<i>n</i> =9)	5.93 \pm 0.55	8.32 \pm 0.89

WCP did not have any effect on apoptosis, which in the colon mucosa is considered a protective factor leading damaged cells towards programmed cell death. As shown in Table 4, this parameter did not seem to be altered after feeding WCP for 90 d.

Table 4. Number of apoptotic cells/crypt in control rats and in rats fed with WCP as indicated. Data are means \pm SE

Groups	Apoptotic cells/crypt
Controls (<i>n</i> =13)	0.254 \pm 0.05
WCP 57 mg/kg (<i>n</i> =14)	0.25 \pm 0.05

We then studied the effects of WCP on carcinogen-induced colon cancer lesions, such as ACF, during the initiation phase. WCP did not influence the number of ACF or ACF dimension (number of aberrant crypts (AC/ACF) or the number of 'large ACF' (supposed to indicate a progression of ACF towards tumour formation) when given 10 d before the carcinogen (Table 5).

Table 5. Number and dimension of ACF in control rats and in animals fed WCP 10 d before the administration of AOM. Data are means \pm SE

Groups	No. of ACF/colon	AC/ACF	Large [#] ACF/colon
Controls	42.94 \pm 3.00	1.67 \pm 0.05	3.84 \pm 1.39
WCP- treated	46.94 \pm 4.96	1.77 \pm 0.09	1.17 \pm 0.27

In these experiments, a large ACF was composed of at least four aberrant crypts.

We finally studied the effect of two compounds that are activated endogenously by mono-oxygenases. DMH, which is transformed into AOM, and 2-amino-3-methylimidazo(4,5-*f*)quinoline, which is further acetylated or conjugated with sulphuric acid into direct acting genotoxic compounds. The possible modulating effect of WCP on the initiation phase of carcinogenesis was studied with the ‘nuclear aberration’ (NA) assay, in which the effect of carcinogens on the colon is measured by scoring the number of acute morphological toxic effects on the colon mucosa cells, 24 h after the administration of a carcinogen. WCP administration did not vary NA frequency produced by treatment with DMH or IQ (Tables 6 and 7).

Table 6. Nuclear aberrations (NA) in colon mucosa of control rats and in animals fed HF diet supplemented with WCP (57 mg/kg) for 10 d before the administration of DMH. NA were scored 24 h after carcinogen administration and are expressed as mean number of NA/crypt ± SE

Groups	NA/crypt
Controls (<i>n</i> =3)	0.26 ± 0.08
Controls + WCP (<i>n</i> =3)	0.34 ± 0.03
Rats treated with DMH (<i>n</i> =14)	3.27 ± 0.43**
Rats treated with DMH + WCP (57 mg/kg) (<i>n</i> =14)	3.91 ± 0.65**

**= *p*<0.01 relative to the respective controls

Table 7. NA in colon mucosa after administration of IQ (250 mg/kg) to control rats and rats fed HF diet supplemented with WCP (57 mg/kg) for 10 d. NA were scored 24 h after carcinogen administration and are expressed as a mean number of nuclear aberrations (NA)/crypt ± SE

Groups	NA/crypt
Controls (<i>n</i> =2)	0.275 ; 0.235
Controls + WCP (<i>n</i> =3)	0.291 ± 0.07
Rats treated with IQ (<i>n</i> =13)	0.996 ± 0.14*
Rats treated with IQ + WCP (57 mg/kg) (<i>n</i> =15)	1.462 ± 0.29*

*=*p*<0.05 relative to controls

DISCUSSION

Dietary antioxidant vitamins are reputed to protect against cancer and ageing by limiting damage to DNA by free radicals, including reactive oxygen species released during cellular respiration. We wanted to verify whether other putative dietary (non-nutrient) antioxidants from tea and wine could protect against induced oxidative damage and gastrointestinal carcinogenesis.

Polyphenols are regular components of the human diet. They occur in vegetables, fruits and in beverages such as tea and wine. Red wine in particular has become a source of interest after the discovery of the so called 'French Paradox' (i.e. the relatively low incidence of cardiovascular diseases in some Mediterranean countries despite the high intake of saturated fat⁴⁵). Phenolic compounds have been suggested to reduce the incidence of myocardial infarction and the risk of death from coronary heart disease⁴⁶. The fact that red wine consumption can prevent the oxidation of low density lipoproteins suggests a possible mechanism for these effects⁴⁷.

It is also established that many flavonoids are scavengers of free radicals, antioxidants, chelating agents, and modifiers of various enzymatic and other biological functions⁴⁸. A recent study reports that green tea protects against liver oxidative DNA damage and hepatotoxicity in rats treated with 2NP⁴. The formation of 8OHdG in the liver induced by 2NP is one of the major mechanisms underlying its carcinogenicity.

However, there is little information on the effects of dietary complex polyphenols. Our results show that pre-treatment with WCP reduced oxidative damage to liver DNA induced in rats by 2NP. In fact, 8OHdG levels after administration of 2NP are significantly decreased by WCP.

We also evaluated the effect of tea and wine polyphenols on DMH-induced colon damage. DMH is a colon-specific methylating carcinogen, but the involvement of superoxide anion in DMH carcinogenesis is also suspected⁴⁹. We report in this paper a protective effect of TR, but not of WCP against the intestinal effect of DMH.

We observed that WCP and, to a lesser extent, TR reduced the basal oxidative damage at the level of the colon mucosa. This is a novel and

interesting observation, which indicates an interesting protective effect of these compounds on the gastrointestinal tract. As a possible mechanism of action for the reduction of basal oxidative damage we suggest an iron chelating effect of polyphenols. Iron, in fact, plays an important role in the formation of hydroxyl radicals through a Fenton reaction.

Contrary to the brilliant effect of WCP and TR on oxidative damage, the action of polyphenols on carcinogenesis was disappointing. In fact feeding F344 rats with complex polyphenols before the administration of AOM did not seem to vary a series of parameters correlated with colon carcinogenesis.

We chose doses of administration higher than ordinary human exposure to WCP (which are of the order of 5 mg/kg for a moderate drinker), since WCP had no toxic effects on the animals. Additionally, such high doses could be considered for human use if some chemopreventive effect were to be found.

Of all the parameters of proliferation analysed (number of cells/crypt, labelling index, distribution of the proliferative activity along the crypt), we observed a decrease only in the number of cells/crypt in rats treated with WCP. With respect to the risk of colon cancer, the significance of variations in this parameter is not clear. The other parameters were not varied compared with controls. The observed differences probably derive from biological fluctuation.

We also controlled for the number of apoptotic cells/crypt in the existing histological specimens. It has been suggested that an increased apoptosis might protect against cancer, eliminating mutated or damaged cells in the mucosa. However, such an effect was not observed in rats fed WCP.

We also carried out assays (induction of ACF or NA) which can predict the influence on the initiation phase of colon carcinogenesis. A potential chemopreventive agent should decrease the number of ACF or of NA. However, we observed none of these effects.

In conclusion, our preliminary data on carcinogenesis did not indicate a preventive action of WCP. However, since anti-tumour effects have been described in the literature^{28,29} using green tea extracts or red wine solids⁵⁰, long-

term carcinogenesis experiments are in process in our laboratory to verify this point.

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Chapter 3

RESVERATROL, A NOVEL INHIBITOR OF CYCLOOXYGENASE-2 GENE EXPRESSION

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INTRODUCTION

Cyclooxygenases (COX)¹ catalyze the synthesis of prostaglandins (PGs) from arachidonic acid. There are two isoforms of COX, designated COX-1 and COX-2. COX-1 is expressed constitutively in most tissues and appears to be responsible for housekeeping functions¹. In contrast, COX-2 is undetectable in most normal tissues but is induced by growth factors, oncogenes, carcinogens and tumor promoters²⁻⁴.

Multiple lines of evidence support the notion that COX-2 is important in carcinogenesis. For example, COX-2 is overexpressed in transformed cells^{2,5,6} and in malignant tissues⁷⁻¹⁰. A null mutation for COX-2 in APC^{Δ716} knockout mice, a murine model of familial adenomatous polyposis, markedly reduces the number and size of intestinal tumors¹¹. Furthermore, treatment with a selective inhibitor of COX-2 caused nearly complete suppression of azoxymethane-induced colon cancer¹². These studies suggest

that targeted inhibition of COX-2 is a promising approach to prevent cancer. Although chemopreventive strategies have focused on inhibitors of COX enzyme activity, an equally important strategy may be to identify compounds that suppress amounts of COX-2¹³⁻¹⁵. Resveratrol, a phytoalexin found in grapes and other foods, has anti-inflammatory and anti-cancer effects¹⁶ (Figure 1).

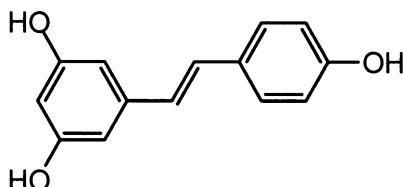


Figure 1. Structure of resveratrol

It inhibits the development of preneoplastic lesions in carcinogen-treated mouse mammary glands, for example; and it blocks tumorigenesis in a two-stage model of skin cancer that was promoted by treatment with phorbol ester (PMA)¹⁶. The anti-inflammatory properties of resveratrol were demonstrated by suppression of carrageenan-induced pedal edema, an effect attributed to suppression of PG synthesis¹⁶. In the current work, we have extended upon prior observations concerning the effects of resveratrol on PG synthesis by determining if resveratrol modulates the expression of the COX-2 gene. Our data show that resveratrol suppresses the activation of COX-2 gene expression by inhibiting the PKC signal transduction pathway. These data provide a mechanistic basis for the chemopreventive and anti-inflammatory properties of resveratrol.

MATERIALS AND METHODS

Materials

MEM medium, PKC assay kits and LipofectAMINE were from Life Technologies, Inc. (Grand Island, NY). Keratinocyte basal medium (KBM) and growth medium (KGM) were from Clonetics Corp. (San Diego, CA). PMA, sodium arachidonate, *trans*-resveratrol, epidermal growth factor, hydrocortisone and o-nitrophenyl-β-D-galactopyranoside were from Sigma Chemical Co. (St. Louis, MO). Enzyme immunoassay reagents for PGE₂

assays were from Cayman Co. (Ann Arbor, MI). [³²P]-CTP was from DuPont-NEN (Boston, MA). Random-priming kits were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Nitrocellulose membranes were from Schleicher & Schuell (Keene, NH). Reagents for the luciferase assay were from Analytical Luminescence (San Diego, CA). The 18S rRNA cDNA was from Ambion, Inc. (Austin, TX). Rabbit polyclonal anti-human COX-2 antiserum was from Oxford Biomedical Research, Inc. (Oxford, MI). Goat polyclonal anti-human COX-1 antiserum was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Western blotting detection reagents (ECL) were from Amersham (Arlington Heights, Ill.). Plasmid DNA was prepared using a kit from Promega Corp. (Madison, WI).

Tissue culture

The 184B5/HER cell line has been described previously¹⁷. Cells were maintained in MEM-KBM mixed in a ratio of 1:1 (basal medium) containing EGF (10 ng/mL), hydrocortisone (0.5 µg/mL), transferrin (10 µg/mL), gentamicin (5 µg/mL), insulin (10 µg/mL) (growth medium). Cells were grown to 60 % confluence, trypsinized with 0.05 % trypsin-2 mM EDTA, and plated for experimental use. MSK Leuk1 was established from a dysplastic leukoplakia lesion adjacent to a squamous cell carcinoma of the tongue in a 46 year old non-smoking female¹⁸. Cells were routinely maintained in KGM and passaged using 0.125 % trypsin-2 mM EDTA. In all experiments, 184B5/HER and MSK Leuk1 cells were grown in basal medium for 24 h prior to treatment. Treatment with vehicle (0.2 % DMSO), resveratrol or PMA was always carried out in basal medium.

PGE₂ Production

5 X 10⁴ cells/well were plated in 6-well dishes and grown to 60 % confluence in growth medium. The cells were then treated as described below. Levels of PGE₂ released by the cells were measured by enzyme immunoassay. Amounts of PGE₂ produced were normalized to protein concentrations.

Western blotting

Analysis was done with a rabbit polyclonal anti-COX-2 antiserum or a polyclonal anti-COX-1 antiserum as described in detail in Ref. 15.

Northern Blotting

Analysis was done with a radiolabeled human COX-2 cDNA as described in Ref. 15.

Nuclear Run-off Assay

2.5 X 10⁵ cells were plated in four T150 dishes for each condition. Cells were grown in growth medium until approximately 60% confluent. Nuclei were isolated and stored in liquid nitrogen. The transcription assay was performed as described previously¹⁵.

Plasmids

The COX-2 promoter construct (-327/+59) was a gift of Dr. Tadashi Tanabe (National Cardiovascular Center Research Institute, Osaka, Japan)¹⁹. The human COX-2 cDNA was generously provided by Dr. Stephen M. Prescott (University of Utah, Salt Lake City, UT). RSV-c-jun was a gift from Dr. Tom Curran (Roche Laboratories, Nutley, NJ). The AP-1 reporter plasmid (2xTRE-luciferase), composed of two copies of the consensus TRE ligated to luciferase, was kindly provided by Dr. Joan Heller Brown (University of California, La Jolla, CA). pSV-βgal was obtained from Promega Corp. (Madison, WI).

Transient Transfection Assays

184B5/HER cells were seeded at a density of 5x10⁴ cells/well in 6-well dishes and grown to 50-60 % confluence. Transfections and analyses were carried out as described previously¹⁵.

Protein Kinase C Assay

The activity of PKC was measured according to directions from Life Technologies, Inc. Briefly, cells were plated in 10 cm dishes at 10⁶ cells/dish and grown to 60 % confluence. Cells were then treated with fresh basal medium containing vehicle (0.2 % DMSO), PMA (50 ng/mL) or PMA (50 ng/mL) plus resveratrol (15 μM) for 30 min. Total PKC activity was measured in cell lysates. To determine cytosolic and membrane bound PKC activity, cell lysates were centrifuged at 100,000 x g for 30 min. The resulting supernatant contains cytosolic PKC; membrane bound PKC

activity is present in the pellet. Subsequently, DEAE cellulose columns were used to partially purify PKC enzymes. Protein kinase C activity was then measured by incubating partially purified PKC with [$\gamma^{32}\text{P}$]ATP (3000-6000 Ci/mmol) and the substrate myelin basic protein for 20 min at room temperature. The activity of PKC is expressed as CPM incorporated/ μg protein.

Statistics

Comparisons between groups were made by the Student's t test. A difference between groups of $P < 0.05$ was considered significant.

RESULTS

Resveratrol inhibits the induction of COX-2 by phorbol esters

We investigated whether resveratrol inhibited PMA-mediated induction of PG synthesis by suppressing the induction of COX-2. Cells were co-treated for 4.5 h with PMA and the indicated concentrations of resveratrol. The medium then was replaced, and the synthesis of PGs was measured in the absence of resveratrol over the next 30 min. PMA in this setting caused about a 2-fold increase in synthesis of PGE₂. This effect was suppressed by resveratrol in a dose-dependent manner (Figure 2).

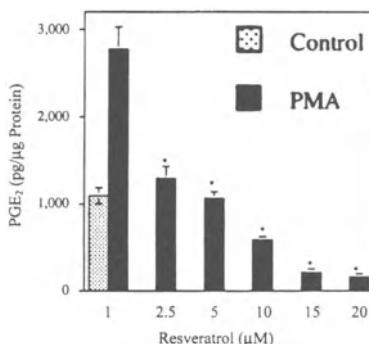


Figure 2. Resveratrol inhibits phorbol ester-mediated induction of PGE₂ synthesis. 184B5/HER cells were treated with vehicle (stippled column), PMA (50 ng/mL, black column) or PMA (50 ng/mL) and resveratrol for 4.5 h. The medium was then replaced with

basal medium and 10 μ M sodium arachidonate. Thirty min later, the medium was collected to measure the amount of production of PGE2. Synthesis of PGE2 was determined by enzyme immunoassay. Columns, means; bars, SD; n=6. *, P<0.001 compared with PMA.

To confirm that these effects of resveratrol were not unique to mammary epithelial cells, we also determined whether resveratrol inhibited PMA-mediated induction of PG synthesis in a premalignant, oral leukoplakia cell line. Treatment of these cells with PMA led to a 2-fold increase in PG synthesis. This effect was inhibited completely by 20 μ M resveratrol (data not shown).

Immunoblotting was performed to determine whether the above effects on production of PGE2 could be related to differences in levels of COX. Fig. 3A shows that PMA induced COX-2 in human mammary epithelial cells. Co-treatment with resveratrol caused a dose-dependent decrease in PMA-mediated induction of COX-2; the maximal drug effect was observed at 15-20 μ M. Neither PMA nor resveratrol altered amounts of COX-1 (data not shown). The ability of resveratrol to suppress the induction of COX-2 was not limited to mammary cells but was also demonstrable in oral epithelial cells (Figure 3B).

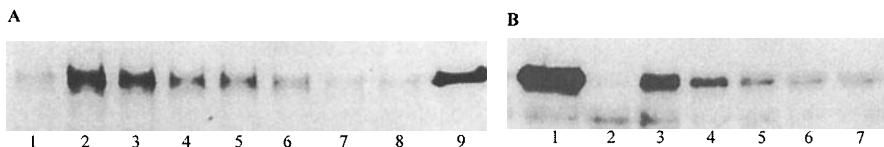


Figure 3. Resveratrol inhibits PMA-mediated induction of COX-2 in human mammary and oral epithelial cells

A - Lysate protein was from 184B5/HER cells treated with vehicle (lane 1), PMA (50 ng/mL, lane 2) or PMA (50 ng/mL) and resveratrol (2.5, 5, 7.5, 10, 15, 30 μ M; lanes 3-8) for 4.5 h. Lane 9 represents an ovine COX-2 standard.

B - Lysates were from premalignant oral epithelial (MSK Leuk1) cells treated with vehicle (lane 2), PMA (50 ng/mL, lane 3) or PMA (50 ng/mL) and resveratrol (10, 20, 30, 40 μ M; lanes 4-7) for 4.5 h. Lane 1 represents an ovine COX-2 standard. Cellular lysate protein (25 μ g/lane) was loaded onto a 10% SDS-polyacrylamide gel, electrophoresed and subsequently transferred onto nitrocellulose. Western blots were probed with antibody specific for COX-2.

To further elucidate the mechanism responsible for the changes in amounts of COX-2 protein, we determined steady-state levels of COX-2 mRNA by Northern blotting. Treatment with PMA resulted in a marked increase in levels of COX-2 mRNA, an effect that was suppressed by resveratrol in a concentration dependent manner (Figure 4). Differences in

levels of mRNA could reflect altered rates of transcription or changes in mRNA stability. Nuclear run-offs were performed to distinguish between these possibilities. As shown in Figure 5, we observed higher rates of synthesis of nascent COX-2 mRNA after treatment with PMA, consistent with the differences observed by Northern blotting. This effect was suppressed by resveratrol.

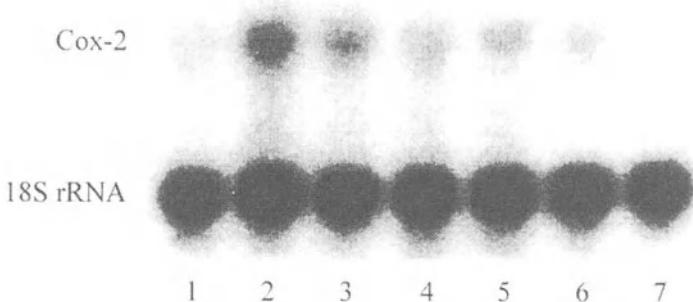


Figure 4. Resveratrol inhibits PMA-mediated induction of COX-2 mRNA.

184B5/HER cells were treated with vehicle (lane 1), PMA (50 ng/mL, lane 2) or PMA (50 ng/mL) and resveratrol (2.5, 5, 10, 15, 20 μ M; lanes 3-7) for 3 h. Total cellular RNA was isolated; 10 μ g of RNA was added to each lane. The Northern blot was hybridized with probes that recognized mRNAs for COX-2 and 18S rRNA. Results of densitometry in arbitrary units: lane 1, 18; lane 2, 225; lane 3, 135; lane 4, 72; lane 5, 45; lane 6, 42; lane 7, 9.

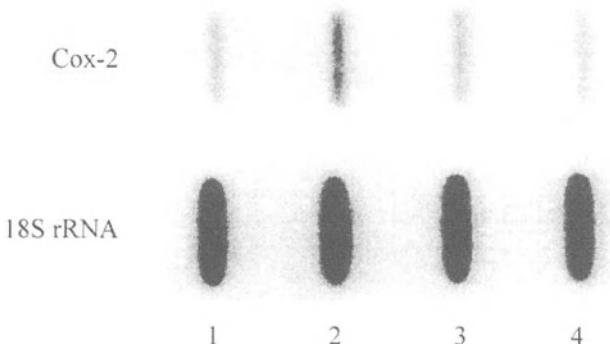


Figure 5. Phorbol ester-mediated induction of COX-2 transcription is inhibited by resveratrol. 184B5/HER cells were treated with vehicle (lane 1), PMA (50 ng/mL, lane 2) or PMA (50 ng/mL) and resveratrol (5 μ M, lane 3; 10 μ M, lane 4) for 3 h. Nuclear run-offs were performed. The COX-2 and 18S rRNA cDNAs were immobilized onto nitrocellulose membranes and hybridized with labeled nascent RNA transcripts. Results of densitometry in arbitrary units: lane 1, 19; lane 2, 44; lane 3, 29; lane 4, 16.

Determining the mechanism by which resveratrol inhibits PMA-mediated induction of COX-2

Tumor promoters like PMA activate PKC and induce AP-1 activity. It was important, therefore, to determine whether resveratrol inhibited PMA-mediated activation of PKC or AP-1. Treatment of cells with PMA stimulated the translocation of PKC activity from cytosol to membrane, an effect that was blocked by resveratrol (Fig. 6).

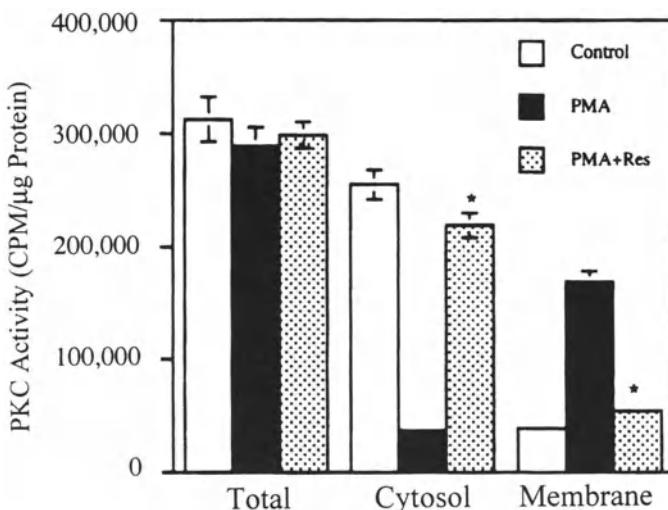


Figure 6. Resveratrol inhibits the redistribution of PKC activity mediated by phorbol ester. 184B5/HER cells were treated with vehicle (open column), PMA (50 ng/mL, black column) or PMA (50 ng/mL) and resveratrol (15 μ M) (stippled column) for 30 min. Total, cytosolic and membrane PKC activities were determined. Columns, means; bars, SD. n=6, *, P<0.01 vs. PMA

Additionally, transiently overexpressing c-Jun, a component of the AP-1 transcription factor complex, caused about a 4-fold increase in COX-2 promoter activity. This effect was blocked by resveratrol (Figure 7A). Resveratrol also suppressed the activation of an AP-1 reporter plasmid by PMA (Figure 7B).

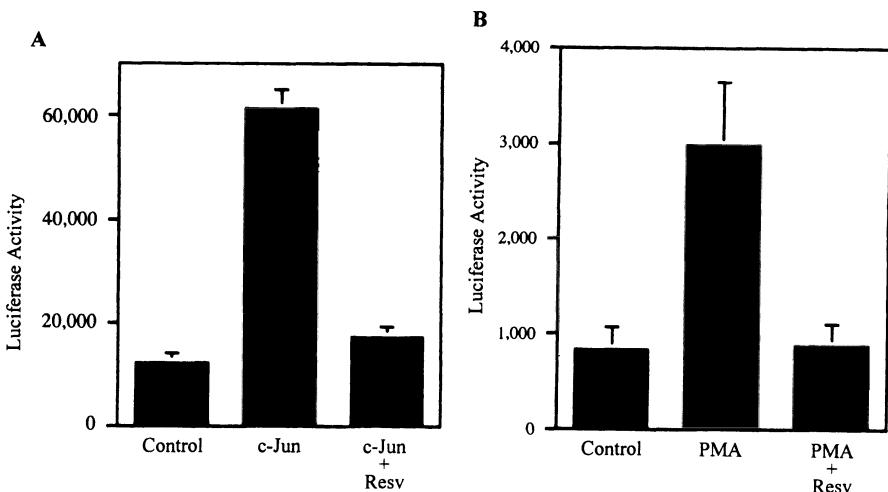


Figure 7. Resveratrol inhibits AP-1-mediated induction of COX-2 promoter activity.

A - 184B5/HER cells were transfected with 0.9 µg of a human COX-2 promoter construct ligated to luciferase (-327/+59) (control) or 0.9 µg of COX-2 promoter construct and 0.9 µg of expression vector for c-jun. All cells received 0.2 µg of pSV β gal. The total amount of DNA in each reaction was kept constant at 2 µg by using empty vector. Twenty four h later, cells were treated with vehicle or resveratrol (15 µM) for 6 h.

B - 184B5/HER cells were co-transfected with 1.8 µg of 2xTRE-luciferase and 0.2 µg of pSV β gal. Twenty four h after transfection, cells were treated with vehicle, PMA (50 ng/ mL) or PMA (50 ng/ mL) and resveratrol (15 µM) for 6 h. Luciferase activity represents data that have been normalized with β -galactosidase activity. Six wells were used for each of the conditions. Columns, means; bars, SD.

DISCUSSION

There is considerable evidence that inhibitors of COX-2 are useful for treating inflammation and preventing cancer^{11,12,20}. Drugs that interfere with the signaling mechanisms that up-regulate COX-2 should also be useful in this regard because they too decrease total COX-2 activity^{13,14}. We have shown in the present experiments that resveratrol suppressed PMA-mediated induction of PG synthesis at least, in part, by inhibiting COX-2 gene expression¹⁵.

Tumor-promoting phorbol esters induce COX-2 gene expression by activating the PKC pathway²¹. A downstream target of activated PKC is the AP-1 transcription factor complex. Resveratrol suppressed PMA-mediated activation of COX-2 transcription by inhibiting the PKC signal transduction pathway at multiple levels. It blocked both PMA-induced translocation of PKC activity from cytosol to membrane (Figure 6), and the increase in COX-2 promoter activity mediated by c-Jun (Figure 7). These inhibitory effects can be explained, in part, by the antioxidant properties of resveratrol since other phenolic antioxidants inhibit both PMA-mediated activation of PKC and AP-1^{22,23}. These results are significant because PKC activity is up-regulated in some cancers and is considered a potential target for anti-cancer therapy²⁴. Additionally, since AP-1 has been implicated in promoting carcinogenesis, these effects are likely to contribute to the anti-tumor activity of resveratrol.

Xie and Herschman showed that the AP-1 transcription factor complex is important for the activation of the murine COX-2 promoter via a cyclic AMP response element (CRE)²⁵. Thus, it is possible that resveratrol blocks PMA-mediated induction of COX-2 by suppressing AP-1-dependent transactivation via the CRE. The anti-AP-1 effect of resveratrol can potentially be explained if resveratrol induced Fra expression like other phenolic antioxidants²⁶. Heterodimers of c-Jun and Fra do not activate AP-1-mediated gene expression as effectively as c-Jun homodimers or c-Jun/c-Fos heterodimers²⁷. Alternatively, resveratrol could suppress PMA-mediated increases in AP-1 activity by inhibiting the induction or phosphorylation of c-Jun.²⁸

We reported previously that retinoids blocked PMA-mediated induction of COX-2 in oral epithelial cells¹³. The same effect of retinoids was observed in the human mammary epithelial cells used in this study. However, whereas resveratrol and retinoids both block PMA-mediated induction of COX-2 transcription, they appear to do so via different mechanisms. Thus, in contrast to resveratrol, retinoids did not block the PMA-induced redistribution of PKC activity from cytosol to membrane (data not shown). Additionally, resveratrol and retinoids antagonize AP-1 activity via different mechanisms. Retinoids antagonize AP-1 activity via a receptor-dependent mechanism²⁹, whereas our data suggest that resveratrol blocks PMA-mediated stimulation of AP-1-activity by inhibiting the PKC signaling cascade. This distinction between resveratrol and retinoids is important for the design of chemopreventive strategies utilizing combinations of drugs that

act *via* different mechanisms.

Finally, based on the finding that resveratrol inhibited COX-2, further studies are warranted to determine how effective this compound or its analogues will be in preventing or treating inflammation and cancer.

FOOTNOTES

This work was supported in part by National Institutes of Health Grant CA68136 (A.J.D.). Data in this manuscript were previously reported in Ref. 15.

The abbreviations used are: COX, cyclooxygenase; CRE, cyclic AMP response element; PGE₂, prostaglandin E₂; PKC, protein kinase C; AP-1, activator protein-1; PMA, phorbol 12-myristate 13-acetate.

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Chapter 4

TRANS-RESVERATROL AND THE LIVER: DEACTIVATION OF LIVER MYOFIBROBLASTS AND INHIBITION OF TUMOR CELL INVASION

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INTRODUCTION

In several situations, mesenchymal fibroblasts acquire a myofibroblastic phenotype and express characteristic cytoskeletal proteins, such as smooth muscle α -actin (α -SMA). Myofibroblasts are present for a limited time in normal wound healing, but persist when tissue repair is abnormal, *i.e.*, during tissue fibrosis and in the stromal reaction to tumors¹. Myofibroblasts are believed to be essential actors of tissue fibrosis. They are involved in tissue contraction, synthesis of extracellular matrix and synthesise cytokines. In the context of cancer, there are less data, but strong evidence point to a role of infiltrating myofibroblasts in invasion².

There are almost no myofibroblasts in the normal liver. In chronic fibrotic liver disease, or in liver primary or secondary cancers, myofibroblasts are numerous³⁻⁶. The origin of liver myofibroblasts is still debated. In fibrotic liver diseases, most authors believe that myofibroblasts derive from the so-called activation of hepatic stellate cells (HSC)⁷. Hepatic

stellate cells (also called Ito cells, perisinusoidal cells, lipocytes, fat-storing cells) are normal liver cells located in the Disse space, between sinusoidal endothelial cells and hepatocytes. In the normal liver, they are involved in storage and metabolism of vitamin A, vasomotricity, and synthesis of the normal extracellular matrix. Many studies, using isolated hepatic stellate cells from rat or human, have shown that these cells, when grown on plastic dishes, undergo a « spontaneous » activation towards a myofibroblastic phenotype (Friedman, 1993). Activation features include morphologic changes, loss of vitamin A, cell proliferation, huge increase in extracellular matrix synthesis, *de novo* expression of α -SMA and β -PDGF receptors, and increased expression of matrix metalloproteinase-2 (MMP-2, or gelatinase A). This *in vitro* model of HSC activation is commonly used as a model that mimics the activation observed in fibrotic liver disease. In addition, several studies strongly suggest that HSC differentiation to myofibroblasts can take place *in vivo*. However, in some cases such as biliary fibrosis, portal fibroblasts can also probably differentiate into myofibroblasts⁸. The origin of myofibroblasts within liver cancer is not clearly known, as these cells could potentially originate from the differentiation of either HSC, or portal fibroblasts. The mainly sinusoidal localization of myofibroblasts in cancer favors however a HSC origin. Using the murine melanoma B16 model, we have shown that injection of tumor cells in the portal vein rapidly led to the formation of liver micrometastases where HSC acquire a-SMA expression⁹. *In vitro*, conditioned medium from B16 cells induced readily the differentiation of rat HSC to myofibroblasts, thus showing that myofibroblasts differentiation from HSC might be under control of tumor cells. We have recently obtained similar results with HCC cells. Thus, conditioned medium from rat HCC cells induced the *in vitro* activation of rat HSC¹⁰. Whether this occurs *in vivo* remains a matter of debate.

Myofibroblasts and extracellular matrix synthesis

We have shown that cultured human myofibroblasts can be used as a model for the ECM-secreting cells in the liver. Our published data indicate that they synthesize collagens type I, III, IV, and V, fibronectin, and laminin¹¹. We have also evidence that they synthesize type VI collagen, undulin/type XIV collagen, thrombospondin, and tenascin. We have recently shown that liver myofibroblasts were also responsible for the synthesis of osteonectin/SPARC, a protein with anti-adhesive properties, involved in the supramolecular organization of the ECM¹². Synthesis of collagens type I, III,

IV, V, fibronectin, laminin and osteonectin, is enhanced in the presence of TGF β 1, a major profibrogenic mediator in the liver^{13,14}.

Liver myofibroblasts are also involved in extracellular matrix breakdown, through the release of matrix metalloproteinase-2 (MMP-2, or gelatinase A), that degrades type IV collagen¹¹.

Myofibroblasts and liver cancer

Myofibroblasts are components of the stroma of most cancers where they can be identified easily through staining with an anti- α -SMA antibody. Several authors have suggested that myofibroblasts can contribute to tumor progression, but the mechanisms involved are poorly understood. Hepatocellular carcinoma (HCC) is the most frequent primary liver cancer. It is characterised by a high rate of local invasion. We have recently shown that exposition of cultured human HCC cell lines to myofibroblasts conditioned medium profoundly affected the tumor cell phenotype¹⁵. Myofibroblasts conditioned medium decreased the proliferation of the HepG2 cell line, while it increased that of the HuH7 cell line. HepG2 cells scattered readily in presence of myofibroblasts conditioned medium, while HuH7 were unaffected. However, invasion of both cell types through Matrigel-coated filters was enhanced up to 100-fold by co-culture with myofibroblasts. We demonstrated that all the above effects were mediated by hepatocyte growth factor (HGF) secreted by myofibroblasts, since they could be blocked by an anti-HGF antibody. Moreover, HGF synthesis by myofibroblasts was clearly shown by Northern blot, Western blot, ELISA and immunoprecipitation, and the tumor cell lines expressed c-met, the specific receptor for HGF. Using RT-PCR, we have also shown that HGF was expressed in human HCC, suggesting that it could play a role in these tumors. Recently, we have begun to unravel the molecular mechanisms of HGF-induced invasion. We observed that exposition of HepG2 cells to HGF led to a very large increase in the expression of urokinase and its receptor¹⁶. Urokinase is a serine-proteinase that can convert plasminogen to plasmin. Plasmin itself is a broad range proteinase able to degrade several extracellular matrix components and thus to promote invasion. In our study, we were able to demonstrate that the induction of urokinase expression was responsible for HGF-induced invasion, since invasion could be blocked by using the specific urokinase inhibitor B428¹⁷.

Trans-resveratrol is a grapevine-derived polyphenol that has undergone a lot of attention recently for several reasons. It is a good anti-oxidant¹⁸. It has been shown to inhibit several steps of the carcinogenesis process¹⁹ and can induce apoptosis of tumor cells²⁰. In this paper, we will describe the effects of *trans*-resveratrol, and related polyphenols on the phenotype of liver myofibroblasts, and on liver cancer cell invasion.

MATERIALS AND METHODS

Culture of human liver myofibroblasts

Myofibroblasts were obtained by outgrowth from explants of non-tumoral liver resected during partial hepatectomy as previously described¹³. The procedure is in accordance with INSERM ethical regulations imposed by French legislation. Isolated cells were characterised as myofibroblasts as previously described in detail. Specifically, the procedure which is based on the selective growth advantage of myofibroblasts in the cultures conditions used, allowed for 100 % pure myofibroblast population, as shown by positive staining for smooth muscle alpha-actin and vimentin and negative staining for von Willebrand factor (an endothelial cell marker), or cytokeratin (an epithelial cell marker).

The HepG2 human HCC cell line was cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal calf serum.

Stilbenes

Trans-resveratrol and *trans*-piceatannol were from Sigma Aldrich. Stilbenes were made as a 35 mM stock solution in ethanol/water (v/v), then diluted into DMEM. In our experiments, the final concentration of ethanol did not exceed 2 mM. *Trans*-piceid was isolated from *Vitis vinifera* cell cultures. The compound was purified from the methanol extract of freeze-dried cells by a combination of chromatographic techniques and characterised with nuclear magnetic resonance as described^{21,22}.

Measurement of cell proliferation

Myofibroblasts were seeded at a density of 20000/well in 24-well plates in DMEM containing 5 % FCS and 5 % HS. On the following day, the

cells were washed 2 times with DMEM and the medium was replaced by DMEM supplemented with 5 % FCS containing *trans*-resveratrol or its solvent. After 4 days, the cells were incubated with DMEM containing 1 mg/mL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) during 2 hours at 37 °C. Elution of the precipitate was performed with DMSO. The rate of proliferation was calculated from absorption values obtained at 540 nm using an automated enzyme-linked immunosorbent assay reader. In the second part of the experiment, the reversibility of the effects of *trans*-resveratrol was studied. Polyphenol was withdrawn on day 4, and the medium was replaced by DMEM supplemented with 5 % FCS.

Net proliferation was expressed as follows. Net proliferation = (B-A)/(C-A), where A represents optical density on day 0 and B and C are respectively the optical densities observed in the presence of *trans*-resveratrol or its solvent.

A similar test was performed with HepG2 cells that were seeded into 96-well dishes at 45×10^3 cells per well, in 2% FCS DMEM with or without *trans*-resveratrol. Cells were incubated for 24 or 48 hr at 37°C before the MTT assay.

Cytoskeletal proteins analysis

1 - Western blot. Myofibroblasts were seeded at a density of 100000/well in 6-well plates. After 4 days of culture with or without *trans*-resveratrol, myofibroblasts were lysed with lysis buffer. Protein concentration was determined with the Bio-Rad protein assay kit. After electrophoresis on a denaturing gel (10 %), 2.5, 5 or 10 µg proteins were transferred to a polyvinylidene difluoride membrane. After blocking non-specific sites, the membrane was incubated with monoclonal antibodies against either smooth muscle α-actin, vimentin, or β -actin, diluted respectively 1/1000, 1/5000 or 1/5250 for 1 hour at room temperature. After washing, the membrane was incubated with a peroxydase-conjugated rabbit anti-mouse antibody for 1 hour. Immunoreactive bands were visualised on Hyperfilm by using ECL reagent. Quantification of protein expression was done following image acquisition on a Macintosh computer, using the NIH Image 1.60 software.

2 - Immuno-fluorescent staining of smooth muscle α-actin. After 4 days of culture on glass coverslips in the conditions described above,

myofibroblasts were washed with Gey's balanced salt solution (GBSS) containing Ca^{2+} and Mg^{2+} , pH 7.4 and fixed with 3.7 % formaldehyde in 0.1 M sodium cacodylate pH 7.4 for 15 min at room temperature. Fixed cells were washed three times with PBS and permeabilized with acetone at -20 °C for 20 min. Cells were then incubated for 1 hour with a FITC-conjugated mouse monoclonal antibody against smooth muscle α -actin diluted 1/400. For amplification of the signal, a secondary antibody conjugated with Oregon Green (1/100) was used. Coverslips were mounted in anti-fade reagent and the cells were observed with a fluorescence microscope.

Northern blot

Myofibroblasts were seeded at a density of 250 000 cells/6.0 cm dish and cultured to confluence, then incubated for 24 hours in serum-free DMEM and treated with or without *trans*-resveratrol in the same medium during 24 hours. Total cellular RNA was isolated using the RNeasy mini kit. Ten μg of total denatured cellular RNA were electrophoresed in a 0.8 % agarose/formaldehyde gel, and transferred to a HybondTM-N+ membrane, using a Vacuum Blotter. Prehybridization and hybridization were carried out at 65°C in $\text{Na}_2\text{HPO}_4/\text{H}_3\text{PO}_4$ 0.5M, pH 7.2, SDS 7 %, EDTA 1 mM, BSA 1g/100 mL, ssDNA 100 $\mu\text{g}/\text{mL}$. The membrane was hybridised with a cDNA probe for human $\alpha 1$ chain of type 1 procollagen labeled with [$\alpha^{32}\text{P}$] dCTP by random priming. The blots were washed in stringent conditions and exposed for autoradiography.

To control for variations in loading and transfer, blots were dehybridised and rehybridised with an oligonucleotide probe to 28 S rRNA (5'-CGC GTC ACT AAT TAG ATG ACG AGC CAT TTG-3'). The probe was labeled with [$\gamma^{32}\text{P}$] ATP using T4 polynucleotide kinase. The autoradiographic signals were quantified as described above.

Cell invasion Assay

A Matrigel invasion assay was performed essentially as described^{15,16}. Briefly, tests were performed with invasion chambers consisting of an 8-mm pore size filter coated with a uniform layer of Matrigel basement membrane matrix (50 $\mu\text{g}/\text{cm}^2$). In the lower compartment of the system, 5×10^4 myofibroblasts were cultured and 4.5×10^4 HepG2 cells were seeded onto the filter. In some experiments, recombinant hepatocyte growth factor (HGF)

(80 ng/mL) was used instead of myofibroblasts in the lower compartment. After a 6-hours incubation, the cells on the upper surface of the filter were wiped with a cotton swab. Filters were fixed for 10 minutes with methanol and stained with hematoxylin and eosin. Cells that invaded the lower surface of the filter were counted under a photonic microscope at a final magnification of 320.

Study of c-met expression by Western-Blot

For c-met detection, cultured cells were directly lysed in loading buffer and 15 micrograms of proteins were resolved by SDS/PAGE on a 7.5% gel then *transferred* to a polyvinylidene difluoride membrane. The membrane was incubated sequentially with an anti-human c-met antibody diluted 1/1000 and a peroxydase-conjugated anti-rabbit IgG antibody. Detection was achieved by enhanced chemiluminescence.

Secreted urokinase assays and zymography

HepG2 cells were seeded in 24-well dishes at 2×10^5 cells per well in DMEM 2 % FCS. After 24 hr, the cells were rinsed once with Gey's balanced salt solution (GBSS) $\text{Ca}^{2+}/\text{Mg}^{2-}$, once with an acid buffer (50 mM glycine, 100 mM NaCl, pH 3) to remove cell surface-bound urokinase, and twice with serum-free DMEM. Finally, cells were incubated in serum-free DMEM for 24 hr. Experiments were made in triplicate. Conditioned media (CM) were first centrifuged at 600 X g for 4 min at 4°C to remove cellular debris, then at 15,000 X g for 5 min at 4°C and stored at -20°C. 6 μL of the supernatants were electrophoresed in a 10% SDS-polyacrylamide gel containing 1 mg/mL gelatine and 12.5 $\mu\text{g}/\text{mL}$ plasmin-free plasminogen. After washing with 2.5% Triton X-100, the gel was incubated at 37°C for 48 hr in 100 mM glycine, 20 mM EDTA, pH 8.3. The gel was finally stained with 0.5% Coomassie blue. Proteolytic activity was detected as a white zone in a dark field. As a control, the same experiment was done in a gel without plasminogen. Purified human urokinase was used as a positive control.

To explore the effect of HGF and *trans*-resveratrol on urokinase synthesis by HepG2 cells, HepG2 cells were seeded as above and incubated in 1 mL serum-free DMEM with or without rhHGF (80 ng/mL) and with or

without *trans*-resveratrol (25 µM). After 24 hr, CM were collected and cells were rinsed twice with 100 µl of serum-free DMEM. Endogenous cell surface-associated urokinase was eluted at room temperature for 3 min with 100 µL of the acid buffer described above. The eluates were neutralised with 25 µL of 0.5 M Tris-HCl, pH 7.8 and centrifuged. The cell layer was finally processed for DNA measurement. Following normalisation for DNA content of the cell layer, urokinase activity in supernatants and eluates was assayed. Urokinase activity on the zymography was quantified with the NIH Image 1.60 software.

RESULTS

***Trans*-resveratrol induces morphological modifications of myofibroblasts**

Treatment of human liver myofibroblasts with *trans*-resveratrol induced typical morphological modifications. Myofibroblasts became slender and bipolar in a dose-dependent manner (Figure 1). These modifications appeared as early as 24 hours following *trans*-resveratrol addition. They persisted as long as resveratrol was included in the culture medium and reverted when it was removed.

On the other hand, *trans*-piceid and *trans*-piceatannol did not modify the morphology of myofibroblasts.

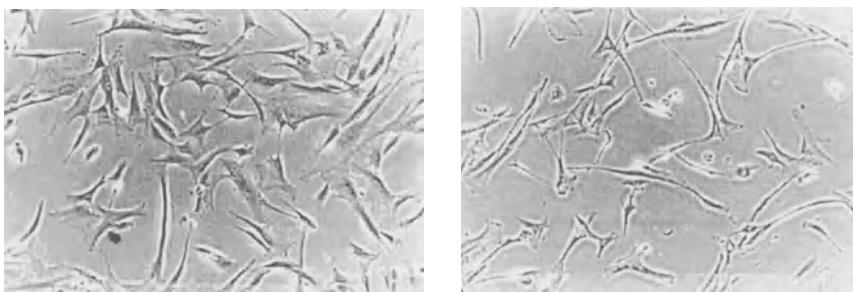


Figure 1. Effect of *Trans*-resveratrol on the Morphology of Cultured Myofibroblasts.

Myofibroblasts were grown either in control medium (left)
or with 100 µM *trans*-resveratrol (right)

Trans-resveratrol has an antiproliferative effect

Treatment of human liver myofibroblasts with increasing concentrations of *trans*-resveratrol inhibited their proliferation in a dose-dependent manner ($ED_{50} = 35 \mu M$). A maximal inhibition was obtained at $100 \mu M$ (Figure 2). A cytotoxicity was excluded on several grounds. First, we did not observe any morphological signs like cells rounding or floating in the medium. In addition, when cultures were exposed to *trans*-resveratrol for 4 days, then switched back to control medium, the cells resumed a normal growth. Finally, trypan blue staining yielded similar results in control and treated cells (data not shown).

On the other hand, piceid, a glycosylated analogue of *trans*-resveratrol, had no effect on myofibroblast proliferation. *Trans*-piceatannol, a hydroxylated analogue of *trans*-resveratrol, had an antiproliferative effect at concentration as low as $25 \mu M$. However, this stilbene was cytotoxic for concentrations ranging from $25-100 \mu M$, we observed rounding and floating cells in the medium.

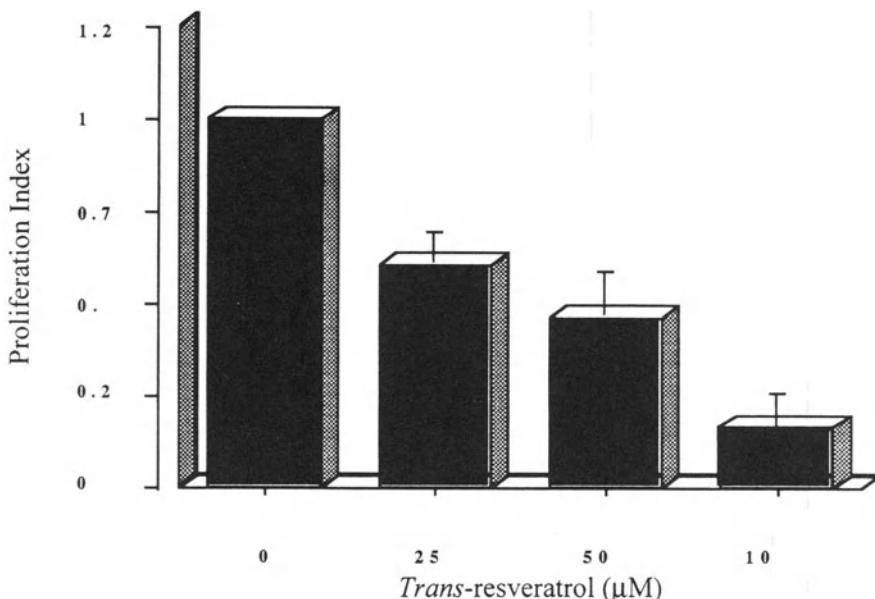


Figure 2. Trans-resveratrol Decreases Myofibroblasts Proliferation. Myofibroblasts were grown for 4 days with the indicated concentrations of trans-resveratrol. Proliferation was assessed by MTT incorporation. Results are expressed as net growth relative to control cells.

Trans-resveratrol decreases expression of smooth muscle α -actin but has no effect on vimentin and β -actin expression.

Smooth muscle alpha-actin expression has been accepted for use as an indicator of myofibroblastic phenotype. Western blot analysis showed that *trans*-resveratrol decreased smooth muscle alpha-actin expression in a dose-dependent manner (ED₅₀= 28 μ M). A maximal inhibition was obtained at 100 μ M. Immunofluorescence staining confirmed these findings with a dose-dependent decrease of smooth muscle alpha-actin expression with *trans*-resveratrol treatment. We observed a decrease in the number of stress fibers and their staining intensity. However, as shown by western blot, *trans*-resveratrol failed to affect the expression of vimentin or β -actin, other cytoskeletal proteins (Figure 3).

Finally neither *trans*-piceatannol nor *trans*-piceid had any effect on smooth muscle α -actin or vimentin expression.

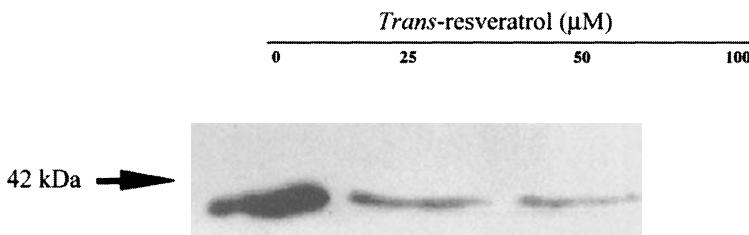


Figure 3. *Trans*-resveratrol Down-Regulates Smooth Muscle α -Actin Expression in Myofibroblasts. Cells were exposed for 4 days to *trans*-resveratrol. Equal amounts of total proteins were analysed by Western blot.

Trans-resveratrol decreases the expression of type I procollagen mRNA

We examined the effect of *trans*-resveratrol on the mRNA level of the $\alpha 1$ chain of procollagen type I. Expression of 28s rRNA was used as an internal standard to control for variations of RNA loading and transfer.

Following treatment with 100 μ M *trans*-resveratrol for 24 hours, we observed a 35 % decrease of the level of pro α 1 (I) transcripts (Figure 4).

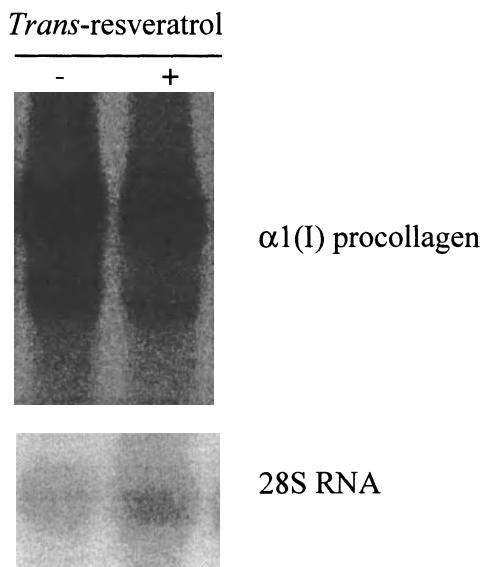


Figure 4. *Trans*-resveratrol down-regulates Collagen Type I mRNA expression in myofibroblasts. Cells were exposed for 4 days to *trans*-resveratrol. Equal amounts of total RNA were analysed by Northen blot with probes to type I collagen and 28S RNA.

Trans-resveratrol decreases myofibroblast migration

Trans-resveratrol dose-dependently decreased the number of migrating myofibroblasts across an experimental wound *in vitro* (Figure 5). Maximal inhibition was roughly 50% at 100 μ M *trans*-resveratrol. This effect was at least in part independent of the growth inhibition, as shown by labelling migrating cells with bromodeoxyuridine (data not shown).

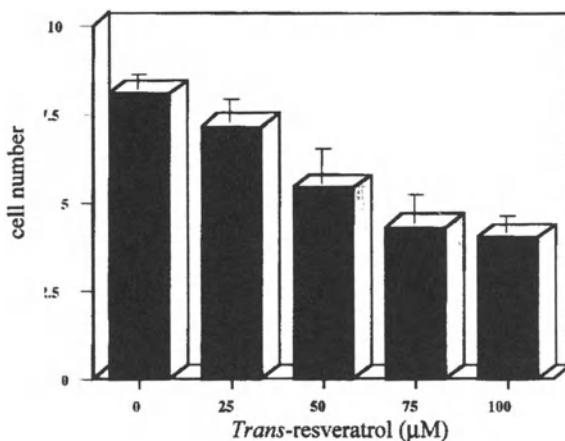


Figure 5. *Trans*-resveratrol Inhibits Myofibroblasts Migration in a Wounding Assay.
Numbers indicate migrating cells after 24 hours.

Effect of trans-resveratrol on tumor cell morphology.

As shown in Figure 6, HepG2 cells grown on plastic in control medium assumed an epithelial morphology forming dense clusters of tightly packed cells. *Trans*-resveratrol (25 and 50 μM) had no effect on HepG2 cells morphology after incubation during 24 or 48 hr. During incubation with recombinant HGF, HepG2 cells rapidly dissociated and many cells showed an elongated shape. This effect was inhibited when cells were incubated during 24 or 48 hr with *trans*-resveratrol (25 and 50 μM).

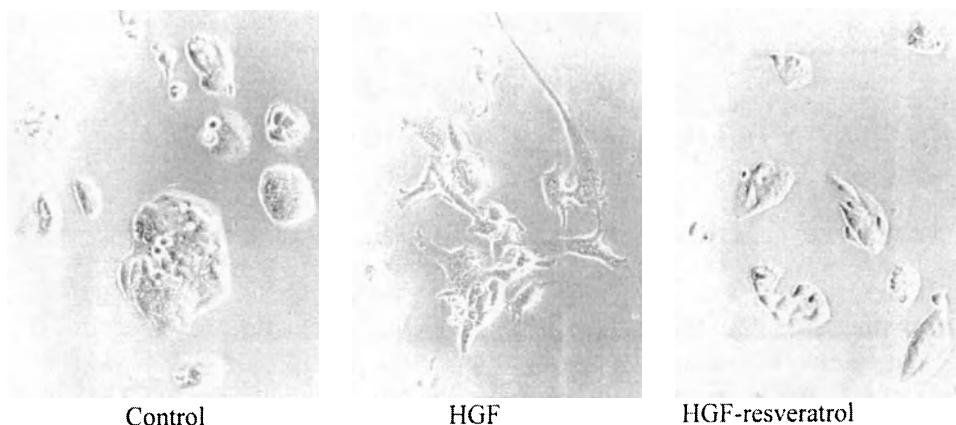


Figure 6. *Trans*-resveratrol Inhibits HGF-Induced HepG2 Cell Dissociation

Effect of trans-resveratrol on tumor cell invasiveness

In control medium, only a few tumor cells could invade Matrigel. In contrast, myofibroblasts present in the lower compartment were able to greatly enhance the invasiveness of HepG2 cells. This increase was inhibited by 63 % after incubation with 25 μ M *trans*-resveratrol. Similar results were obtained when invasion was induced by recombinant HGF instead of myofibroblasts.

Effect of trans-resveratrol on c-met (HGF receptor) expression

As shown in Figure 7, *trans*-resveratrol (25 μ M) had no effect on c-met expression by HepG2 cells. Exposure to HGF induced a 50 % decrease in the 145 kD (mature) isoform together with a 200 % increase in the 170 kD (precursor) isoform expression. Addition of *trans*-resveratrol together with HGF induced a further decrease in the amount of mature c-met, and reduced by 50% the HGF-induced-increase in the 170 kDa precursor form.

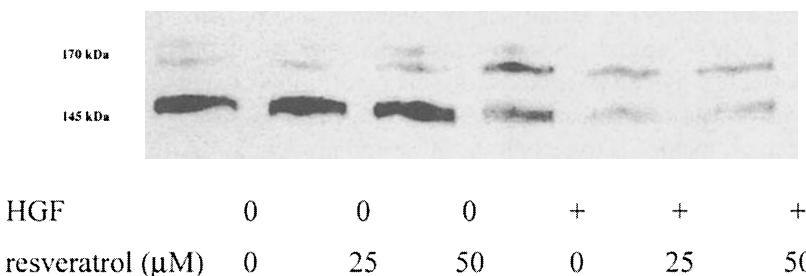


Figure 7. Effect Of Trans-Resveratrol on C-Met Expression. HepG2 cells were exposed for 24 hours to HGF and/or *trans*-resveratrol. Identical amounts of protein were analysed by Western blot.

CONCLUSION

In this study, we show that *trans*-resveratrol might have interesting possibilities as a therapeutic agent in the liver since it is able to deactivate liver myofibroblasts, key actors of liver fibrosis and most likely liver cancer

progression, as well as to directly inhibit tumor cell invasion. However, many issues remain to address.

1 - What is the mechanism of myofibroblast deactivation? *Trans*-resveratrol is a known anti-oxidant molecule. It has been shown that anti-oxidant agents, such as vitamin E, were able to prevent the activation of hepatic stellate cells into myofibroblasts²². Whether this will prove true in the case of established myofibroblasts remains to be established. It has also been shown that *trans*-resveratrol was an inhibitor of several enzymes that could be involved in cell proliferation. Thus, *trans*-resveratrol strongly inhibits the activity of purified ribonucleotide reductase²³. There are however no data showing that this is true at the level of the intact cell. On the other hand, *trans*-resveratrol inhibits the activity of several tyrosine kinases in living cells²⁴. Furthermore, Kawada et al. have shown that *trans*-resveratrol reduces the tyrosine phosphorylation of several proteins, likely including the platelet-derived growth factor receptor, in cultured hepatic stellate cells²⁵. This could provide part of the explanation of the mechanism of action of *trans*-resveratrol on myofibroblasts.

2 - What is the mechanism of the anti-invasive effect? The same type of explanation could be offered. However, our preliminary data suggest that *trans*-resveratrol does not act by inhibiting the tyrosine phosphorylation of the HGF receptor, since several signals transduced via this receptor are unaffected by *trans*-resveratrol (data not shown). Our hypothesis is that *trans*-resveratrol selectively inhibits one pathway, downstream of the HGF receptor, that leads to cell dissociation and invasion.

ACKNOWLEDGMENTS

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Chapter 5

UNDERSTANDING THE COLOUR OF RED WINES: FROM ANTHOCYANINS TO COMPLEX PIGMENTS

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INTRODUCTION

The colour of red wine depends, to a great extent, on its phenolic composition. During maceration diverse phenolics are extracted from the solid parts of the grape, especially hydroxycinnamic esters and flavonoids, the most significant of these being the anthocyanins, primarily responsible for colour in young red wines, and the flavanols (catechins and proanthocyanidins), which communicate astringency and influence the evolution of the wine colour (Figure 1).

As a consequence of their reactivity, in the course of conservation and ageing, the anthocyanins disappear gradually, being involved in different reactions, at the same time that more stable complex pigments appear, which are responsible for the colour in aged wines.

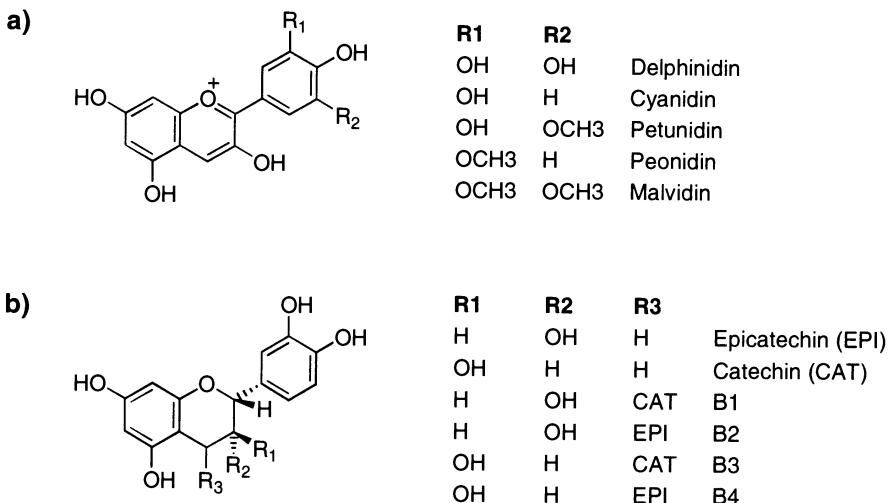


Figure 1. Structures of some Anthocyanidins and Flavanols found in Grapes and Wine

Since the earliest works by Somers⁵⁴, these are commonly referred to as polymeric pigments and among the mechanisms proposed to explain their formation and the stabilisation of the colour of red wine are the processes of copigmentation^{21,40}, the browning of flavanols^{13,14,19,24,34,41}, the direct condensation between anthocyanins and flavanols^{38,39,46,51,59}, or the reactions of anthocyanins and/or flavanols with products such as acetaldehyde^{4,19,44,45,51,59}, pyruvic acid²⁷, or glyoxylic acid²⁶. All of these may occur simultaneously in wine, at variable rates and extensions in relation to the quantity and type of phenolic compounds present, the conditions of pH and temperature, the content of SO₂ and the availability of acetaldehyde and O₂. The difficulty of studying all these reactions directly in wine, due to the variety of elements that can have an influence, have lead researchers to study them in model media where different combinations of substances are introduced in controlled conditions of reaction, to allow the evaluation of the importance of each factor separately. In spite of the evidence obtained in model assays, difficulties exist for the demonstration and isolation of the pigments actually formed in red wine and only very recently the presence of some of them has been demonstrated directly in wine^{2,12,60}, although most of them still remain unidentified.

A new, interesting item of discussion in relation with grape and wine anthocyanins and flavanols is their possible protective role with regard to chronic diseases⁶, which has conferred additional importance to the study of their presence and modifications in red wines.

In the following pages, the current knowledge about the processes involved in the formation of new pigments in red wines is reviewed and some results obtained in our laboratory are contributed.

ANTHOCYANIN-FLAVANOL COPIGMENTATION

In aqueous and hydro-alcoholic solutions, at acidic pH values, the anthocyanins occur as an equilibrium mixture of four main structural forms: the coloured flavylium cation and quinonoidal anhydro-bases, the colourless carbinol bases and the colourless or pale yellow chalcones (Figure 2). In strongly acidic media ($\text{pH} < 2.5$), anthocyanins exist as flavylium cations, whose colour is usually red or orange. As the pH increases the bluish anhydro-bases are formed first, but they rapidly fade to form the hemiketal structure by hydration. Thus, at the usual pH value of the wine (3.2 to 4) the highest proportion of anthocyanins occurs as colourless or slightly coloured structures.

A process that may be of the greatest importance to oppose the negative effect of weak acidity and, thus, contribute to the maintenance of the red colour in young red wines, where the anthocyanins are still the primary components of the colour, is copigmentation. This is a molecular association occurring between the coloured forms of the anthocyanins and other compounds, whether phenolics or not, to form non-covalent complexes vertically stacked, which are maintained by hydrophobic interactions between aromatic nuclei and further stabilised by superimposed hydrophilic sugars³¹. The main role of the copigmentation is to control the extent of the hydration reaction; anthocyanins involved in the formation of complexes cannot be approached by water molecules, thus preventing them from hydration. The usual results are hyperchromic and bathochromic shifts in the visible band of the absorption spectrum of the solution. Different substances may act as anthocyanin copigments, such as flavonoids (including the anthocyanins themselves), amino acids, organic acids, polysaccharides, etc. Copigments do not need to be coloured, but they must possess a flat and polarisable part in their structure that allows them to stack with the

anthocyanins^{10,17}. These interactions help explain the variety of colour hues and intensities in flowers and fruits, at pH values where anthocyanins are usually colourless¹, but they were not thought to be important in wines, due to their relatively low concentrations of anthocyanins, when compared with those existing in vacuoles, and the disrupting effect of the alcohol on the copigmentation complexes¹¹. However, in recent times, more attention has been focused on copigmentation as a mechanism for the stabilisation of the red colour in young red wines and because it has been postulated as the first step that could lead to an ulterior covalent reaction which would give rise to the formation of condensation compounds among anthocyanins and colourless molecules, such as flavanols^{9,39}.

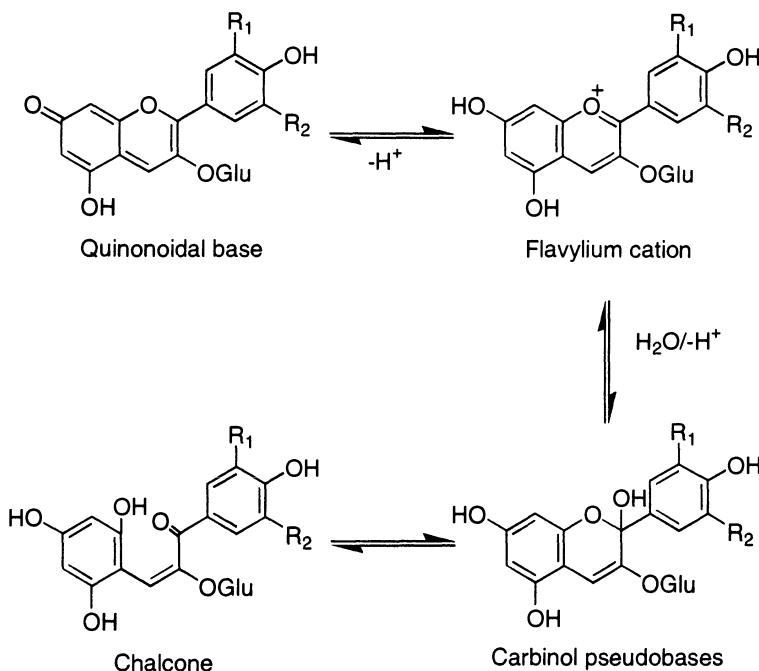


Figure 2. Structural equilibria of anthocyanins in aqueous solutions

Among the substances that may act as anthocyanin copigments are the flavanols, catechins and proanthocyanidins, which are usually found, to a greater or lesser extent, in red wines. The efficiency of some common grape flavanols (catechin (cat), epicatechin (epi) and procyanidin dimers B2 and B3) as copigments of malvidin-3-monoglucoside, the most usual

anthocyanin in wines, has been studied in our laboratory²⁰. On recording the absorption spectrum of equilibrated and weakly acidic (pH 3.2) aqueous solutions of Mv3g (10^{-4} M) with increasing concentrations of these flavanols, in the range from 5 to 50 copigment/pigment molar ratio, the hyperchromic and bathochromic displacements, characteristic of copigmentation, were observed (Figure 3).

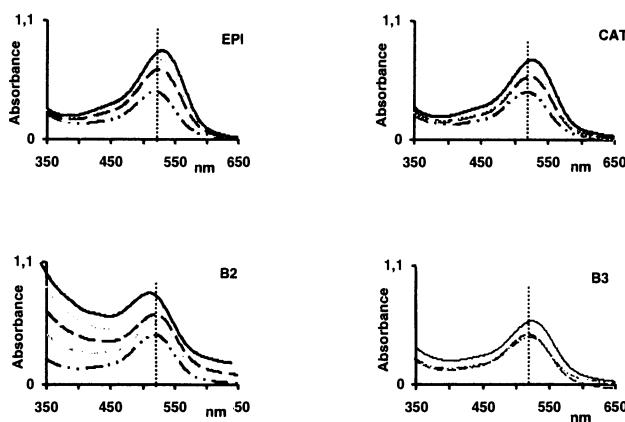


Figure 3. Changes observed in the Spectrum of Mv3g Aqueous Solution after Adding Increasing Concentrations of Catechin (CAT), Epicatechin (EPI), and Procyanolins B2 and B3 in the Range from 5 to 50 Copigment/Pigment Molar Ratio (— · · : 5; - - - : 20; - - - - : 30; - - - - - : 40; — : 50).

The constants of copigmentation (K_c) at 25°C and 0.5M ionic strength between the flavylium ion and each flavanol, calculated according to Dangles and Elhajji²⁰ and accepting an 1:1 stoichiometry, were: $108 \pm 13 \text{ M}^{-1}$ (cat); $274 \pm 14 \text{ M}^{-1}$ (epi); $202 \pm 12 \text{ M}^{-1}$ (B2), and $87 \pm 4 \text{ M}^{-1}$ (B3). In accordance with the values of the K_c , epicatechin behaved as the best copigment of Mv3g, followed by B2, cat and B3. In studies carried out with malvin¹¹, it had already been found that epicatechin associated more strongly to the anthocyanin than its 3-stereoisomer catechin. The explanation offered was that in catechin the 3-hydroxy group and the B-ring are situated on different sides of the A-ring plane; on the contrarily, in epicatechin both groups are situated on the same side of the plane, which allows it a closer

approach to the anthocyanin. The fact that B2 and B3 had Kc values inferior to those of their constituent monomers (epicatechin and catechin, respectively) might be due to their greater size, which would make their approach to the anthocyanin more difficult.

Two outstanding conclusions were drawn from this study. Firstly, the fact that the copigmentation effect was observed in all the range of concentrations assayed, which leads to the supposition that even low amounts of flavanols may have an effect on the colour of young red wines. Secondly, the very distinct behaviour showed by the solution where B2 was added. The solutions containing catechin, epicatechin or B3 had, visually, a red hue, with variable intensity depending on the type and concentration of the flavanol; however, the addition of B2 clearly displaced the colour towards orange hues, more intense as the concentration of the procyanidin increased. Thus, this flavanol is that which caused the most dramatic changes in the colour of the Mv3g solution, although the greatest Kc value was obtained for epicatechin. It is necessary to take into account that the copigmentation constants were calculated from the variation of the absorption at 520 nm produced in the Mv3g spectrum by the addition of increasing amounts of the copigments, in spite of the whole visible region of the spectrum being affected. In other words, the copigmentation not only modifies the colour of the anthocyanin solutions in a quantitative way, on producing a hyperchromic effect on the maximum wavelength of absorption, but it may also provoke important qualitative effects. Thus, it can be supposed that red wines could show notable differences in their colour intensity and hue depending not only on their anthocyanin composition but also on the type and concentration of the flavanols present.

DIRECT CONDENSATION AND BROWNING

In solutions that contain mixtures of anthocyanins and flavanols a progressive browning is produced, due to the disappearance of the anthocyanins and the formation of new pigments, which have maximum absorption in their visible spectra around 430-460 nm^{24,46}. It has been speculated that these pigments could have xanthylum structures, which would be the result of the reorganisation of the products of the direct condensation between anthocyanins and flavanols^{39,46}, in accordance with the mechanism shown in Figure 4.

The formation of a xanthylum derivative (1,3,6,8-tetrahydroxyxanthylum ion, Figure 5) in solutions containing catechin and a

synthetic anthocyanin (5,7-dihydroxy-3,4'-dimethoxyflavylium ion) was found by Escribano-Bailon *et al.*¹⁹. This same pigment had also been previously found in stored grape juice³⁶. However, Escribano-Bailon *et al.*¹⁹ also indicated that this xanthylum pigment was liable to be formed upon degradation of the flavylium ion and that the reactivity towards catechins is reduced in anthocyanins showing an OH group at C-5, such as grape anthocyanins.

In recent studies carried out by our group^{48,49}, the formation of a colourless dimer with the same structure as the intermediary product speculated in Figure 4 was demonstrated in solutions containing anthocyanins and flavanols. However, this product was stable in the assay conditions and it did not rearrange to give brown or yellowish pigments. What is more, most of the pigments whose formation was observed in these solutions and that led to their browning did not, in fact, require anthocyanin for their formation, but they were shown to derive directly from the flavanols²⁴.

The structures of two yellowish pigments (3 and 4) derived from flavanols have recently been postulated¹¹. These resulted from the condensation between catechin units mediated by glyoxylic acid produced in the oxidation of tartaric acid, according to the chain of reactions shown in Figure 6. The same two pigments were also shown to be the major coloured products formed in solutions containing mixtures of Mv3g and catechin^{43,39}; molecular ions corresponding to the intermediary structures involved in their formation, catechin dimer linked through a carboxy-methine bridge (1) and lactone (2), were also found⁴⁹, thus confirming the validity of the mechanism suggested by Fulcrand *et al.*²³.

The speculated structure of another major yellowish pigment formed in catechin+Mv3g model solutions, besides pigments 3 and 4, is shown in Figure 7. This structure, deduced from its mass spectrum, would derive from the glyoxylic acid-mediated condensation of catechin and phloroglucinol which was believed to be produced in the degradation of the Mv3g^{29,42}. Other yellowish catechin-derived pigments have also been found in these solutions whose formation seemed to involve acetaldehyde⁴⁹. These observations permit the affirmation that the principal pigments responsible for the browning of the solutions containing flavanols and anthocyanins derive from the flavanol and involve products from the oxidation of tartaric acid (i.e., glyoxylic acid) and ethanol (i.e., acetaldehyde). The anthocyanins

may also participate indirectly in the formation of some of these pigments through products of their degradation, such as phloroglucinol.

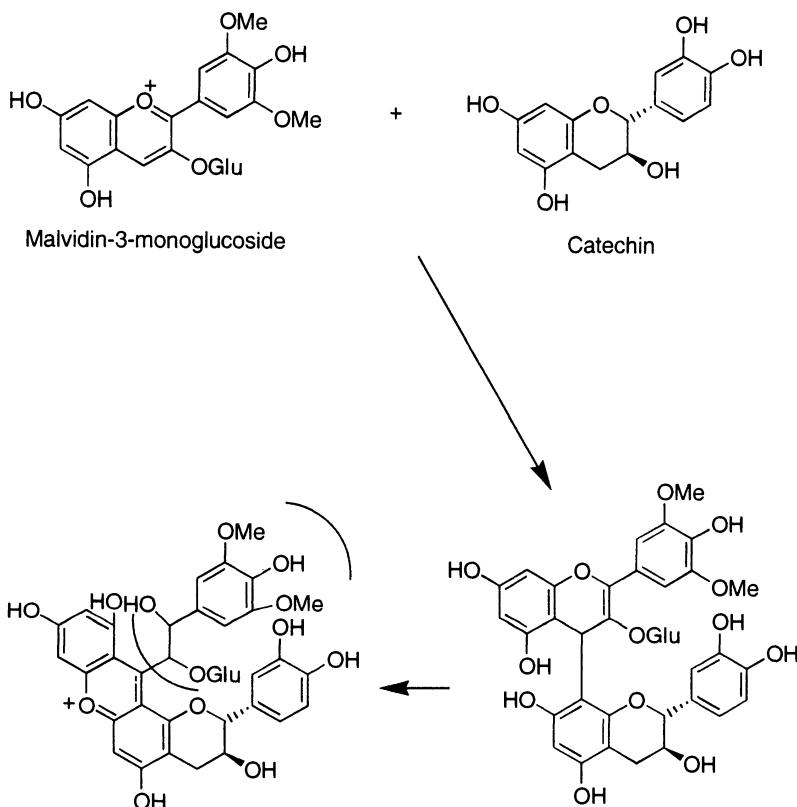


Figure 4. Mechanism proposed for the formation of xanthlyium structures after anthocyanidin-flavanol direct condensation

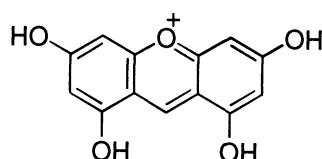


Figure 5. Xanthlyium ion found in anthocyanin-flavanol solutions¹⁹ and in stored grape juice³⁶

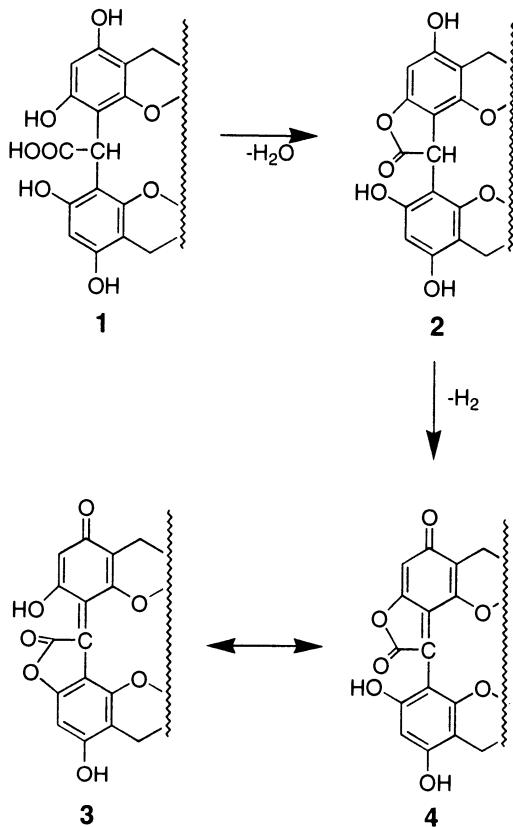


Figure 6. Postulated chain of reactions leading to the formation of yellowish pigments in wine-like catechin solutions²⁸

In model media, epicatechin units seem to be more sensitive to direct browning, while those of catechin demonstrate a greater tendency to condense with the anthocyanin, yielding more colourless condensation products^{24, 48}. This different behaviour, linked to the fact that most of the assays are usually conducted using catechin as a model flavanol, may be one of the reasons that have led different authors to draw erroneous conclusions about the importance of the anthocyanin-flavanol direct condensation as a mechanism to explain the changes of colour observed in wine-like model solutions, as well as about the nature of the pigments formed.

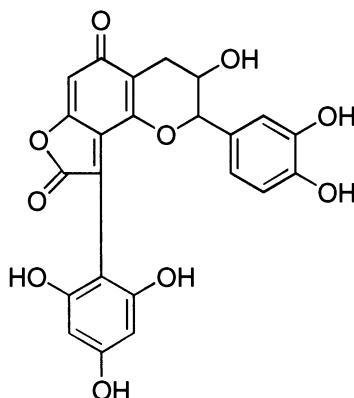


Figure 7. Speculated structure of a yellowish pigment found in wine-like flavanol+mv3g solutions^{48,49}

A factor that greatly influenced the formation of pigments and subsequent changes of colour in the model solutions is the ratio between flavanol and anthocyanin concentrations. In solutions containing relatively high flavanol concentrations, where a greater initial copigmentation effect exists, anthocyanins are more protected from degradation and flavanols are less liable to browning. Thus, in better copigmented solutions less brown pigments are formed and the red colour is maintained longer⁴³. Therefore, it could be foreseen that the existence of relatively high concentration of flavanols, especially of those that, like epicatechin, are better anthocyanin copigments, must confer to red wines protection against the reactions which lead to their browning. On the other hand, it has also been seen that greater quantities of colourless flavanol-anthocyanin condensation products are produced on increasing the concentration of flavanol⁴³, which supports the suggestion of Brouillard and Dangles⁹ that the copigmentation further favours the covalent union between flavanols and anthocyanins.

In spite of the evidence obtained in model assays, the presence of this kind of brown-yellowish pigments has not been shown in wine. However, a red pigment has recently been found in red wine^{47,60}, which, according to its molecular ion (*m/z* at 781 in positive ion mode), might result from the Mv3g-(epi)catechin direct condensation (Figure 8). This kind of structure might derive from the oxidation of the anthocyanin-catechin colourless dimers found in model solutions (see above), even though this process have not been observed in these media, possibly due to their lower oxidative

potential. The formation of these pigments was postulated by Somers⁵⁵, though no reference has been made to their actual detection in wine.

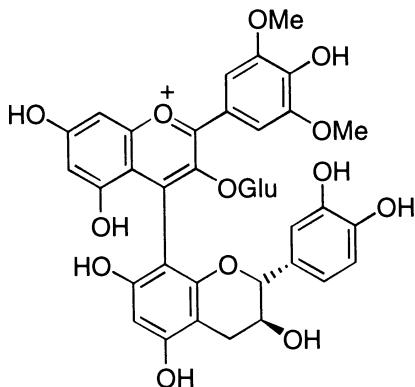


Figure 8. Proposed structure for a red pigment found in wine⁶⁰

ACETALDEHYDE-MEDIATED CONDENSATION

Acetaldehyde is a product of the greatest importance for the colloidal stability and the evolution of the colour in red wines. In its presence, the flavanols condense among themselves by establishing ethyl bridges (Figure 9) between the nucleophilic positions of their phloroglucinol ring (C-6 and C-8), to give rise to colourless products^{51,52}.

This kind of reaction seems to be more favoured for proanthocyanidins than for monomeric catechins²². The products of the condensation may further depolymerise, yielding ethyl-flavanol adducts that can react with the original condensed products, thus increasing their size, or with anthocyanins to form anthocyanin-ethyl-flavanol condensed pigments^{32,44}. The anthocyanins do not seem to be likely to condense among them by this mechanism⁷.

The pigments resulting from the acetaldehyde-mediated condensation between anthocyanins and flavanols (Figure 10)³⁹ are more violet than the original anthocyanins, probably through stabilisation of its anhydrobase

form. They exhibit appreciable colour at low acidic pH where the anthocyanins are usually colourless and they are also more stable regarding the bleaching by bisulphite^{43,56,59}.

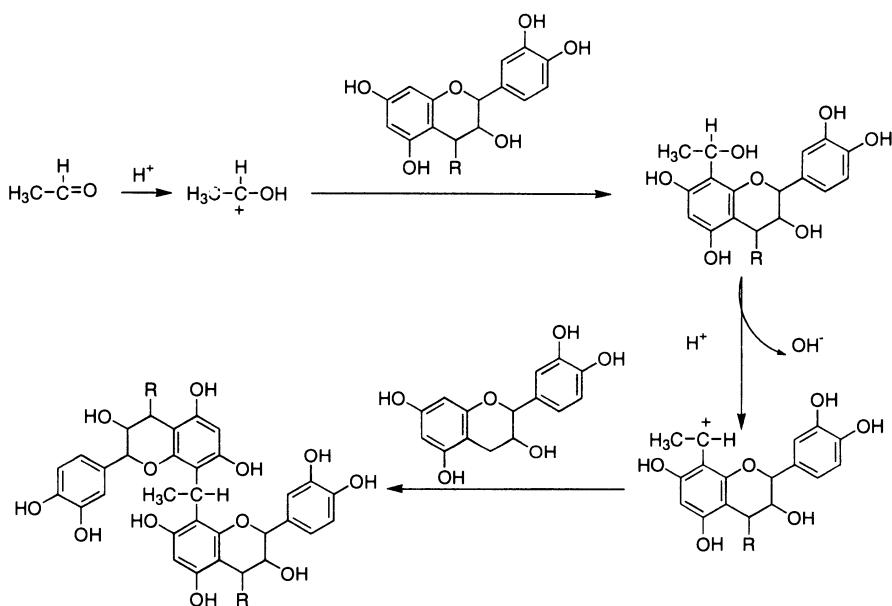


Figure 9. Mechanism of the acetaldehyde-mediated condensation

Their colour and greater stability have been attributed to the reduced reactivity of the C-2 of the anthocyanin residue, due to the perturbation of its electronic environment and/or sterical hindrances derived from the presence of the flavanol residue, which makes them less liable to hydration and more easily stabilised by deprotonation to the violet anhydrobase⁵⁹.

In model media containing Mv3g and (epi)catechin two major pigments are formed as a result of the acetaldehyde-mediated condensation^{4,44,45}. Both pigments were shown to have identical molecular weight (809) by FAB-MS⁴, which was first explained by the possibility of linkage through different nucleophilic positions. However, following NMR

experiments (long-range NOE correlations), it was determined that the position of the ethyl bridge was established between C-8 of both catechin and flavylium moieties and, thus, the two pigments only differed by the configuration of the asymmetric carbon of the ethyl bridge¹⁹. The formation of similar pigments involving procyanidin dimers^{16,23} and trimers^{47,60} has also been found.

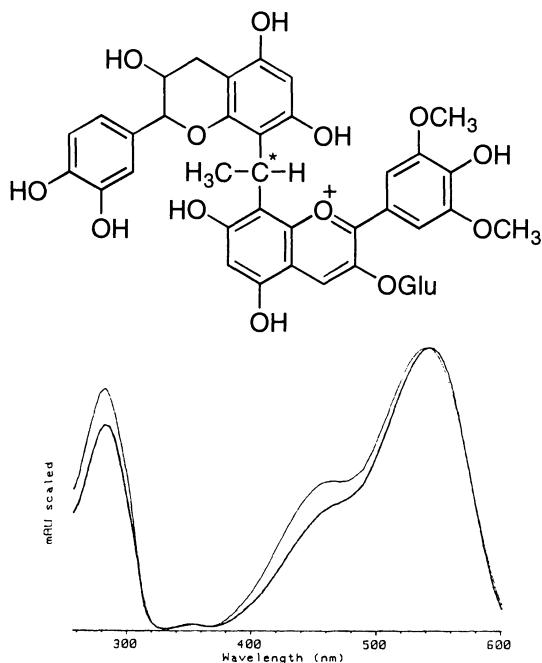


Figure 10. UV-vis Spectra and structure of the pigments formed in the acetaldehyde-mediated condensation of mv3g and catechin

A very influential factor on the acetaldehyde-mediated condensation is the acidity. In the range of pH from 2 to 5, more rapid formation of pigments was seen to occur as the pH decreased^{30,44}, which was attributed to the greater facility of the acetaldehyde to form the cation necessary for the reaction taking place⁴⁴. At sufficiently low pH, in the presence of acetaldehyde, the reaction progresses very rapidly and large polymers are formed that further precipitate causing a loss of colour⁴⁴. Temperature is another determining factor; at low temperatures the formation of condensed pigments is slower, but the products formed are more stable, since they

reduce their tendency to polymerise, thus allowing them to be accumulated in greater amounts^{5,44}. What is more, low temperature increases anthocyanin copigmentation and reduces their degradation, which contribute to the maintenance of the red colour and make more anthocyanin available for the formation of condensed pigments⁴⁴.

In wine, acetaldehyde is produced during fermentation, which causes an important loss of anthocyanins at this stage. Acetaldehyde is also formed in further stages by non-enzymatic oxidation of the ethanol^{57,61}, possibly coupled to the autoxidation of the catechol ring of catechins and procyanidins⁶². The production of acetaldehyde by ethanol oxidation and the subsequent formation of ethyl-bridged condensation products have been demonstrated in hydro-alcoholic solutions containing catechins and/or anthocyanins without added acetaldehyde^{24,46}. Nevertheless, during wine maturation and ageing, the amount of acetaldehyde available to participate in condensation reactions is limited by the presence of free SO₂ and insufficient acidity^{15,58} and, therefore, in these stages, these kinds of reactions take place more slowly than in model solutions and smaller amounts of condensation products are usually formed. This might be the reason why these products either colourless⁵³ or coloured^{47,60} have not been found in wine until very recently. The importance of the acetaldehyde-mediated condensation for the evolution of the red wine colour during maturation and ageing is still controversial. Some authors assign them a key role in the formation of new pigments⁴³, while others confer more interest to other kinds of reactions, taking into account the low availability of acetaldehyde^{5,57}.

OTHER ANTHOCYANIN-DERIVED PIGMENTS

In 1996, Cameira-dos-Santos *et al.*¹² isolated two red-orange pigments from the material absorbed in polymeric membranes used for the cross-flow microfiltration of wine whose structure was later identified by Fulcrand *et al.*²⁵ as resulting from the cycloaddition of the double bond of a 4-vinylphenol residue over the C-4 and the OH group at C-5 of Mv3g and its *p*-coumaroyl derivative, respectively (Figure 11).

Pigments with similar colour characteristics were later identified by Bakker and Timberlake² and Bakker *et al.*³ in Port wines, which received the generic name of "Vitisins" (Figure 12). Vitisin A has recently been synthesised by Fulcrand *et al.*²⁷ by reaction between pyruvic acid and Mv3g (Figure 13) and its structure revised according to NMR data. These same

authors indicated that vitisin B could derive from a similar reaction between Mv3g and ethanal.

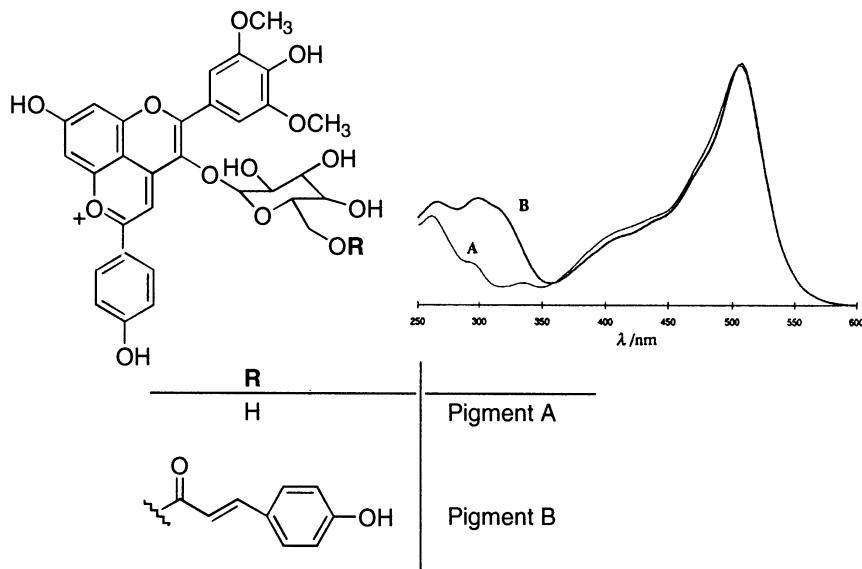


Figure 11. Structures and UV-vis spectra of two anthocyanin derived pigments identified by Fulcrand *et al.*²⁵

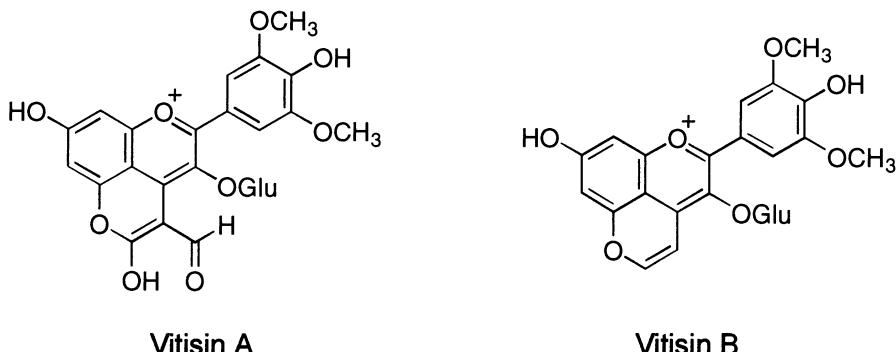


Figure 12. Structures of: Vitisin A and Vitisin B according to Bakker *et al.*³ and Bakker and Timberlake²

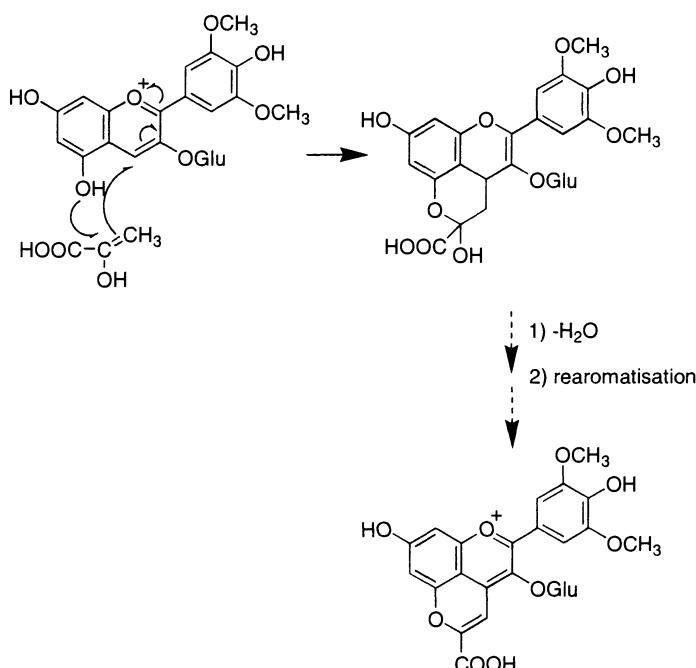


Figure 13. Vitisin A: Structure and mechanism of formation according to Fulcrand *et al.*²⁷

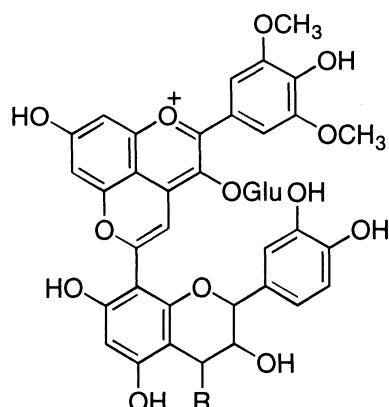


Figure 14. Structure proposed for the vitisin-like pigments found by Francia-Aricha *et al.*²³ in solutions containing Mv3g and flavanols in the presence of acetaldehyde.

R = H or (epi)catechin unit

The formation of vitisin-like pigments was also found in model media containing Mv3g and flavanols in the presence of acetaldehyde, where they appeared together with the characteristic products of the acetaldehyde-mediated condensation²³. The molecular mass of these pigments, established by LC-MS, was coherent with a vitisin-B structure possessing a flavanol residue (Figure 14). Acetaldehyde seems to be involved in the formation of this kind of pigments, which may result from the reaction of an ethyl-flavanol adduct over C-4 and hydroxyl group at C-5 of Mv3g, as for the formation of the pigment shown in Figure 11. The ethyl-flavanol adduct would derive either from the direct reaction between flavanol and acetaldehyde or from the depolymerisation of the products of the acetaldehyde-mediated condensation of flavanols.

A structural feature common to the vitisin-like pigments is that they contain two heterocycles conjugated with the ring-A of the anthocyanin, which makes it likely that they exist in different mesomeric forms (Figure 15). The data available show that this family of pigments have a colour more stable to pH changes and fading by SO₂ than the original anthocyanins^{2,23,50}. Their greater stability linked to the fact that they have an orange-red tone similar to that which red wine acquires during ageing, leads to the consideration that they could play a crucial role in the changes of colour that are produced in red wine during maturation and ageing.

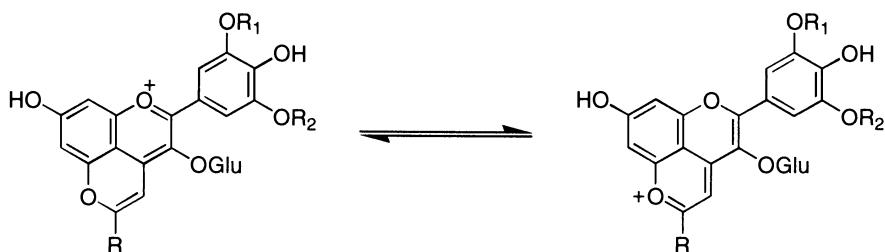


Figure 15. Mesomeric forms of the vitisin-like pigments

In diverse mature red wines analysed by LC-MS in our laboratory the presence of various vitisin-like pigments has been detected, derived from Mv3g as well as from other anthocyanins (e.g., delphinidin and petunidin 3-monoglucoside and their acetyl and p-coumaroyl esters)^{33,47,60}. Furthermore, the formation of vitisin B and related pigments have been induced by adding

acetaldehyde to red wine⁶⁰, supporting its role as a precursor, in accordance with previous suggestions^{23,27}.

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Chapter 6

Physiological Properties of Resveratrol Isomers in Wine: Compositional Changes during Processing

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INTRODUCTION

Resveratrol is a polyphenolic compound that has a C6-C2-C6 structure, it is a stilbene. Stilbenes are ethylene derivatives substituted by two phenyl rings. Ring A usually carries two hydroxyl groups in *m*-position, while ring B is substituted by hydroxy and methoxy groups in the *o*-, *m*-, and *p*- position. They are synthesised from the cinnamic acid derivatives¹.

They are widely distributed in liverworts and higher plants², in monomeric form and as dimer, trimer and polymeric stilbenes, the so-called viniferins.

Stilbenes are synthesised by a wide range of plant species, including *Dipterocarpaceae*, *Cyperaceae*, *Gnetaceae*, *Pinaceae*, *Leguminosae*, *Myrtaceae*, *Moraceae*, *Fagaceae*, *Liliaceae*, and *Vitaceae*; however, they are usually found in the not edible parts of the plant, such as roots, barks, rhizomes, and leaves. Resveratrol has been quantified in grapes and peanuts and in their by-products. Therefore, until now, we can say that the major resource of resveratrol and piceid in the human diet are grapes and peanuts. In folk medicine, humans also have resveratrol from the use of medicinal plants³, for example the roots and rhizomes of *Polygonum cuspidatum*, *Veratrum*

formosanum, that are usually used in the traditional Chinese medicine for the treatment of several ailments.

The production of the polyphenol, *trans*-resveratrol (*trans*-3,5,4'-trihydroxystilbene) is positively correlated with resistance of the wine to cryptogamic⁴⁻⁶, and it is also considered as a good marker for grey mold resistance⁷. In grapevine, this compound is synthesised in leaves³, roots⁹, and grape skins^{110,11}.

Resveratrol is synthesised in response to microbial infection or stress^{4,12}. When the infestation is 10 %, the wines have higher resveratrol content¹³. However, resveratrol is degraded by the pathogen *Botrytis cinerea* in highly infected grapes, due to the presence of laccase activity¹⁴. Resveratrol synthesis is also induced by abiotic elicitors, after chemical treatments, such as herbicide, fungicide¹⁵, following the application of the inductors, carbohydrates and galacturonic¹⁶ and by UV-light exposure^{11,12,17,18}.

In grape products in addition to the aglycone, *trans*-resveratrol, *trans*-piceid, can also be quantified (Figure 1)¹⁹⁻²². In grape berries, these compounds, are primarily located in the skin cells and they are absent or low, in the fruit flesh¹⁰. They are present in two isomeric forms, *cis* and *trans*; however the *cis*-resveratrol is a by-product of fermentation²¹, and it is rarely found in grapes²³.

PHYSIOLOGICAL PROPERTIES

Antioxidant

Trans-resveratrol, as antioxidant, is more effective than butylhydroxytoluene (BHT), quercetin or tocopherol on lipid peroxidation in liposomes and in rat liver²⁴. Resveratrol can inhibit LDL oxidation²⁵⁻²⁷. *Trans*-resveratrol mainly acts by reducing the copper-catalysed oxidation, while flavonoids are better scavengers of free radical. However, resveratrol cannot chelate ferrous ions. Fouconneau *et al.*²⁸ noted that the presence of 4'-hydroxy in B ring and the *meta* hydroxy structure in the A ring are essential for the antioxidant activity of the stilbenes to prevent metal-induced lipid peroxidation in microsomes and LDL. *Cis*-resveratrol possesses also physiological activity inhibiting oxidation of human LDL²⁹; as antioxidant the activity of the *cis* isomer is half that of *trans* isomer.

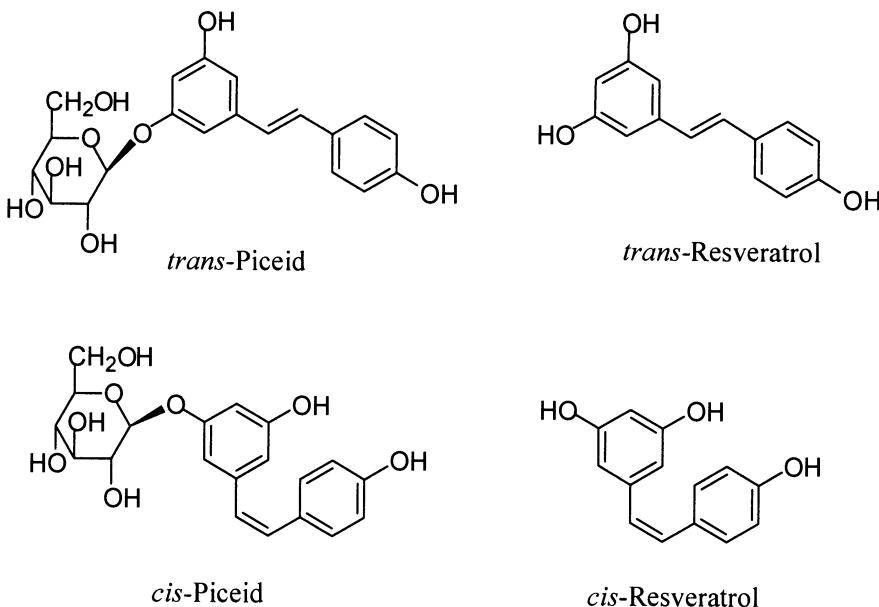


Figure 1. Chemical structures of resveratrol and piceid isomers

Polygonum cuspidatum root is used in folk medicine for the treatment of some cardiac ailments, including atherosclerosis and inflammation. The main active compounds in these roots are piceid and resveratrol. Piceid reduces the serum triglyceride and LDL-cholesterol concentrations as well as the ratio of total cholesterol to HDL-cholesterol³⁰.

However, when *trans*-resveratrol is studied by itself, there are some contradictory results. While Soleas *et al.*³¹ observed a decrease in the intracellular concentration of apolipoprotein B with the increase of *trans*-resveratrol concentrations (1-50 µmol/L), they also observed a lower secretion of cholesterol esters and triglycerides in a dose-dependent manner. Wilson *et al.*³² found that when resveratrol is administered at a dose of 0.6 mg/Kg during

the first 5 days and 1.0 mg/Kg from 6 to 60 days, to hypercholesterolemic rabbits, it promotes aorta atherosclerotic lesions, rather than having a beneficial protective effect. Turrens *et al.*³³ report that resveratrol has no significant effect on lipoprotein profile nor does it prevent the oxidation of plasma lipid *in vivo*, when it was injected to rats at 20 and 40 mg/Kg body weight.

Antiaggregation properties

Trans-resveratrol can block ADP, collagen and thrombin induced platelet aggregation^{34,35}. Resveratrol also has the ability to inhibit arachidonate metabolism^{34,35}, specifically by inhibiting thromboxane A2 formation (or thromboxane B2 and HHT), which are stable products formed from thromboxane A2 and lipoxygenase, effects which could moderate thrombotic events and antinflammatory process. Resveratrol mediates the antiinflammatory process mainly by inhibiting cyclooxygenase- and hydroperoxidase functions. *Cis*-resveratrol inhibits protein-tyrosine kinase and platelet aggregation^{3,36}. Piceid isomers have also similar properties to resveratrol, inhibiting platelet aggregation^{3,37-39}. On the other hand, in a manner less active than *trans*-resveratrol, piceid also inhibits eicosanoid synthesis.⁴⁰.

Chen and Pace-Asciack⁴¹ observed that *trans*-resveratrol cause nitric-oxide relaxation of precontracted endothelium-intact rat aorta, however Fitzpatrick *et al.*⁴² find no vasorelaxation activity in assays with *trans*-resveratrol.

Cancer chemopreventive and chemotherapeutic agent

Resveratrol inhibits the three major stages of carcinogenesis: initiation, promotion, and progression. It has antiinitiation activity because of its antioxidant and antimutagenic effects, and it inhibits the induction of phase II drug-metabolizing enzymes. It possesses antipromotion activity because of its antiinflammatory effects, causes inhibition of production of arachidonic acid metabolites catalyzed by either COX-1 or COX-2, and chemical carcinogen-induced neoplastic transformation of mouse embryo fibroblast, and resveratrol antiprogression activity is demonstrated by its ability to induce human promyelocytic leukemia (HL-60) cell differentiation^{43,44}. It is a remarkable inhibitor of ribonucleotide reductase⁴⁵. Resveratrol significantly inhibits the inducible nitric oxide synthase as well as mRNA expression. Clement *et al.*⁴⁶ report that resveratrol induce the apoptosis cell death.

Breast Cancer

Studies *in vitro* have been carried out using the estrogen-dependent human breast cancer cell line, MCF-7. Exposure of MCF-7 to concentrations of resveratrol ranging from 5 to 40 µg/mL led to a decrease in the rate of exponential growth. Resveratrol is a potential chemopreventive agent for both hormone responsive and non-responsive breast cancers⁴⁷. The relevance of the MCF-7 model remains a matter of conjecture and further studies would be useful in this context.

Phytoestrogen

Resveratrol has a similar structure to diethylstilbestrol, it can bind to the estrogen receptor (ER) *in vitro*, it may exhibit estrogenic activity, so it may produce cardioprotective effect; however it may produce undesirable side effects, resveratrol could exert a growth stimulating estrogenic effect on human breast carcinomas⁴⁸. It can also antagonize the effects of 17-beta-estradiol⁴⁹. Resveratrol is a partial ER by itself ; however it acts as an ER antagonist in the presence of estrogen leading to inhibition of human breast cancer cells⁵⁰.

Resveratrol in dementia

According to Tredici *et al.*⁵¹, resveratrol possesses many of the properties of the drugs used for preventing or treating Alzheimer's disease, resveratrol increases ERK2 phosphorylation⁵², such us ampakines, it has antinflammatory, antioxidant, estrogenic and hypolipemic properties.

In 1997 and 1998, Sun group^{53,54} published two papers in where they demonstrate that resveratrol protects brain from damage caused by oxidative stress, implying a beneficial value for preventing neurodegenerative diseases in general and Alzheimer's disease. Resveratrol is an amphipatic molecule and is able to provide more effective oxidative protection for cellular and subcellular components than other food antioxidants, such us ascorbic acid or tocopherol.

Resveratrol absorption

In the absence of more human data, it is necessary to refer to work carried out on animal models. When rats are administered with *trans*-resveratrol (2 mg/kg), the stilbene is quantified, resulting in a plasma

concentration of 0.175 mg/L at 15 min⁵⁵. However other peaks with the same spectra as resveratrol appeared in the chromatogram, probably due to resveratrol conjugation in the liver or the small intestine: to glucuronide, sulfate or methylated forms. Once it has been absorbed, resveratrol tends to accumulate in the liver and kidney⁵⁶.

The β-glycosidic bond of piceid is resistant to the hydrolysis by pancreatic enzymes and the conjugation with glucose will enhance the absorption from the small gut^{57,58}. Microorganisms in the colon can release the aglycon, but at the same time they can degrade the free aglycon.

RESVERATROL IN WINES

Assays of resveratrol and piceid

GC/MS

Most GC methods require a pre-derivatization with bis-[trimethylsilyl]-trifluoroacetamide (BSTFA) prior to column application with detection by flame ionization or mass-spectrometry (MS). Although GC-MS techniques have many advantages in the assay of phenolic compounds in wine, including higher sensitivity and identification by mass spectral characteristics. The disadvantages of GC methods for stilbenes compared to the liquid chromatography methods are⁵⁹:

- Time required for the extraction or for the derivatization (60 min).
- Trans* to *cis* isomerization may occur during derivatization⁶⁰.
- Trans*-polydatin is converted to the free isomers⁶⁰, resulting in an overestimation of the aglycone.

HPLC

HPLC can be used in routine analysis because it is rapid and does not require any sample pretreatment. In 1995, Lamuela-Raventos *et al.*²⁰ published a method that allowed the quantification not only of the resveratrol isomers, but also both piceid isomers simultaneously. In the direct injection method, red and rosé wine samples can be chromatographed immediately after filtration.

In wines, the levels of resveratrol will depend on the grape variety, climatic conditions of the harvest, and ecological procedures employed.

Resveratrol levels change in a significant manner from one variety to another. Bavaresco *et al.*⁶¹ and Jeandet *et al.*⁷ reported differences in the production of resveratrol in wine varieties, since the presence of this compound is an indicator of the resistance of the plant to infections. Romero-Pérez *et al.*⁶² found that wines obtained from different wineries, vintages and appellations could be grouped within varieties by the amount of resveratrol and piceid present. These compounds have been shown to be chemotaxonomic markers of wines, distinguishing varieties in white wines from different appellations, wineries, and vintages.

Red wines are the highest in *trans*-resveratrol content, with the average level of all four compounds *trans*- and *cis*- resveratrol and piceid, approximately 8 mg/L depending on the grape variety²⁰. In rosé wines⁶³ the levels range between 1.38 to 2.93 mg/L. White and sparkling wines are usually made from free-run juices, so they have much lower resveratrol content than the red ones, due to minimal skin contact associated with white wine production. The levels are between 0.1 for Chardonnay to 1.2 mg/L for Sauvignon Blanc and Xarel.lo⁶³. When, Jeandet *et al.*²³, fermented the white variety, Chardonnay with pomace contact, the levels of resveratrol increased 10 times. Because maceration with skins and seeds, during fermentation, is the main factor that contributes to the presence of stilbenes in wines. Resveratrol requires relatively long maceration time on the skins to be extracted^{23,63-66}. However, the amount present will also depend on other enological techniques employed: yeast strains⁶⁷, the fining agents used⁶⁷⁻⁶⁹, and the time of ageing in oak⁶⁹.

Changes during processing

Manipulations that can be applied during wine making to increase the levels of stilbenes, as well as other phenolics, include treatment of juice with commercial pectolytic enzymes. This can increase resveratrol levels by 50 % (see Figure 2). The enzymes breakdown the hypodermis of the skins, releasing flavour compounds and phenolic compounds⁷⁰. While, fining agents and filter treatments can decrease the levels of stilbenes in wines. Tobella and Waterhouse⁶⁸, in 1996, studied the effect of six fining agents: polyvinylpyrrolidone (PVPP), gelatine, agar, egg albumen, bentonite, and carbon and they observe that PVPP and carbon are the most effective in

removing resveratrol; however the other four fining agents did nor affect the levels of these compounds. Similar results were obtained by Vrhovsek *et al.*⁶⁷, they note that gelatine did not affect either the concentration of free or glucosides forms of resveratrol, while PVPP decreased the concentration of all resveratrol forms (up to 90 %). Some other treatments can also decrease resveratrol levels, Lamuela-Raventós *et al.*²⁰ also observed that some membranes can remove more than 60 % of resveratrol. However, Soleas *et al.*⁶⁹ note that the most aggressive difference occurred with the use of the filter pad.

Resveratrol appears relatively stable in wine during bottling. Jeandet *et al.*¹³ studied the resveratrol content of wines vinted by three producers throughout 13 years and they did not observe a decrease of resveratrol content during ageing in the bottle. Some aged wines have more resveratrol content than young wines. When wines are aged in oak, major losses of resveratrol isomers occurred even when the wines were kept cool and without light⁶⁹.

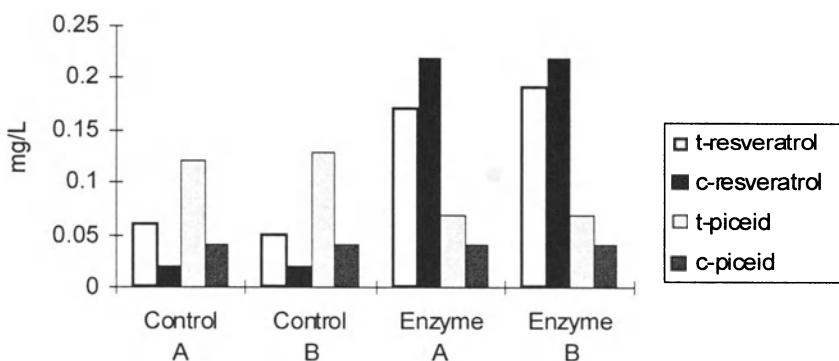


Figure 2. Resveratrol and piceid levels in control and treated juices with pectolytic enzymes

RESVERATROL IN GRAPE JUICES

In grape juices, the total resveratrol content in red juices is between 0.69 and 14.47 mg/L (mean 4.73 mg/L) and in white ones is between not detected and 1.44 mg/L (mean 0.49 mg/L). Levels of total resveratrol in red and white grape juices, are similar to those described previously in red and white wines. The levels of the four compounds in red juices average ten-fold higher than in the white juices. In juices, the glycosides account for 90 % or more of the total

resveratrol content in juices⁷¹. It is interesting to note that the fresh juices from the winemaking grape varieties had a total average resveratrol content of 0.72 mg/L, more than two-fold higher than the commercial juices at 0.29 mg/L. It is not possible to tell whether this difference is due to the use of different varieties, changes during storage, or differences in juice production.

As has been commented previously, the different content of resveratrol between white and red samples is also observed in wines. The lower levels found in white wines are due to the absence of skin contact during the fermentation process. However, in juices the differences in juice resveratrol content between white and red grape juices have to be attributed to the juice processing technology (*i.e.* pressing and skin colour extraction procedures) since the levels of *trans*-resveratrol in grape skins are similar for red and white grape berries⁷².

Thus, grape juice, in particular red grape juice, may be an alternative dietary source to wine to achieve the beneficial effect of resveratrol. Grape juice lacks alcohol, which in moderation has some beneficial properties, but it cannot be present in foods for particular populations such as for children or patients with hepatic diseases.

CONCLUSIONS

Resveratrol is a molecule with very interesting physiological properties, our major source of this compound from diet are grape products, such as red wine and red juice. However, many studies have focused on *in vitro* effects, we still need studies on bioavailability and clinical trials in humans and interactions with other antioxidant molecules, synergism effects. In juice and wine resveratrol is not the only phenolic present, there are other phenolic components which we still not know physiological activities and they may increase resveratrol effects. In any case, we drink wine because it as a pleasant drink to have with meals, as it has been used traditionally in the Mediterranean diet, so when we drink wine, in moderation with meals, we will be having not only pleasure but also healthy nutrients for our body.

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Chapter 7

The French or the Mediterranean Paradox? Biological Mechanisms Potentially Involved in a Protective Effect of Wine Phenols against Clinical Manifestations of Atherothrombosis

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INTRODUCTION

Cardiovascular events are the first cause of mortality and morbidity in industrialized countries. Most of the arterial cardiovascular events are due to the atherothrombotic disease. The word "atherothrombosis" associates several fundamentals:

- Extension of the disease, affecting the arteries of medium diameter of the entire arterial tree.
- Similarity of the physiopathogeny whatever the localization of the disease over the arterial tree.

The overall physiopathogeny of the disease associates:

- atherosclerosis (*i.e.* the lesions of the arterial walls implicating the cellular infiltration by monocytes, lymphocytes...),
- the tissular development (migration and proliferation of medial smooth cells, of adventitial fibroblastic cells, oversynthesis of extracellular matrices...)

- and the lipidic infiltration (extracellular intracellular lipid deposits, lipidic remnants of apoptotic cells...).

The lesions of the arterial wall are progressive and are associated and intricate with thrombotic events which explain the concept of atherothrombosis. The thrombotic mechanisms are implicated at every step of the atherosclerotic disease : the thrombotic process takes place in the tissue inside the arterial wall before the first deposits of lipidic material, and some theories explain that a fibrin network (i.e. a transformation of fibrinogen into fibrin by the enzymatic coagulation cascade) inside the arterial wall retains the first deposits of oxidized lipoproteins. The intratissular fibrinolytic systems are associated with all the mechanisms of cell migration, cell proliferation, conjunctive tissue metabolisms...

This lesion evolves until the rupture of the fibrous cap due to the growth of the lipidic core which destabilizes by activation of mechanisms closely linked to inflammation (macrophage and monocyte infiltration and activation) and due to the catabolic weakening of the fibrous cap which constrains the lesion on the luminal side until rupture. This rupture of the fibrous cap involves the fibrinolytic systems which among other effects activates several matrix metalloproteinases implicated in collagen metabolism. This rupture of the cap of atherosclerotic lesion triggers a thrombotic reaction by exposition to the flowing blood of thrombogenic materials (collagen and matricial molecules which activate the platelets, tissue factor which initiates the coagulation cascade).

This thrombotic reaction:

- can remain limited as a scaring mechanism of the lesion (but which induces a rapid evolutionary step of the lesion),
- or can reach an occlusive size with subsequent ischaemic clinical manifestations

Whatever its ultimate size (limited or occlusive), the thrombus will stabilize. But during the time required for stabilization, it evolves during hours, days or weeks, scattering small debris from the ruptured lesion (cholesterol emboli) and parts of the thrombus which will destroy the downstream capillary bed. The ischaemic tissues explain the dual evolution of the disease : evolution of the upstream main arterial lesion and evolution of the downstream tissular and capillary disease. This progressive

destruction of the down stream tissues by this microembolic process is most of the time clinically silent. For example in the coronary bed, it leads to myocardial insufficiency.

Apart from the atherosclerotic plaque rupture, others mechanisms can trigger a thrombotic reaction :

- Erosion of the atherosclerotic plaque,
- Stenosis of the arterial lumen by the lesion which changes flow conditions. Very high shear stress flow conditions can also activate the thrombotic reaction directly in the blood flow (platelet and cellular activation, activation of molecules such as von Willebrand's factor).

The atherothrombotic disease is the association of blood and vascular wall mechanisms which ultimately lead to the clinical ischaemic cardiovascular manifestations.

DISCUSSION

These cardiovascular complications are less frequent than expected in the populations of the south of Europe (compared to the North of Europe and to North American populations¹). What has been called as the French Paradox should be called the Mediterranean paradox². The incidence of cardiovascular events in France is between one third and one forth of the incidence in the northern part of Europe in Belfast but is similar to neighboring countries (Switzerland) and slightly higher than southerner countries (Italy, Spain)¹. Among the three French observational regions of the Monica Study, there is also a small North – South gradient in France with a lower incidence in Toulouse compared to the two Northern and Eastern regions. These differences in the incidence of atherothrombotic complications in various populations depend on genetically determined characteristics of the populations and on the multiple environmental conditions, among which the nutritional conditions are predominant. One of the characteristics the Mediterranean diet compared to Northern European populations is a regular intake of polyphenols (from the fruits and vegetables) and also from a regular consumption of wine. The problem is to determine if this regular intake of wine can in part explains the

Mediterranean Paradox and what are the components of wine potentially modify a so complex disease as atherosclerosis.

One of the difficulties in evaluation of the potential effects of wine is that wine is a highly complex mixture of components which evolve with time and which differ significantly from one wine to another. One of these compounds is alcohol and the role of alcohol is also highly complex since it bears numerous deleterious effects and some potentially beneficial ones which depend on the amount and type of absorption. A recent study³ has evaluated prospectively the health risk of wine and beer drinking in middle-aged men in Eastern France, and sustain the hypothesis that a moderate and regular intake of wine reduces the risk of cardiovascular death. Some of this benefit was also achieved by a moderate drinking of beer. But more interestingly moderate drinking of wine (and only of wine) was associated with a lower all-cause mortality, demonstrating for the first time that a moderate drinking of wine could have an overall benefit on total population mortality. The results of this type of study which compares in the same French genetic background the specific effect of the type of drinking among the other environmental factors, are convincing because of the high number of subjects included. However, other studies criticize the hypothesis to rely the lower incidence of cardiovascular events in the French population on the regular absorption of wine⁴ should not rely on older epidemiological statistics which were misleading, should not focus on a French specificity (which doesn't exist) but should envisage a Mediterranean paradox². It must be considered that many other environmental and genetic characteristics of the Mediterranean population can explain this lower incidence of the atherosclerotic disease in the Southern European populations.

As regular and moderate wine intake could bear some health benefit, it has to be determined if these effects are directly linked to the absorption of wine compounds or indirectly linked to the numerous conditions (nutritional, environmental, socio-economical, psychological...) accompanying the regular wine consumption.

Among the wine components, the phenols have been mainly studied because they are a major class of wine components, and they have several effects (anti-oxidative, anti-proliferative...) observed in *in vitro* conditions which are potentially beneficial among the numerous mechanisms involved in the atherosclerotic disease. A bunch of studies, clinical and

experimental, sustain this hypothesis but questions remain on the absorption, the metabolism and the mode of actions of phenols compounds on their potential anti-atherothrombotic properties. The links between nutritional phenols, antioxidant activity, reduced evolution and complication from atherothrombosis are sustained by concordant evidences but have not been definitively proven. *In vitro* wine derived phenols express potent antioxidant activities⁵ which allow to reduce LDL peroxidation⁶. They act as scavengers for free radicals⁷. This antioxidant activity of the phenols is due to their chemical structure. *In vivo*, in human volunteers, the absorption of nutritional phenols derived from tea⁸, from olive oil⁹, from liquorice¹⁰, and from wine reduces the oxidability of LDL and increases the plasma anti-oxidative capacities¹¹. But besides these antioxidant characteristics, phenols are potentially involved at each of the steps of the atherothrombotic disease. Each type of phenols has several types and potency of activities. Wines are complex mixtures of various types of phenols evolving with maturation. These various phenols from wine are differently metabolized and absorbed after wine after wine intake. This multistage complexity explains the difficulty to demonstrate a global beneficial effect of wine and to isolate a compound which could be responsible for the effect of wine.

Phenols from a global wine extract exert a direct effect on endothelial cells and stimulate the secretion of nitric oxide (and of other cytoprotective and vasorelaxative agents). When wine derived tannins and anthocyanins are applied on endothelium, they induce a vasorelaxation dependent form the integrity of the endothelial layer¹² but even more interestingly, oral administration of these wine derived phenols have a global hypotensive activity on the animal¹². This systemic effect appears also related to NO since it is abolished by the administration of an antagonist of nitric oxide synthase¹². Among its numerous effects, NO is a potent antagonist of platelet activation. This activity could be related to the antiplatelet activity and to the global antithrombotic activity demonstrated with wine derived phenols¹³. Besides these types of experimental animal data and *in vitro* data on isolated platelets or leukocytes sustaining an antithrombotic of the wine phenolic compounds there are few experiments on *ex vivo* coagulation parameter profile either in healthy volunteers receiving a relatively short term (few days to few weeks) wine supplementation or epidemiological data relating wine intake and haemostatic, coagulation and/or thrombotic parameters.

These types of data have shown a decrease platelet reactivity correlated with the wine intake¹⁴⁻¹⁶. Taken together, all these elements suggest that the anti-atherothrombotic activity of wine derived phenols could result both of anti-atherosclerotic and antithrombotic activities.

The second type of difficulties and uncertainty about nutritional phenols is their absorption and bioavailability. Wine phenols are characterized by:

- their low solubility,
- their absorption which is only partial, variable, modified by interactions with food mainly with proteins¹⁷,
- and by their metabolism in the intestinal tract (conjugated in the intestinal mucosa catabolised by the colic bacterial flora¹⁸⁻²⁰).

Until now, non of the studies have taken into consideration these numerous and complex interactions.

One additional type of difficulties in experimental studies is due to the species specificity which are due not only to the physiological differences between the animal and human species but also from the health conditions of the animal, from the diet. Even so numerous studies have been conducted in various animal species. Animal models of atherosclerosis are either genetic or acquired models. Among genetic models one of the most representative are the mice knock out for the Apo E gene which develop hypercholesterolemic and arterial lesions overloaded with lipids which are not identical to human atherosclerotic models but are considered as representative²¹. It has been shown that these mice, fed with global wine extract, were *ex-vivo* characterized by decrease sensitivity of their LDL to oxidation²² and more interesting by a decrease in the atherosclerotic lesions they developed²³. Ourselves in a model of localized atherosclerotic reaction (Figure 1) triggered by a peri-adventitial stimulation by surgical application of a silastic collar around the carotid in the pig²⁴ we have shown that an important to moderate but chronically (3 months) consumption of red wine extract induced a trend toward an inhibitory effect against the development of a neo-intimal thickening in our acute model of adventitial stimulation of the pig carotid artery (data not published).

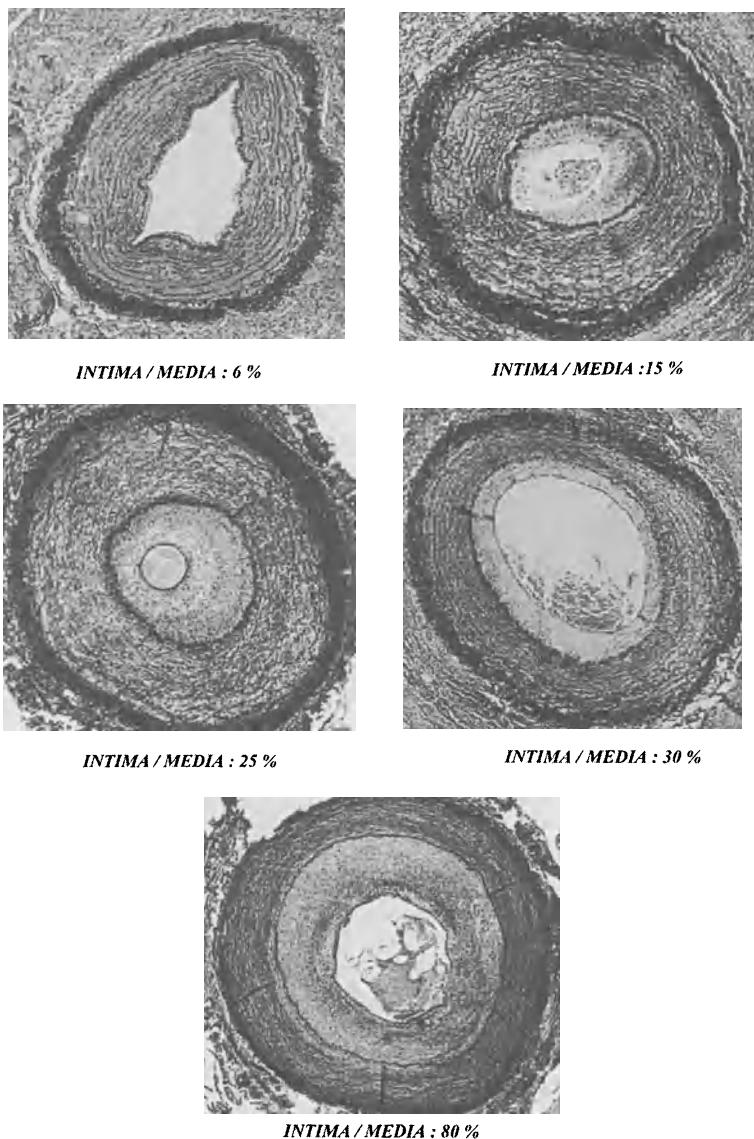


Figure 1. Intimal proliferation in pig carotid artery following chronic (1 month) peri adventitial stimulation by a silastic collar

This effect could be mediated by an effect on smooth muscle cell proliferation and was not accompanied by any significant effect on the thrombotic reactivity. The association of ethanol to the wine extract didn't influence significantly the effect of this phenolic extract on both neo-intimal thickening and thrombus development. But interestingly, the plasma titration of the catechins measured as an indicator of phenol absorption it was shown that alcohol accelerated the kinetics of absorption of catechins (as well from wine as the regular pig chow) but the alcohol adjunct did not increase the maximum plasma concentration. Besides the effect of the phenolic compounds of the wine extract, the effect of the glycerol which is another quantitatively significant constituent of wine mainly some type of white wine. The effect of the glycerol content appeared to be different from that of other phenolic components present in the wine extract since it reduced the number but not the size of the intimal lesions perhaps by a protective effect against the mechanisms involved (at endothelial level ?).

CONCLUSION

Even if numerous questions are still pending, polyphenols (and specially grape seed phenols) are already used and publicized as nutritional complements efficient in prevention of cardiovascular events.

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Chapter 8

ALCOHOL AND CARDIOVASCULAR DISEASES: EPIDEMIOLOGICAL STUDIES

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INTRODUCTION

There is hardly a month without the publication in one of the major medical journals of an article on alcohol. Depending on the subject, alcohol is presented either as a hard drug or as a protective factor. Beyond medicine, major commercial interests are at stake. Thus, it is not surprising to find numerous articles dealing with alcohol in newspapers and magazines. The newspapers tend to favour a moderate alcohol intake because of its particular psychological properties (loss of inhibitions). Important scientific surveys seemed to find positive relationships between moderate alcohol consumption and health. A great number of studies have been carried out in the cardiovascular field. These studies investigated the different aspects of cardiovascular diseases.

In this review, we are going to describe firstly the relationships between alcohol and different clinical events. Secondly, we will consider the complex relationships between alcohol and cardiovascular diseases in countries with low cardiovascular incidence such as in France.

ALCOHOL AND TOTAL MORTALITY

The relationship between overall mortality and alcohol is complex. The overall mortality curve is U-shaped or J-shaped¹⁻³. In other terms, moderate drinkers are relatively protected by a regular alcohol intake. Conversely, abstainers and heavy drinkers are at relatively high mortality risk. In men, the lowest risk is observed for an intake of 10 g/day. For women, the values of the protective intake are lower. For men, risk increases when values are around 40 g/day; in women, this risk appears when these values are around 20 g/day only.

ALCOHOL AND STROKE

The relationship between alcohol and stroke is particularly difficult to analyse since strokes correspond to a great number of clinical entities^{3,4}. Strokes can be categorised as ischaemic, lacunar, embolic or haemorrhagic. In other words, it seems more accurate to analyse the relationships between alcohol and strokes according to their types. A linear positive correlation between alcohol consumption and the risk for haemorrhagic stroke was found. Conversely, a J-shaped correlation was found between alcohol consumption and ischaemic strokes. Because of a relative number of haemorrhagic and ischaemic strokes³, the number of ischaemic strokes that are saved should be far greater than the number of haemorrhagic strokes that occur at mild to moderate alcohol consumption.

ALCOHOL AND BLOOD PRESSURE

The relationship between alcohol and blood pressure (BP) is difficult to analyse because of putative confounders. These putative confounders correspond to age, gender, body mass index, smoking, coffee, physical activity, type A/B behaviour and educational level. Even after adjustment for these putative confounders, the same estimate was found³. In fact, only randomised trials would be able to assess the relationship between alcohol and BP definitely. As for overall mortality or stroke, what kind of relationship can be found between alcohol intake and BP levels? More particularly, what are the effects of moderate and light alcohol consumption?

According to some authors there is a threshold phenomenon³. This threshold phenomenon would be around 30 g/day. For other authors, J-shaped relationships were found. In this case, lower BP levels were found in moderate alcohol drinkers. In fact, alcohol is not harmless to BP. It is admitted that above 30 g/day, an increment of 10 g/day increases systolic BP by 1 or 2 mm Hg and diastolic BP by 1 mm Hg.

ALCOHOL AND CORONARY HEART DISEASE

The relationship between alcohol and coronary heart disease (CHD) is particularly interesting to study since CHD is the main cause of death in industrialised countries. At light to moderate levels of alcohol consumption, CHD mortality decreases. At higher drinking levels, the number of non-coronary causes of cardiovascular deaths, that is to say cardiomyopathy, sudden death or haemorrhagic stroke increases. This complex relationship accounted for the U-shaped relationship of alcohol with cardiovascular death¹⁻³. In other words, alcohol has a protective effect against CHD, and cardiac events (cardiomyopathy, sudden death) or cerebrovascular events (haemorrhagic stroke) appear only when alcohol intake is high.

What are the plausible mechanisms by which alcohol reduces the risk of CHD? The mechanisms which contribute to the cardioprotective effect of alcohol will be discussed later. Among these mechanisms, some can be found at the haemostatic level; more recently, authors have been interested in phenolic substances in red wine. Actually, several works have shown an inhibition of oxidation of LDL associated with phenolic compounds in red wine. Despite all these very interesting mechanisms which should be taken into consideration, a majority of authors thinks that the protective effect of alcohol against CHD is mainly due to lipid variables^{3,5,6} (Tables 1 and 2).

Table 1. Effect of Total Alcohol Intake in Logistic Regression of Selected Predictors on Myocardial Infarction: The ECTIM Study, 1988-1991

Predictor variable*	Model 1		Model 2	
	Odds ratio	95 % CI†	Odds ratio	95 % CI†
Age (years)	1.31	1.12-1.53	1.34	1.14-1.56
Alcohol (g/day)	0.80	0.66-0.97	0.90	0.74-1.10
Smoking (cigarettes/day)	1.60	1.45-1.76	1.52	1.35-1.71
Body mass index‡	0.96	0.68-1.36	0.64	0.44-0.93
Hyperlipidemia	2.57	1.91-3.46	2.48	1.83-3.37
Diabetes mellitus	1.35	0.91-2.02	1.36	0.89-2.06
Hypertension	1.36	1.02-1.80	1.32	0.98-1.78
HDL† cholesterol (g/litre)			0.63	0.56-0.70

* Adjusted for study centre. Continuous variables were entered in the units noted. Odds ratios based on continuous variables were calculated for the following variations; 10 years of age, 50 g of alcohol/day, 10 cigarettes/day, 10 units of body mass index, and 0.10 g/litre of HDL cholesterol. Odds ratios based on dichotomous variables were computed using the following reference categories: dyslipidemia, no; diabetes, no; hypertension, no, † CI, confidence interval; HDL, high density lipoprotein, ‡ Weight (kg)/height² (m²)

Reproduced from Marques-Vidal *et al.* *Am. J. Epidemiol.* 1996⁵

Table 2. Effect of Wine-derived and Non-wine-derived Alcohol Intake in Logistic Regression of Selected Predictors on Myocardial Infarction: The ECTIM Study, 1988-1991

Predictor variable*	Model 1		Model 2	
	Odds ratio	95% CI†	Odds ratio	95% CI†
Age (years)	1.31	1.12-1.53	1.35	1.15-1.58
Alcohol (g/day)				
Wine	0.74	0.55-0.99	0.86	0.64-1.15
Non wine	0.82	0.67-0.99	0.90	0.74-1.10
Smoking (cigarettes/day)	1.60	1.45-1.76	1.52	1.35-1.71
Body mass index‡	0.96	0.68-1.37	0.67	0.46-0.97
Hyperlipidemia	2.58	1.92-3.47	2.48	1.83-3.37
Diabetes mellitus	1.36	0.91-2.03	1.36	0.89-2.07
Hypertension	1.36	1.02-1.81	1.32	0.98-1.78
HDL† cholesterol (g/litre)			0.63	0.56-0.70

* Adjusted for study centre. Continuous variables were entered in the units noted. Odds ratios based on continuous variables were calculated for the following variations; 10 years of age, 10 g of wine- or non-wine-related alcohol/day, 10 cigarettes/day, 10 units of body mass index, and 0.10 g/litre of HDL cholesterol. Odds ratios based on dichotomous variables were computed using the following reference categories: dyslipidemia, no; diabetes, no; hypertension, no, † CI, confidence interval; HDL, high density lipoprotein, ‡ Weight (kg)/height² (m²) - Reproduced from Marques-Vidal *et al.*, *Am. J. Epidemiol.* 1996⁵

Alcohol raises total HDL as well as both HDL₂ and HDL₃. In the relationship between alcohol and CHD, HDL cholesterol must not be considered as a confounding variable. In fact, HDL cholesterol is in the causal pathway between exposure and disease. To analyse the impact of HDL cholesterol on the relationship between alcohol and CHD, some studies could be chosen for demonstration: the LRC Study, the Honolulu Study, the MRFIT Study and the Boston Area Study³. The first three studies are cohort studies and the last one is a case-control study. In these four studies, when the relationship between alcohol and CHD is analysed, the relative risk is lower than 1 in favour of a protective effect of alcohol (relative risk: 0.80 in the LRC Study, 0.83 in the Honolulu Study, 0.89 in the MRFIT Study and 0.60 in the Boston Area Study). When HDL cholesterol is included in the multivariate analysis, the relative risk is close to 1. When the variation of the alcohol coefficient is calculated in percentage, the value approximates 50 % (55 % in the LRC Study, 47 % in the Honolulu Study, 45 % in the MRFIT Study and 60 % in the Boston Area Study). In other words, HDL cholesterol levels account for more than half of the causal relationship between alcohol and CHD.

ALCOHOL AND HAEMOSTATIC FACTORS

A great number of studies have studied the relationship between fibrinogen levels and alcohol consumption³. Eight studies out of twelve have shown an inverse association between alcohol and fibrinogen levels. In the last representative population survey carried out by the Toulouse MONICA Project, adjusted fibrinogen levels in men were 3.50 g/L in total abstainers *vs* 3.23 g/L in those who drink more than 80 g/day ($p < 0.01$). In women, adjusted fibrinogen levels were 3.62 g/L in total abstainers *vs* 3.18 g/L in those who drink more than 20 g/day ($p < 0.0001$).

Studies which have analysed the relationship between Factor VII and alcohol are very few. Two studies out of three reported an inverse association between Factor VII concentration or Factor VII activity and alcohol³. In recent studies, authors have put into evidence a positive association between alcohol and tissue-type plasminogen activator antigen. Lastly, platelets may play a significant role in the relationship between alcohol and cardiovascular disease. In the Caerphilly Heart Study, alcohol was inversely associated with the response of platelets to aggregation induced by collagen and ADP (not thrombin); those consuming a diet high in saturated fats were most affected by alcohol³.

WINE, BEER, SPIRITS AND CORONARY HEART DISEASE

When we analyse the previous chapters, an inverse association between moderate alcohol consumption and CHD seems to be well established. Men and women who drink one or two drinks a day have the lowest risk of CHD. The possible additional benefits of wine and especially of red wine have received a considerable attention in the media. Thanks to published epidemiological studies and a systematic and excellent review by Rimm *et al.* ⁷, we are going to review the respective benefits of the three mainly consumed alcoholic beverages: wine, beer and spirits.

ECOLOGICAL STUDIES (TABLES 3, 4)

Up to now, twelve ecological studies^{8,18} can be analysed in the light of the different patterns of alcohol consumption. The authors found a strong inverse association between average per capita consumption of wine and mortality from CHD. The inverse association was pronounced for wine in both men and women, less strong for spirits and non-existent for beer.

However, ecological studies have important limitations⁷. In some countries, a small proportion of the population may consume a large proportion of a specific type of alcoholic drink so that average per capita consumption may be an inaccurate representation. In other countries such as in France, average per capita consumption of wine may be more representative as it is drunk by most of the population. Furthermore, what may appear as a moderate or low level of consumption of spirits on the basis of per capita per day may mask excessive consumption by a small proportion of the population.

CASE-CONTROL STUDIES (TABLE 5)

At least five case-control studies^{5,6,19-21} have analysed the relationship between different types of alcohol and CHD. Taken together, these case-control studies did not suggest that one specific type of drink may be more cardioprotective. In a recent case-control study⁶, wine, beer and spirits drinkers were defined as those who consumed one half or more drinks per day with more than half of their consumption as wine, beer or spirits. Compared to non-drinkers, after adjustment for age and sex, reductions in risk of myocardial infarction were similar. Adjustment for total HDL cholesterol attenuated the protective effect in all groups.

Table 3. Ecological studies of consumption of specific types of alcoholic drink and correlation with mortality from heart disease

Study	Correlation				<i>Adjusted for meat consumption</i>	<i>Adjusted for age</i>
	Wine	Beer	Spirits			
St Leger <i>et al</i> 1979 ⁸ : per capita consumption (by county) v ischaemic heart disease mortality, 1970. Men and women aged 55-64 in 18 developed countries	Men -0.70 Women -0.61	Men 0.23 Women 0.31	Men -0.26 Women -0.32			
La Porte <i>et al</i> 1980 ⁹ : per capita consumption (by country) v coronary heart disease mortality, 1972. Men aged 55-64 in 20 countries	-0.62	0.15	-0.29			
Werth 1980 ¹⁰ : per capita consumption (by state) v coronary heart disease mortality, 1969-78. Men and women in USA	-0.49 to -0.58 (p<0.001)	Not given	Not given			
La Porte <i>et al</i> 1981 ¹¹ : per capita consumption (by state) v coronary heart disease mortality, 1970. Men and women in USA	White men 0.05 White women 0.17	White men 0.11 White women 0.09	White men 0.19 White women 0.24			
Schmidt <i>et al</i> 1981 ¹² : per capita consumption (by state) v coronary heart disease mortality, 1970. Men and women in USA	-0.28 (p<0.05)*	-0.06 (NS)*	-0.26 (p<0.05)*			
Nanji 1985 ¹³ : per capita consumption (by country) v ischaemic heart disease mortality, 1970. Men in 27 developed countries	-0.75 (p<0.001)	0.60 (p<0.0010)	No association			
Nanji <i>et al</i> 1986 ¹⁴ : change in per capita alcohol intake (by country) v change in ischaemic heart disease mortality, 1970-80. Men in 22 countries	-0.50 (p<0.01)	0.32 (p<0.05)	Not given			

* Interstate correlations - Reproduced from Rimm *et al. BMJ* 1996 7

Table 4. Ecological Studies of Consumption of Specific Types of Alcoholic Drink and Correlation with Mortality from Heart Disease

Study	Correlation			<i>Adjusted for saturated and polyunsaturated fat. Predictive equation almost identical when total alcohol used instead of each drink type</i>
	Wine	Beer	Spirits	
Hegsted 1988 ¹⁵ : per capita consumption (by country) v coronary heart disease mortality, 1971, 1973. Men in 18 countries	p<0.01 (inv.)	NS	NS	
Renaud et al 1992 ¹⁶ : per capita consumption (by country) v coronary heart disease mortality, 1987. Men and women aged 35-64 in 17 countries	-0.87 (p<0.0001)	NS	NS	<i>Adjusted for dairy/fat intake</i>
Artaud-Wild et al 1993 ¹⁷ : per capita consumption (by country) v coronary heart disease mortality, 1977. Men aged 55-64 in 40 countries	-0.16 (NS)			
Criqui et al 1994 ¹⁸ : per capita consumption (by country) v coronary heart disease mortality, 1965, 1970, 1980, 1988. Men and women aged 35-74 in 21 countries	1965 p=0.07 (inv.) 1970 p>0.01 (inv.) 1980 p>0.01 (inv.) 1988 p=0.12 (inv.)	NS p=0.09 (inv.) NS NS	NS NS NS	<i>Total alcohol significantly correlated with mortality for all countries ($r=0.35$) but significantly inversely correlated with mortality in countries with high cholesterol and saturated fat intake</i>
La Porte et al 1980 ¹⁹ (time trend analysis): per capita consumption (by state across years) v coronary heart disease mortality, 1950-75. Men and women in USA	-0.41	-0.61	-0.07	<i>After incorporating 5 year lag, change in consumption of beer and cigarettes were strongest predictors of changes in mortality</i>

Reproduced from Rimm et al. BMJ 1996⁷

Table 5. Case-control studies of consumption of specific types of alcoholic drink and relative risk of coronary heart disease

Study	Wine			Beer			Spirits			<i>Adjusted for:</i> cigarette smoking, previous hospitalisation for congestive heart failure, religion, and relative weight
	Total alcohol (oz/day)	RR*	Total alcohol (oz/day)	RR*	Total alcohol (oz/day)	RR*	Total alcohol (oz/day)	RR*	Total alcohol (oz/day)	
Hennekens <i>et al</i> 1979 ¹⁹ : 1136 men aged 30-70 (568 died from coronary heart disease, 568 controls) in Boston, USA	0 ≤ 2 > 2	1.0 0.3 (p<0.001) 1.0	0 ≤ 2 > 2	1.0 0.3 (p<0.001) 1.0	0 ≤ 2 > 2	1.0 0.2 (p<0.001) 1.1	0 ≤ 2 > 2	1.0 0.2 (p<0.001) 1.1	0 ≤ 2 > 2	<i>Results not materially altered when crude odds ratio adjusted for:</i> <i>confounders. Women who drank more than one drink type had relative risk of 0.6</i>
Rosenberg <i>et al</i> 1981 ²⁰ : 1431 women aged <50 (513 with myocardial infarction, 918 hospital controls) in Boston, New York, and Philadelphia, USA	None Only drink wine	1.0 0.4 (p<0.001)	None Only drink beer	1.0 0.8	None Only drink beer	1.0 0.8	None Only drink spirits	1.0 0.9	None Only drink spirits	
Kaufman <i>et al</i> 1985 ²¹ : 3151 men aged <55 (2170 with non-fatal myocardial infarction, 981 hospital controls) in north-eastern USA	0 < 5 5-9 10-19 ≥ 20	1.0 1.2 1.8 2.4 ≥ 20	0 < 5 5-9 10-19 ≥ 20	1.0 1.3 1.1 1.2 1.1	0 < 5 5-9 10-19 ≥ 20	1.0 1.3 1.1 1.2 1.1	0 5-9 10-19 ≥ 20	1.0 1.1 1.3 1.7 1.0	0 5-9 10-19 ≥ 20	<i>Adjusted for:</i> <i>age and smoking</i>

* Relative Risk - 1 oz alcohol = 28.3 g - Reproduced from Rimm *et al. BMJ* 1996⁷

COHORT STUDIES (TABLES 6-8)

Eleven separate cohort studies²²⁻³⁵ have provided results on risk of CHD in association with specific types of alcoholic drink. Five studies reported a significant inverse association between wine consumption and CHD, five reported such an association for beer, and five reported an association for spirits. Two studies found wine to have the strongest inverse association with CHD, none found this for beer, and two found it for spirits. The significance for any single type of drink was partly a function of the distribution of intake of different drinks in the population studied⁷. In short, almost all cohort studies found a strong inverse association between total alcohol intake²³⁻²⁷ and CHD, but no consistent pattern has emerged for specific types of drink.

ALCOHOL IN LOW-RISK POPULATION FOR CORONARY HEART DISEASE: THE EXAMPLE OF FRANCE

The French paradox

Despite a high level of risk factors (total cholesterol, hypertension, smoking, a high intake of saturated fat), the incidence of CHD is lower in France than in USA or in Northern European countries. A large number of scientific works¹⁶⁻¹⁸ has been undertaken to study this contrast between risk factors, similar to those found in the neighbouring countries, and the particularly low CHD mortality rates, and has been largely debated in the mass media. For instance, the following sentence was written in the Sunday Times: "*An international study revealed that plump burghers from the Toulouse area of France, despite tucking into daily four-course meals dripping with foie gras, butter, cream and other cholesterol-laden delicacies, were four times less likely to have a heart attack than their more*

Table 6. Cohort studies of consumption of specific types of alcoholic drink and relative risk of coronary heart disease

Relative Risk (95% Confidence interval) - ** Consumption of alcohol calculated as consumption of each drink type without conversion to alcohol. In this population consumption of wine and spirits was small compared with beer: average monthly consumption was 1-2 glasses of wine, 1-2 glasses of spirits, and 2±12 oz glasses of beer - † Relative risk (age adjusted) derived from rates of coronary heart disease for each category of alcohol consumption divided by rate for abstainers - Reproduced from Rimm *et al*, BMJ 1996

Table 7. Cohort studies of consumption of specific types of alcoholic drink and relative risk of coronary heart disease

Study	Wine			Beer			Spirits		
	drinks/day† in non- smokers	RR* (p=0.07)	drinks/day† in non- smokers	RR* (p=0.15)	drinks/day† in non- smokers	RR* (p=0.21)	drinks/day† in non- smokers	RR* (p=0.21)	drinks/day† in non- smokers
<i>Friedman et al 1986²⁷:</i> 2016 men aged 30-59 (24 year follow up) in Framingham heart study									
Stampfer et al 1988 ²⁸ : 87526 women aged 34-59 (200 with fatal or non-fatal myocardial infarction in 4 years follow up) in nurses' health study	0 <5 ≥5	1.0 0.9 0.4	0 <5 ≥5	1.0 0.3 1.0	0 <5 ≥5	1.0 1.1 0.7	0 0.7 (0.4 to 1.3)	1.0 1.1 (0.7 to 1.8)	alc. (g/day)
Rimm et al 1991 ²⁹ : men aged 40-75 (350 with fatal or non fatal heart disease or revascularisation in 2 years follow up) in health professionals follow up study	0 2 (0.64 to 1.50)	1.0 0.98 (0.54 to 1.18)	0 2 (0.54 to 1.18)	1.0 0.80 (0.54 to 1.18)	0 2 (0.54 to 1.18)	0 2 (0.39 to 0.77)	alc. (g/day)	alc. (g/day)	drinks/day‡:
									drinks/day‡:
									drinks/day‡:

Adjusted for systolic blood pressure, serum cholesterol conc., haemoglobin conc., left ventricular hypertrophy, relative weight

Adjusted for family history of coronary heart disease, menopause, hormone replac. therapy, age, smoking, body mass index, hypertension, high cholesterol conc., exercise, intake of dietary fat and cholesterol

Adjusted for family history of coronary heart disease, smoking, age, body mass index, profession, diabetes, hypertension, high cholesterol concentration, intake of dietary fat, fibre, and cholesterol

* Relative Risk (95% Confidence interval) - † Alcohol content per drink was defined as 4 oz for wine (16.75% alcohol), 8 oz for beer (5% alcohol), and 2 oz for spirits (50% alcohol). We calculated relative risks from published (coefficients from a Cox proportional hazards models which accounted for all three drink types simultaneously - ‡ Drinks of each type defined as a 4 oz glass of wine (10.8g alcohol), a 12 oz glass of beer (13.2g alcohol), and a shot of spirits (15.2g alcohol) - Reproduced from Rimm et al. BMJ 1997

Table 8 Cohort Studies of consumption of specific types of alcoholic drink and relative risk of coronary heart disease

Study	Wine		Beer		Spirits	
	RR*	RR*	RR*	RR*	RR*	RR*
Quintiles of mean alc. intake (g/d)						
Farchi <i>et al</i> 1992 ³⁰ ; 1563 men aged 45-64 (166 died from cardiovascular disease in 15 years follow up) in Italian rural cohort study	22.7	1.0				
	56.4	0.77 (0.34 to 1.76)				
	77.8	0.67 (0.29 to 1.58)	Adjusted for: age, smoking, and occupation. Men with prevailing cardiovascular disease at baseline were excluded			
	108.2	1.31 (0.64 to 2.66)				
	164.7	1.61 (0.79 to 3.31)				
drinks/w [†]						
Klatsky <i>et al</i> 1992 ¹⁹⁹³ , 1990, 1986 ³¹⁻³³ ; 129170 men and women (600 died from coronary heart disease in 7 years follow up) in Kaiser Permanent study	< 2	1.0	< 2	1.0	< 2	1.0
	≥ 2	0.5 (0.4 to 0.7)	≥ 2	0.7 (0.5 to 0.9)	≥ 2	0.6 (0.5 to 0.8)
drinks/w [†]						
Gronbaek <i>et al</i> 1998 ³⁴ ; 7217 women and 5633 men aged 30-70 (1119 died from coronary heart disease in 12 years follow up) in Copenhagen city heart study	Never	1.0	1.0	1.0	1.0	1.0
	Monthly	0.69 (0.62 to 0.77)	0.79 (0.69 to 0.91)	0.79 (0.85 to 1.06)		
	Weekly	0.53 (0.45 to 0.63)	0.87 (0.75 to 0.99)	1.08 (0.93 to 1.26)		
	1-2/day	0.47 (0.35 to 0.62)	0.79 (0.68 to 0.91)	1.16 (0.98 to 1.39)		
	3-5/day	0.44 (0.24 to 0.80)	0.72 (0.61 to 0.88)	1.35 (1.00 to 1.83)		
Beverage type (continuous variable)						
Klatsky <i>et al</i> 1997 ³⁵ ; 128934 men and women (3931 persons hospitalised for coronary disease) in Kaiser Permanent study	0.8 (p<0.01)	0.7 (p<0.001)	0.9 (p<0.001)	0.9 (p<0.001)	0.9 (p<0.001)	0.9 (p<0.001)
Adjusted for: age, sex, race, body mass index, smoking, marital status, education, coffee use and baseline coronary risk						

* Relative Risk - [†] The amount of alcohol per drink was not defined - [‡] Drinks defined to contain 12 g alcohol on average - Reproduced from Rimm *et al. BMJ* 1996⁷ and Klatsky *et al. Am. J. Cardiol.* 1997⁵

ascetic counterparts around Belfast", and in Newsweek: "*One reason the inhabitants of Toulouse, France, (big cheese-lovers), have only half the incidence of heart disease of their compatriots in Lille and Strasbourg may be the difference in the dairy fats they consume*". In fact, the French paradox is a very complex subject since it implies not only classical risk factors but also far less studied risk factors such as nutritional or behavioural factors (physical activity or socio-economic status). It is thus interesting to study epidemiological surveys carried out in France.

Alcohol and death in a cohort study in Eastern France

Between January 1978 and December 1983, Renaud *et al.*³⁷ have collected data from 34014 consecutive French patients, aged 40 to 60 years, who had reported their consumption of alcoholic beverages and their smoking habits. In 1993, ten to fifteen years after their first visit, they evaluated the vital status for all subjects. The cohort experienced 418068 person-years of follow-up through 1993. They obtained cause of death information for only 1529 out of the 2642 men who died. Cox proportional hazards model was used to estimate the relative risk when adjusting for age, education, smoking, systolic BP, serum total cholesterol and body mass index.

Most of the subjects (77 %) drank wine and wine constituted 82 % of the total alcohol consumption. Beer represented 11.6 % of the total alcohol intake and aperitifs and liquors, 6.4 %. Aperitifs and liquors were consumed by 67 % of the men. Beer was consumed by 28 % of the subjects, mostly in addition to wine.

The crude death rate by total alcohol showed a J-shaped curve (Table 9). When adjusted for possible confounders, an intake of 1 to 76 g/day of alcohol was associated with an 18 % to 30 % lower all cause mortality rate and by contrast, the authors found a 40 % increase in relative risk with a daily intake of alcohol of more than 128 g/day.

Both cardiovascular diseases and coronary heart disease (Table 10) show similar patterns in relation to alcohol intake that is to say a decreased risk (from 27 to 39 %) at any level of alcohol higher than 22 g/day. By contrast for cancer mortality (Table 11), the authors found a J-shaped curve. Violent death was increased in each category of alcohol, but it did not increase in relation to the amount of alcohol. The authors³⁷ conclude that a moderate intake of alcohol, mostly in the form of wine, seems to protect from death, not only from CHD and cardiovascular diseases but also from

other causes. This study seems to confirm the speculation that the so-called "French paradox" is due, at least in part, to a regular consumption of wine.

Table 9. Crude and adjusted death rates for men (ages 40-60 years) according to total alcohol intake

Alcohol intake (g/day)	Men (N)	Deaths (N)	Crude Rate/1000 Person-years	Adjusted Relative Risk*	
				RR	95 % CI
0 - Occasionally	3748	298	6.61	1.00	Referent
1 - 21	3277	198	4.92	0.80	0.67-0.96
22 - 32	5362	298	4.49	0.70	0.59-0.82
33 - 54	9034	578	5.17	0.76	0.66-0.87
55 - 76	3208	231	5.84	0.82	0.69-0.98
77 - 128	7665	746	7.91	0.94	0.82-1.08
> 128	1720	293	14.21	1.37	1.16-1.61
Total	34014	2642	6.32		

* Evaluated by Cox proportional hazards model, adjusted for age, education, smoking, systolic blood pressure, serum total cholesterol, and body mass index

Reproduced from Renaud *et al.* *Epidemiology* 1998⁷

Alcohol and carotid intima-media thickness in a representative sample

CHD risk factors have been consistently related to an increase in carotid intima-media thickness (IMT) in selected populations. However, few studies were population-based and furthermore little attention has been given to the influence on CHD risk factors of IMT in low-risk populations for CHD. We therefore examined the association between carotid IMT and CHD risk factors in a large ($n = 1013$) and representative sample of middle-aged men and women in one of the European populations with the lowest CHD risk³⁸. Alcohol consumption was quantified in grams of alcohol per day with a 7-day recall method. A high-resolution B-mode ultrasonography of the common carotid arteries was performed. The IMT was measured at 6 points on each side, on the far wall exclusively, which made altogether 12 sites on the common carotid artery. The means at the 12 sites were combined to produce an overall mean common carotid IMT. Forward multiple regression

Table 10. Adjusted relative risk for death from selected causes, according to alcohol intake

Alcohol intake (g/day)	Cardiovascular Diseases			Coronary Heart Disease		
	N	Relative Risk*	95% confidence interval	N	Relative Risk*	95% confidence interval
0 - Occasionally	54	1.0	Referent	37	1.0	Referent
1 - 21	39	0.83	0.55-1.25	29	0.89	0.54-1.44
22 - 32	52	0.65	0.45-0.95	36	0.65	0.41-1.03
33 - 54	105	0.72	0.52-1.00	71	0.70	0.47-1.04
55 - 76	39	0.73	0.48-1.10	25	0.66	0.40-1.11
77 - 128	106	0.67	0.48-0.94	67	0.61	0.41-0.92
> 128	32	0.75	0.48-1.18	19	0.65	0.37-1.15
Total	427			284		

* Evaluated by Cox proportional hazards model, adjusted for age, education, smoking, serum total cholesterol, systolic blood pressure and body mass index. Cause of death could be obtained for only 1529 men - Reproduced from Renaud *et al.* *Epidemiology* 1998

Table 11. Adjusted relative risk for death from selected causes, according to alcohol intake

Alcohol intake (g/day)	All Cancers			Violent Death		
	N	Relative Risk*	95% confidence interval	N	Relative Risk*	95% confidence interval
0 - Occasionally	75	1.0	Referent	13	1.0	Referent
1 - 21	47	0.78	0.54-1.13	16	1.37	0.66-2.86
22 - 32	86	0.81	0.59-1.11	22	1.19	0.60-2.36
33 - 54	177	0.93	0.71-1.22	34	1.05	0.56-2.0
55 - 76	68	0.98	0.71-1.37	21	1.77	0.88-3.57
77 - 128	257	1.32	1.02-1.72	35	1.19	0.62-2.27
> 128	85	1.64	1.20-2.26	10	1.43	0.62-3.32
Total	795			151		

* Evaluated by Cox proportional hazards model, adjusted for age, education, smoking, serum total cholesterol, systolic blood pressure and body mass index. Cause of death could be obtained for only 1529 men - Reproduced from Renaud *et al.* *Epidemiology* 1998

was used to examine which variable was best at explaining IMT. In forward multiple regression in men, age, smoking, systolic BP, HDL cholesterol, alcohol and the interaction between age and alcohol were independently associated with IMT ($R^2 = 0.33$).

The role of alcohol in the IMT predictive model in men must be considered in the light of the relationship between alcohol and atherosclerosis. Anyhow, in our IMT predictive model, we have shown the interaction between age and alcohol consumption. This stresses the fact that alcohol cannot prevent the consequences of ageing on atherosclerosis, that is to say the combined effect of other risk factors, measured or not. Finally, a regular alcohol consumption may also indicate a different nutritional behaviour. A regular and moderate alcohol consumption is associated in France with a regular fruit and vegetable consumption, rich in antioxidant vitamins and in polyphenols such as red wine.

Types of alcoholic beverages and lipid levels in 3224 individuals

The aim of this study was to examine the relationship between lipid levels and consumption of different types of alcoholic beverages among 3224 individuals (1630 men and 1594 women)³⁹. The MONICA project was an international ten-year program co-ordinated by the World Health Organisation. Its aim was to monitor CHD deaths, myocardial infarction, coronary care and cardiovascular risk factors. In France, 3 MONICA collaborative centres were created: one in the North (Lille), one in the East (Strasbourg) and one in the Southwest (Toulouse). These centres conducted 3 representative cross-sectional surveys in 1994-1996. Alcohol consumption and types of alcoholic beverages (wine, beer, aperitif, liquor, cider) were quantified in grams of alcohol per day with a 7-day recall method.

In men, the average alcohol consumption was 31.6 g/L and the median consumption was 24 g/L (Table 12). In women, the average alcohol consumption was 9.4 g/L and the median consumption was 3.8 g/L. In men, the average wine consumption was 21.2 g/L and the average beer consumption was 6.9 g/L. In women, the average wine consumption was 6.3 g/L and the average beer consumption was 1.8 g/L. It was interesting to study the different drinking patterns in men in the different regions in France (Table 13). Mean daily grams of alcohol was 26.8 g/day in the South of France and 38.7 g/day in the North of France. Moreover, wine represented

83.3 % of alcohol consumption in the South whereas it represented 53.7 % in the North. Beer represented 27.2 % of alcohol consumption in the North of France. When alcohol intake was analysed in the different regions (Table 14), striking differences appeared. In men, 53 % of the subjects who drank no alcohol at all were in the South of France. Conversely, 54 % of the male subjects with an excessive alcohol intake (> 80 g/day) were in the North.

Table 12. Mean and median daily grams of alcohol consumed in three French representative surveys

	Men (n=1630)		Women (n=1594)	
	Mean	Median	Mean	Median
Alcohol	31.6	24	9.4	3.8
Wine	21.2	13.7	6.3	1.7
Beer	6.9	0	1.8	0
Aperitif	3.2	1.0	1.2	0
Cider	0.1	0	0	0
Liquor	0.4	0	0.1	0

Table 13. Drinking pattern in men in different regions in three French representative surveys

	North (n=556)	East (n=473)	South (n=601)
Mean daily grams of alcohol consumption	38.7	29.5	26.8
% Wine	53.7	63.6	83.3
% Beer	27.2	26.9	4.0
% Aperitif	18.3	7.7	12.5

Table 14. Drinking pattern in men in different regions in three French representative surveys

	Daily grams of alcohol consumption				
	None (n=251)	<20 (n=477)	20-40 (n=394)	40-80 (n=395)	≥ 80 (n=113)
North	25%	31%	35%	38%	54%
East	23%	33%	32%	29%	19%
South	53%	37%	34%	33%	27%

A series of multivariate analysis was carried out after adjustment for age, centre, income, educational level, smoking, physical activity, waist-to-hip ratio, heart rate, BP, hematocrit and glycaemia (Table 15). In men, an increasing daily alcohol intake was associated with higher levels of triglycerides, from 1.36 mmol/L to 1.55 mmol/L ($p < 0.05$). Conversely, HDL cholesterol levels increased significantly when alcohol consumption increased, from 1.20 mmol/L to 1.50 mmol/L ($p < 0.0001$). In men, a significant increase was also observed for apolipoprotein A1 levels when alcohol consumption increased. Similar trends were observed for HDL cholesterol and apolipoprotein A1 levels in women.

Table 15. Daily grams of alcohol consumption and adjusted* lipid levels in men in three French representative surveys

	Daily grams of alcohol consumption					
	None (n=251)	<20 (n=477)	20-40 (n=394)	40-80 (n=395)	≥ 80 (n=113)	p
Total cholesterol [†]	5.72	5.84	5.86	5.94	6.17	<0.01
Triglycerides [†]	1.36	1.33	1.35	1.42	1.55	<0.05
HDL cholesterol [†]	1.20	1.29	1.34	1.38	1.50	<0.0001
LDL cholesterol [†]	3.90	3.95	3.91	3.92	3.96	NS
Apolipoprotein B [‡]	1.26	1.25	1.26	1.27	1.32	NS
Apolipoprotein A1 [‡]	1.52	1.60	1.64	1.68	1.79	<0.0001

* Adjustment for age, centre, income, educational level, smoking, physical activity, waist-to-hip ratio, heart rate, blood pressure, hematocrit, glycaemia - † mmol/L - ‡ g/L

Men who were regular alcohol drinkers were assigned to three groups: men who drank only wine, men who drank alcohol but no wine, and men who drank both wine and other alcohols (Table 16). Multivariate analyses were carried out after adjustment for the same confounding factors as in the previous analysis and for total alcohol daily consumption. This analysis showed higher triglycerides levels in the group which drank alcohol but no wine when compared to the groups which drank wine (1.58 mmol/L vs 1.44 or 1.37 mmol/L, $p < 0.05$). HDL cholesterol levels were significantly lower ($p < 0.05$) in the group which drank no wine when compared to the groups which drank wine (1.25 mmol/L vs 1.34 mmol/L). When the drinking patterns were analysed according to total alcohol consumption in French male drinkers, valuable information was obtained. Indeed in men, when alcohol consumption increased, wine intake remained predominant; but, a

significant increase in other alcohol intake was observed too. In conclusion, wine consumption was associated with potential protective effects. However, among men who consumed alcohol in France, 92 % drank wine, so the meaning at a population level of the results found in the non-wine drinking group remains speculative.

Table 16. Drinking pattern and adjusted* lipid levels (mmol/L) in male drinkers in three French representative surveys

	Wine only (n=221)	Alcohol without wine (n=105)	Other drinkers (n=1053)	p
Total cholesterol	5.94	5.79	5.91	NS
Triglycerides	1.44	1.58	1.37	<0.05
HDL cholesterol	1.34	1.25	1.34	<0.05

* Adjustment for age, centre, income, educational level, smoking, physical activity, waist-to-hip ratio, heart rate, blood pressure, hematocrit, glycæmia and total alcohol consumption (0-20 g/d; 20-40 g/d; ≥40 g/d)

Catechin in the Mediterranean diet

Flavonoids from vegetable and fruit intake appears to be inversely related to CHD mortality. Flavonoids from red wine have been shown to strongly inhibit low density lipoprotein oxidation both *in vitro* and *in vivo* but also to reduce platelet aggregation *i.e.* significant steps in reducing CHD mortality. In the same way, (+)-catechin, a naturally occurring flavonoid, has been demonstrated to prevent human plasma oxidation and to inhibit oxidation of low density lipoprotein. Thus, flavonoids may partly explain the protective effects of the Mediterranean diet rich in vegetable, fruit and wine against CHD. The first step in evaluating this hypothesis is to determine to what extent each of these foodstuffs contributes to antioxidant flavonoid levels in the blood. The aim of this study was to determine which type of diet contributes most to plasma concentration of (+)-catechin⁴⁰.

A cross-sectional study was carried out from June 1996 to April 1997. A sample of 1183 men and women aged 35 to 65 years and living in the region of Toulouse (Southwest of France), an area with low CHD mortality and morbidity rates, was recruited. The sample was selected at random from the polling lists available in each town hall. Response rate reached 60 % of the people contacted. Subjects were screened in a health centre administered by the social security system. From the initial sample of 1183 people, 182 (15.4 %) consecutive subjects (100 men and 82 women) were included in the

specific study to assess (+)-catechin concentration in plasma in relation to dietary patterns.

Subjects were asked to have their evening meal between 7 p.m. and 11 p.m. They were asked not to eat or drink (except water) after 11 p.m. and to comply with a minimum fasting period of 10 hours before the blood collection on the next morning between 8 and 10.30 a.m. Subjects were instructed not to change anything in their nutritional habits. The average fasting period was 11 hours and 55 minutes (ranging from 10 to 15 hours). Food intake was assessed using a food recall method to test the relationship between dietary catechin intake and (+)-catechin concentration in plasma. After blood collection, a qualified dietitian interviewed subjects concerning their last evening meal. A blood sample was collected after a 10 hour fasting period and (+)-catechin measurement in plasma was performed by high-performance liquid chromatography method using fluorescence detection. Furthermore, identification of catechin in plasma by Mass spectrometer was realised after collection of the HPLC peak, dry evaporation and adjustment for methanol.

Taking fruit, vegetable and wine consumption into account, four types of diet were identified as shown below:

Diet	Vegetable	Fruit	Wine
I	0	0	0
II	+	+	0
III	0	0	+
IV	+	+	+

Diets III and IV contained wine while diet I and II were characterised by no wine intake. The average consumption of red wine represented 87.5% of the total wine intake, whereas white and rosé wines represented 2.9 % and 9.6 % of the total wine consumption respectively. Diet II and IV included vegetable or/and fruit whereas diet I and III did not. Diet I was characterised by no fruit, no vegetable and no wine intake. Vegetable, fruit and wine were included in diet IV.

The figure shows mean plasma concentration of (+)-catechin according to different types of diet. The differences found in (+)-catechin concentrations in plasma between the four diets were statistically significant (overall test $p < 0.001$). The highest concentrations were found in diets III (577.4 $\mu\text{g/L}$) and IV (673.4 $\mu\text{g/L}$) and the lowest in diet I (133.9 $\mu\text{g/L}$). Diet II had an intermediate plasma level (456.1 $\mu\text{g/L}$). After adjustment for age,

sex, smoking, body mass index, waist-to-hip ratio and total energy intake, the lowest concentration of (+)-catechin in plasma was found in diet I (131.6 µg/L). (+)-Catechin level was 3-fold higher in plasma when vegetable and fruit (449.5 µg/L) were consumed (diet II) and 4-fold higher when only wine (598.5 µg/L) was consumed (diet III). When consumption of vegetable, fruit and wine was combined, the (+)-catechin concentration had the most elevated level (637.1 µg/L). When plasma concentration of (+)-catechin was based on MJ energy intake, differences between the four diets were smaller. Plasma concentrations of (+)-catechin were: 125.8 µg/L, 209.1 µg/L, 190.1 µg/L and 225.2 µg/L in diets I, II, III and IV respectively.

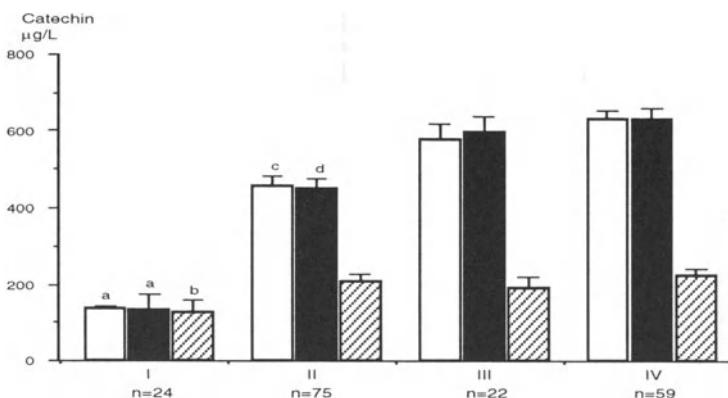


Figure 1 Plasma concentration of (+)-catechin according to four types of diet among 180 subjects (means with SEM)

- Crude values, (ANOVA analysis, overall test, $p<0.001$)
- Adjusted for age, sex, smoking habits, body mass index, waist-to-hip ratio and total energy intake (ANCOVA analysis, overall test, $p<0.001$)
- Plasma concentration of (+)- catechin based on MJ consumed, adjusted for age, sex, smoking habits, body mass index and waist-to-hip ratio (ANCOVA analysis, overall test, $p<0.001$, after log transformation)

I - without vegetable, fruit and wine - II - with vegetable and fruit - III - with wine and without vegetable and fruit - IV - with wine, vegetable and fruit

^a Significantly different from corresponding bar of diet II, III and IV, $p<0.001$ (Scheffe test)

^b Significantly different from corresponding bar of diet II, IV, $p<0.001$ and III, $p<0.05$ (Scheffe test)

^{c, d} Significantly different from corresponding bar of diet IV, ^c $p<0.001$, ^d $p<0.01$ (Scheffe test)

This study demonstrates that in subjects consuming Mediterranean foodstuffs, the highest concentration level of (+)-catechin in plasma was

observed when wine, fruit and vegetable were consumed. Among these vegetal foodstuffs, red wine appears to be the most effective to produce this effect in a sample of free-living population in the South of France. If, as reported, antioxidant flavonoids, especially catechin, have a significant protective effect against CHD, red wine and some fruit and vegetables, owing to their flavonoids, may provide the highest protection among all the Mediterranean foodstuffs which have been tested.

DISCUSSION

Very few subjects in cardiovascular epidemiology are so well documented as the relationship between alcohol and cardiovascular diseases³. A great number of results from descriptive, case-control and cohort studies are available. The epidemiologist's dream at the end of this review would be a randomised study. Its aim would not be the analysis of the effects of the different types of alcohol on biological parameters but it would be aimed at assessing the long-term effect of the different types of alcohol consumption on cardiovascular events. For ethical reasons, it is highly improbable or even not desirable that such randomised trials should be carried out.

A great number of studies have tried to understand mechanisms from which originated the protective effect of alcohol. First, it is highly probable that HDL cholesterol levels which are higher in regular drinkers mainly induce this protective effect. Other mechanisms than its effects on HDL levels have been proposed to explain the other half of the protective effects of alcohol against CHD. More particularly, other important mechanisms could be the inhibition of platelet aggregation, the reduced plasma fibrinogen levels, the increase in fibrinolytic activity, the inhibition of oxidation of LDL associated with phenolic compounds in red wine. The main conclusion drawn from these analyses is that moderate alcohol intake protects against CHD.

The problem which is not solved so far is whether the different types of alcohol have a differential effect on CHD^{5-7,36-40}. This question is extremely complex. Indeed, statistical analyses are liable to multiple confounding factors. These confounding factors may be attributable to the differences in diets, physical activity or socio-economic levels. The drinking patterns differ between countries. In a collaborative work between the French and the Northern Irish MONICA centres⁴ it was not difficult to show

that the drinking behaviours of the two populations differed in many respects. In France, alcohol consumption was regular and wine consumption was predominant. In Northern Ireland, alcohol consumption was concentrated during the weekend and beer consumption prevailed. Can these distinct drinking patterns account for the differences in CHD occurrence ?

In the light of epidemiological studies analysed in this review, it appears that ethanol provides the best protective effect against CHD. It seems also likely that some surveys have omitted to analyse important confounding factors. It is difficult to record correctly data on socio-economic and nutritional factors or on physical activity. At the moment, no clear conclusion has been drawn concerning the differences between wine and beer consumption^{3,7}. We can postulate that phenolic substances that is to say flavonoid and non-flavonoid phenolic compounds, flavonols, anthocyanins and tannins, abundant in red wine, may partly explain its protective effect. These added effects that may be attributed to wine properties are quite difficult to put into evidence in a sample greater than 1000 subjects. Nevertheless, it would be very accurate for the future to collect the maximum of socio-economic and environmental variables to carry out a perfect statistical analysis enabling a non-ambiguous discussion on the differential effects of wine, beer and spirits.

CONCLUSION

Moderate alcohol intake, whatever its origin, is a protective factor against cardiovascular disease. At heavy dose, it becomes dangerous, even for cardiovascular disease. This message is very difficult to convey in terms of Public Health. Indeed, we are all aware that the average alcohol consumption in the population is closely connected to the percentage of heavy drinkers. This is why it would not be wise to promote alcohol consumption in the population. We must not forget that alcohol is highly responsible for a great number of violent deaths or traffic road accidents. Consequently, public health messages must respect moderate drinkers and not incite abstainers to start a potentially deleterious alcohol intake.

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Chapter 9

POLYPHENOLS AND CANCER PREVENTION

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INTRODUCTION

Cancer prevention must be a leading public health objective, if we are to reduce cancer morbidity and mortality in the world. Cancer chemoprevention is defined as the use of chemical agents to prevent cancer and eliminate or reduce preinvasive, intraepithelial, precursor lesions that progress to invasive cancer. There are several thousand single agents and a large number of defined and undefined mixtures in the literature which either have epidemiological evidence of preventing cancer, have been shown to prevent cancer in experimental animals, to prevent cancer-related endpoints or to exhibit activity either *in vivo* or *in vitro* that are mechanistically related to cancer prevention.

The selection and prioritization of agents from initial *in vitro* screening to final human clinical trials is an applied science problem with major implications¹⁻⁵. Drug discovery and development efforts to identify the best chemical agents which have the greatest potential to make

significant reductions in human cancer incidence is a key strategies to make rapid progress in the most efficient manner possible. It is only in the last several years that published clinical trials using cancer chemoprevention strategies are effective have appeared in the literature. There are published results of a human trial where 13-cis-retinoic acid prevented the recurrence of cancer in patients with prior head and neck cancer⁶. The 83 % reduction in new cancers with treatment represented 10 patients who were cancer free at the end of this small trial. Since then there have other published reports of human cancer preventing activity for Sulindac in colon⁷, all-*trans*-retinoic acid for cervical cancer⁸. Tamoxifen for breast cancer⁹, and 13-*cis*-retinoic acid oral mucosa^{10,11}.

Strategies to develop chemopreventive drugs for human use have been evolving for over 15 years^{1,4,12}. The NCI Chemoprevention Program has developed as a linear array strategy by which agents enter and flow through the system in stepwise pattern. This developmental plan starts with drug discovery and ends with FDA New Drug Approval. Agents can enter the system at any point, depending upon the amount, type, and quality of information available¹³. Currently there are over 30 agents in clinical trials sponsored by our program and over 500 agents in the preclinical testing phase at NCI.

GRAPE PHYTOCHEMICALS AND CHEMOPREVENTION

Epidemiological evidence has linked decreased cancer risk to increased consumption of phytochemicals and lignans in a vegetarian diet¹⁴. NCI's "5-A- Day" effort has the goal of increasing public awareness of the importance of eating 5 or more servings of fruits and vegetables every day for better health. The scientific basis of the 5-A-Day concept comes from the over 150 epidemiologic studies which demonstrated that people eating 5 or more servings of fruits and vegetables per day had a relative risk of digestive and respiratory cancer 0.5 compared to populations consuming 2 or less servings per day.

Chemicals found in grapes, including antioxidant, antimutagenic have demonstrated various biological activities relevant to chemoprevention, signal transduction, and antiinflammatory activity¹⁵⁻¹⁷. Jang *et al.*¹⁸ showed that resveratrol inhibited the hydroperoxidase activity of COX-1 and COX-2, demonstrated antiinflammatory activity, inhibited free-radical formation,

inhibited DMBA-induced mouse mammary lesions *in vitro*. Based on these large number of relevant chemopreventive mechanisms and *in vitro* studies, resveratrol was entered into the chemoprevention development process in the two cell culture assays and in the aberrant colon crypt study. Resveratrol is a naturally-occurring phytoalexin which has been identified in various human dietary sources, but concentrated in grapes.

PHENYL PROPANOIDS

A unique enzyme found in plants is phenylalanine ammonia lyase. This enzyme catalyzes the conversion of basic amino acids to cinnamic acid. Cinnamic acid is the precursor of a majority of plant phenolics. These phenolic compounds give plants many of their basic phenotypic characteristics. Phenolic compounds are utilized by plants in a variety of ways including basic reproduction and survival. Plant polyphenols can serve to attract birds or insects for cross pollination. Other polyphenolics function as insecticides, fungicides, and to preventive attacks by plant eating animals. The major class of polyphenols in grapes is the catechins. Catechins are found in a wide variety of plants including tea plants.

The phenylpropanoids are a class of compounds found in grapes and in many other plants. As mentioned above the enzyme, phenylalanine ammonia lyase, produces cinnamic acid. Cinnamic acid in turn can be converted to either coumaric acid or coumarin. Coumarin in turn can be converted to either caffeic acid or umbelliferone. In our program, caffeic acid has been shown to inhibit azoxymethane-induced aberrant crypts in rat colons, methyl nitrosourea (MNU) -induced and 7,12-dimethylbenz[a]anthracene (DMBA)-induced mammary cancer in rats (unpublished results). Caffeic acid may act as a cancer preventive agent by a number of mechanisms including activity as an antiinflammatory, Ornithine decarboxylase inhibitor, antioxidant, arachidonic acid metabolism inhibitor, cyclooxygenase inhibitor, and free radical scavenger¹⁹. Caffeic acid is converted to esculetin, which has similar properties including being a potent lipoxygenase inhibitor²⁰ and an ODC inhibitor and free radical scavenger¹⁹. Caffeic acid is also converted into ferulic acid which has similar mechanisms of action as caffeic acid. In addition ferulic acid has been shown to inhibit azoxymethane-induced aberrant crypts in rat colons²¹. Ferulic acid can be further metabolized into scopoletin.

Curcumin is a plant polyphenolic compound found abundantly in the spice tumeric. It is actually formed by the esterification of two ferulic acid

molecules. Curcumin has many potential chemopreventive mechanisms including antioxidant, cyclooxygenase inhibitor, antiinflammatory, lipoxygenase inhibitor, ornithine decarboxylase inhibitor, and free radical scavenger^{19,22}. Curcumin also has a wide range of efficacy in preventing azoxymethane induced aberrant crypts in rats, methyl-azoxymethane-induced colon cancer in mice, and methylnitrosourea-induced mammary cancer in rats²¹. Curcumin is currently being developed for human clinical trials by the NCI²³.

TANNINS

The tannins are another class of polyphenolics which have cancer preventing potential. The major types of tannins are hydrolyzable and condensed. Gallotannins are hydrolyzed into gallic acid subunits. Ellagitannins are also hydrolyzable polyphenols and yield ellagic acid as subunits. Ellagic acid inhibits thymidine kinase, increases glutathione, and inhibits the binding of carcinogens to DNA¹⁹. In an NCI study (unpublished) ellagic acid has inhibited azoxymethane-induced cancer of the colon in rats. Previous studies have shown that it also inhibits N-nitrosomethylbenzylamine induced esophageal carcinogenesis in rats²⁴. Ellagic acid has no effect on methylnitrosourea or 7,12-dimethylbenz[a]anthracene-induced mammary cancers in rats (unpublished data), it did inhibit benzo[a]pyrene and NNK-induced mouse lung adenomas. There is also evidence of some activity against skin cancers and liver cancers (unpublished data).

The catechins are one more variety of polyphenols which are potent antioxidants. In an effort to determine the cancer chemopreventive activity of tea compounds, initially conducted mechanistic assays and *in vitro* cell transformation assays to measure efficacy of these compounds (Table 1). The tea polyphenols showed high activity in most mechanistic assays which measured: ornithine decarboxylase (ODC) and NAD(P)H:quinone reductase (QR) activity. Moderate activity was seen in inhibition of free radical formation, glutathione-S-transferase (GST) induction, and little activity in the enhancement of glutathione (GSH). In the *in vitro* assays tea polyphenols inhibited morphological transformation in rat tracheal epithelial (RTE) cells, inhibited anchorage independence in human lung tumor (A427) cells, and inhibited hyperplastic alveolar nodule formation in mouse mammary organ cultures (MMOC)²⁵ (Table 2). Following the observation of significant *in*

vitro activity animal efficacy studies in the rat azoxymethane (AOM)-induced colon and *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal carcinogenesis models were begun²⁶⁻²⁸ with these compounds. In the AOM-induced colon cancer model in rats no efficacy was observed with either black or green tea extracts, black or green tea polyphenols, theaflavins, or epigallocatechin-gallate. However moderate efficacy was seen with black tea theaflavins in the esophageal model, but no effect by the other tea extracts. However there have been many published positive effects of tea polyphenols in many systems²⁹.

FLAVONOIDS

Another major class of polyphenols in grapes is the flavonoids. There are several major classes of flavonoids: Flavones, Flavanones, Isoflavones, and Flavonols. Examples of the flavones include apigenin and luteolin. Apigenin and luteolin are antioxidants, antimutagens, and share many of the common properties of polyphenols listed above. Apigenin has shown efficacy mainly in inhibiting mouse skin papillomas following topical treatment with DMBA/TPA³⁰. Luteolin showed some protection against 20-methylcholanthrene-induced cancer in mice³¹. Naringenin is a flavanone which our Chemoprevention Program has tested for efficacy to prevent mammary and colon cancers in experimental animals. DMBA-induced and MNU- induced mammary cancer was prevented in Sprague-Dawley rats fed naringenin in the diet. In the DMBA rat model 10 grams/kg diet was necessary to lower tumor incidence significantly, while in the MNU model only 4 grams per kilogram of diet was necessary for lowering tumor multiplicity as a measure of efficacy. No effect of naringenin was seen when tested in the rat AOM-induced aberrant colon crypt model. Genistein, an isoflavone, has had much testing in experimental animals (reviewed²³). In addition our testing program showed genistein inhibited the formation of AOM-induced aberrant crypts³², but not colon tumors³³. Further work is in progress with isoflavone mixtures for human clinical trials.

Table 1. Effect of tea extracts on chemoprevention-related biochemical endpoints

AGENT	Prescreening biochemical assays				
	GSH ^A	ODC	QR	GST	FR
Black tea, caffeinated	NE ^B	NE	NE	+++	+
Black tea polyphenols	+	NE	+++	++	NE
Theaflavins	NE	NE	NE	NE	+
Green tea, caffeinated	++	+++	+++	NE	++
Green tea polyphenols	NE	+	++	++	+
Epicatechin	NE	+++	+++	NE	NE
Epicatechin gallate	NE	+	++	++	++
Epigallocatechin	NE	+++	++	++	++
EGCG	NE	NE	NE	+++	NE

^A Abbreviations are: GSH, induction of glutathione; ODC, inhibition of TPA-Induced ornithine decarboxylase activity; QR = induction of NADPH:quinone reductase, GST = induction of glutathione-S-transferase; FR = free radical inhibition

^B Relative Activity: NE= no effect; + = low; ++ = moderate; +++ = high

Table 2. Activity of tea polyphenols in *in vitro* cell transformation assays

Test Material	<i>In Vitro</i> Transformation Assay ²		
	A427	RTE	MMOC
Black tea, caffeinated	+	+	+
Black tea polyphenols	+	+	NE
Theaflavins	+	+	NE
Green tea, caffeinated	NE	+	NE
Green tea polyphenols	+	+	+
Epicatechin	+	NE	NE
Epicatechin gallate	+	+	NE
Epigallocatechin	+	NE	NE
EGCG	+	+	+

^aA427 = Human cell line anchorage independent growth inhibition; RTE= rat tracheal epithelial transformation inhibition, MMOC = mouse mammary organ culture alveolar nodule inhibition.

^b + = effective (IC₅₀ in µg/ml); NE = not effective

FLAVONOLS

There are three major flavonols found in fruits and to some extent in grapes. Kaempferol, quercetin and myricetin. Kaempferol and other flavonoids moderately inhibited the increase in ornithine decarboxylase activity caused by 12-O-tetradecanoylphorbol-13-acetate in mouse skin and subsequently also decreased the incidence of DMBA/TPA-induced skin cancers in mice^{34,35}. Quercetin, a close structural analogue of kaempferol, has been studied extensively and has mixed cancer modulating results. In a study of rat intestinal and bladder cancer, quercetin induced cancers in both organs whereas the animals fed control diets had none³⁶. Pancreatic precancerous lesions were also induced by quercetin in an NMU-induced rat model³⁷. In this study the pancreatic tissue showed increased cell proliferation and decreased apoptosis. However quercetin inhibited the growth of human laryngeal cancer cell lines and primary laryngeal tumors³⁸. Also a 2% supplemented quercetin diet inhibited the formation of AOM-induced rat colon aberrant crypts³⁹. This decrease was found both the stressed and nonstressed animals.

Myricetin, another flavonol, also acts as an antioxidant, a cyclooxygenase inhibitor, a free radical scavenger, and a lipoxygenase inhibitor. Chang⁴⁰ found that myricetin inhibited benzo[a]pyrene-7,8-diol-9,10-epoxide induced lung cancer in mice. Another study found activity against 7,12-dimethylbenz[a]anthracene and benzo[a]pyrene induced mouse skin tumors⁴¹.

PHYTOALEXIN

Resveratrol, 3,4',5-trihydroxystilbene, is a phytoalexin and found in the grape skins and stems in abundant quantities. It is now thought it is natural fungicide against molds that attack grapes. However it has a number of properties which make it an attractive cancer preventive agent. Among these properties are inhibition of cyclooxygenase and aromatase, and free radical inhibition. In assays measuring induction of 7,12-dimethylbenz[a]anthracene hyperplastic alveolar nodules in organ cultures of mouse mammary gland, resveratrol greatly reduced the percentage of glands that were affected by the carcinogen¹⁸. In that same report the authors present evidence that 7,12-dimethylbenz[a]anthracene/12-O-

tetradecanoylphorbol-13-acetate induced mouse skin cancers were inhibited by this natural phytoalexin. Resveratrol inhibited benzo[a]pyrene-induced morphologically transformed rat tracheal epithelial cells in culture⁴² (Table 3). In the same paper it is reported that resveratrol inhibited anchorage independent growth of human A427 lung cancer cells by over 60 % (Table 4). Further studies using azoxymethane-treated rat colons, resveratrol significantly inhibited aberrant crypt formation⁴² (Table 5). Further studies are planned including inhibition of colon tumors in long term carcinogenesis studies.

Table 3. Inhibition of anchorage independent growth in human A427 cells by resveratrol

Test agent	Concentr. (μ M)	Observed colonies per well	Corrected frequency	Percent control	Percent inhibition
Resveratrol	0.03	139	163	72	28
	0.10	139	150	66	34
	0.30	105	134	59	41
	1.00	117	117	52	48
	3.00	111	114	50	50
	10.00	66	87	39	61
	DMSO	226	226	100	0
13-cis-retinoic acid	15.0	126	194	67	33

Table 4. Inhibition of benzo[a]pyrene-induced morphologically altered foci in rat tracheal epithelial cells by resveratrol

Concentration (μ M)	Foci per dish	Percent transformed	Corrected percent transform.	Percent B[a]P	Percent inhibition
Resveratrol					
0.03	40	21	15	88	12
0.1	35	19	13	75	25
0.3	40	21	15	88	11
1.0	36	19	13	78	22
3.0	42	22	16	93	7
DMSO	13	6	0	0	100
B[a]P	53	23	17	100	0
B[a]P+RA	10	4	-1.5	-8.8	109

Table 5. Inhibition of azoxymethane-induced aberrant colon crypts in rats by resveratrol

Group	#Animals	Dose g/kg diet	<u>Aberrant crypts / colon *</u>		
			Mean ± S.E.M	% Control	Result
1	10	0	201 ± 8	100	
2	10	1.0	194 ± 12	97	No effect
3	10	2.0	164 ± 11	82	Positive**

* Mean of two independent scorers

** Statistically different ($p < 0.05$)

DISCUSSION AND CONCLUSIONS

Polyphenols are one of the most abundant phytochemicals in vegetables and fruits, including grapes. Such phytochemicals, especially the catechins, flavones, isoflavones, flavanones, flavanols, tannins, and phenyl propanoids, have great promise as cancer preventing agents. The tannins appear active against skin, lung, liver, and esophageal cancer. Skin and mammary cancer may be inhibited by the flavonoids. The efficacy for isoflavones appears questionable with weak activity in mammary.

In many cases the isolated, purified chemical moieties representing these classes have shown efficacy in *in vitro* and in whole animals. However large scale use in humans is, in many cases, economically impractical, since many are very expensive. For the general population, consumption of a diet containing at least 5 servings of fruits and vegetables per day, is the most economical and perhaps most efficient way to assure ample intake of these valuable disease-preventive chemicals. High risk populations, such as those who expose themselves to tobacco smoke, or work in hazardous conditions, are genetically predisposed, or who have precancerous lesions or have had one cancer removed, may likely take pharmacological doses of concentrated phytochemicals. The safety of consumption such large doses will need to be verified.

The inhibition of cancer by grape derived phytochemicals likely occurs through multiple mechanisms. Among the potential activities of polyphenols in blocking the carcinogenic process are: antioxidation, free radical scavenging, carcinogen detoxification, inhibition of ornithine decarboxylase, cyclooxygenase inhibition, and aromatase inhibition. Most of these activities block DNA damage and/or cell proliferation.

Polyphenols effectively act as electron donors which eliminate free radicals. Free radicals are molecules that possess an atom with a single electron in the surrounding orbital. Since these orbitals are unstable with one electron and only stable with two electrons, the atom seeks to capture an additional electron. In the cell the most common free radicals are carbon, oxygen and nitrogen free radicals. Once the polyphenols donate an electron to free radicals, they themselves become free radicals. However due to their conjugated (alternating single and double bonds) they do not seek to capture an electron. The unpaired electron is "delocalized" and undergoes "resonance stabilization". Thus the polyphenol breaks the free radical chain reaction in cells and limits damage to important biomacromolecules such as DNA. This elimination of intracellular free radicals can be responsible for the antimutagenic, antiinflammatory, and antipromotional properties of polyphenols.

Some of the anti estrogenic activities of isoflavones may be linked to their physical structure. These "phytoestrogens" may interact with the estrogen receptor or the enzyme aromatase and decrease activity. Genistein is one isoflavone which may have anticarcinogenic properties due to its structure.

Grapes and wine that comes from grapes are rich in polyphenols and other beneficial agents which hold much promise in our efforts to prevent cancer of all types in humans.

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