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Natural astringency in foodstuffs — A molecular interpretation

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NATURAL ASTRINGENCY IN FOODSTUFFS — A MOLECULAR INTERPRETATION

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"I myself started with the 'information' that tannins were waste products deposited in the wood of trees because the plants had nothing else they could do with them; a horrible thought! Convinced that they had a function, I set out to try and find it by way of their systematic distribution linked to the idea of their astringency."

E. C. Bate-Smith

I. INTRODUCTION

A. Astringency and Astringents

The objective evaluation of the taste and flavor of foods and beverages still depends largely upon sensory perception. Although the methodology of testing has been greatly refined and systematized, a fundamental understanding of the physiology and chemistry underlying the sensation of taste is still lacking. This is certainly the case with respect to that quality of foods and drink generally referred to as astringency — this despite the fact that it has long been recognized that the acceptability of many fruits and fruit products (such as wines and fruit juices) is critically dependent on the type and concentration of astringent principles which are present.[1]

The word astringent is derived from the Latin *ad* (to) and *stringere* (bind); thus, astringency is properly defined as a binding reaction. Indeed, astringents in medicine and pharmacology are recognized as substances that bind to and precipitate proteins. They are used, for example, to control hemorrhage and diarrhea and to inhibit mucous secretions.[2-4] In this context, it is therefore of particular interest to note that many Japanese and Chinese folk medicines frequently employ, as antidiarrheic and hemostatic agents, plants rich in that group of secondary metabolites known as polyphenols or vegetable tannins. Typical of the very many members of this group of herbal medicines are Geranii Herba[5] (Japanese name — "Gen-no-shoko" — the herb of *Geranium thunbergii*) and the root of *Paeonia lactiflora,* which is one of the most important Chinese crude drugs.[6,7] The outer skin of the root of the tree paeony (Mudan) is used medicinally to cure disorders of the bloodstream, including high blood pressure. Its usage dates back some 2000 years and demand for "Dan pi", as its root bark is called, ensures that it is still planted by the acre in some Chinese states. Many herbal remedies likewise derive from plants of the family Rosaceae,[8,9] and in European culture today several remedies based on such plants persist. The astringent and stimulant character of raspberry (*Rubus idaeus*) leaf tea is well documented[10] as is its use as a medicament during parturition. Extracts of hawthorn (*Crataegus* sp.) find usage in Western medicine similarly in the treatment of heart disorders. Vegetable tannins (polyphenols) are generally regarded as the active principles of these herbal remedies.[5-9]

As a sensation of taste, that of astringency — generally recognized as a feeling of extreme

dryness or puckeriness — is not confined to a particular region of the mouth or tongue, but is experienced invariably as a diffuse stimulus.[1] Moreover, it may take a significant time to develop. A mucous membrane covers all the exposed surfaces of the mouth which are moistened by secretions of the salivary glands. According to Bate-Smith,[11,12] the primary reaction whereby astringency develops is via precipitation of proteins and mucopolysaccharides in the mucous secretions. This is believed to be caused by combination with the astringent principle(s). These astringents include salts of multivalent cations (Al, Cr, Zn, Pb, Ca, B), dehydrating agents such as alcohol and dimethyl ketone, mineral acids, and naturally occurring vegetable tannins. Polyphenols (syn. vegetable tannins) thus have a harsh astringent taste and produce in the palate a feeling of constriction, dryness, and roughness. These effects, it is believed, render many plant tissues unacceptable as food sources to potential predators and, on this basis, they are thought to constitute one of the most important groups of higher plant defensive secondary metabolites.[13,14]

Insofar as understanding the mechanism of the astringent response of plant tissues is concerned, then attention must inevitably focus on polyphenols and their interactions with proteins and polysaccharides. Parenthetically, it should also be noted that these distinctive properties and characteristics of polyphenols not only substantially influence the taste and palatability of many foodstuffs and beverages, but in like manner they determine the nutritional value of many forage crops[15,16] — particularly legumes — and livestock feeds such as the green pods of *Ceratonia siliqua* and sorghum (*Sorghum vulgare*). Similarly, the peculiar geographical distribution of esophageal cancer world-wide has been attributed to the use as beverages of plant decoctions rich in polyphenols.[17,18] In the U.S. and Europe, epidemiology has shown an analogous correlation of the occurrence of esophageal cancer with the consumption of particular alcoholic beverages. The most significant correlation is with apple-based drinks. Apples used for making ciders are selected for a high tannin content which lends the beverage both bitterness and astringency. It seems possible, on the basis of present evidence, that the frequent passage of beverages with a high tannin content through the esophagus may cause damage to the esophageal epithelium and increase its susceptibility to known carcinogens.

These and numerous related phenomena point increasingly to the need for a far more comprehensive understanding at the molecular level of the nature of polyphenol complexation with proteins, polysaccharides, nucleotides and nucleic acids, and various alkaloids. It is, of course, conceivable that such studies may also ultimately throw some light on the fundamental enigma of the role of polyphenol metabolism in plants. This review summarizes the present state of knowledge in this area of science.

B. Natural Plant Polyphenols: Vegetable Tannins

The word tannin has a long established and extensive usage, particularly in the botanical literature, but a firm definition of what constitutes a vegetable tannin is not easy to give.[19-21] The original implications of the word clearly indicate a plant material that produces leather from hide. Probably the most acceptable definition is still that of Bate-Smith and Swain,[20] formulated in 1962. They adopted the earlier ideas of White[19] and classified vegetable tannins as "water-soluble phenolic compounds having molecular weights between 500 and 3000 and, besides giving the usual phenolic reactions, they have special properties such as the ability to precipitate alkaloids, gelatin, and other proteins". Many still prefer the description "tannin" which they find valuable simply because of its lack of precision. Scientifically and terminologically, plant polyphenol is to be preferred for this class of higher plant secondary metabolite if attempts are to be made to interpret their diverse properties at the molecular level.[22]

Plant polyphenols are broadly divisible into two major groupings: the proanthocyanidins and the polyesters based on gallic and/or hexahydroxydiphenic acid and their derivatives.[22]

Table 1
SOME "TANNIN"-CONTAINING PLANTS THAT (AS LEAF, FRUIT, BARK, ROOT, ETC.) HAVE BEEN USED AS FOODSTUFFS, FORAGE CROPS, LIVESTOCK FEEDS, AND IN BEVERAGES AND HERBAL PREPARATIONS

Proanthocyanidins

Apple (*Malus* sp.); persimmon (*Diospyros kaki*); grape (*Vinus vinifera*); strawberry (*Fragaria* sp.); blackberry, dewberry, raspberry (*Rubus* sp.); plum, cherry (*Prunus* sp.); bilberry, cranberry (*Vaccinium* sp.); gooseberry, black and red currant (*Ribes* sp.); quince (*Cydonia* sp., *Chaenomeles chinensis*); cocoa bean (*Theobroma cacao*); kola nut (*Cola acuminata*); pear (*Pyrus* sp.); hawthorn (*Crataegus* sp.); rose hip (*Rosa* sp.); Chinese gooseberry (*Actinidia chinensis*); yam (*Dioscorea alata*); sorghum (*Sorghum* sp.); barley (*Hordeum vulgare*); sainfoin (*Onobrychis viccifolia*); herbaceous legumes (*Lotus* sp., *Trifolium* sp., *Coronilla varia, Lespedeza cuneata, Lathyrus pratense*); heather (*Calluna vulgaris*); wattle (*Acacia* sp.); rhubarb (*Rhei rhizoma*); Myricaceae bark (*Myrica rubra*); *Polygonum multiflorum* root

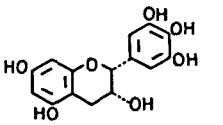
Galloyl and Hexahydroxydiphenoyl Esters

Blackberry, dewberry, raspberry (*Rubus* sp); walnut (*Juglans* sp.); strawberry (*Fragaria* sp.); carob pods (*Ceratonia siliqua*); rose flower, hip (*Rosa* sp.); pomegranate (*Punica granatum*); acorn (*Quercus* sp.); tea (*Camellia sinensis*); Uva-ursi (*Arctostaphylos uva ursi*); paeony root (*Paeonia* sp.); geranium root — geranii Herba (*Geranium* sp.); smoke-tree (*Cotinus coggyria*); cloves-flower buds (*Eugenia caryophyllata*); witch hazel (*Hamamelis* sp.); kinimizuhiki (*Agrimonia japonica*); ohebi-ichigo (*Potentilla kleiniana*); kibushi-leaves, fruit (*Stachyrus praecox*); rhubarb (*Rhei rhizoma*); *Casuarina (Casuarina stricta*); sweet gum-leaves (*Liquidambar* sp.); Pistacio (*Pistacia vera, P. chinensis*); guava (*Psidium guava*); Nupharis Rhizoma (*Nuphar japonicum*); *Acacia* leaves (*Acacia milotica*); *Bergenia* leaves, roots (*Bergenia crassifolia, B. cordifolia, B. purpurascens*); Myricaceae bark (*Myrica rubra*); persimmon (*Diospyros kaki*)

As they were recorded in the earlier literature, the former category corresponds with that of the condensed tannins[19,21] and the latter to the hydrolyzable tannins.[19,21] Plant polyphenols are members of that body of natural substances, generally referred to now as secondary metabolites,[23] which occur sporadically throughout nature and which appear (as yet) to have no explicit role in the economy of the organism that produces them. As secondary metabolites, plant polyphenols possess several distinctive molecular characteristics which set them apart. Not only do they carry a multiplicity of phenolic groups, but their molecular weights encompass a wide range. Proanthocyanidins have been described up to 20,000 in molecular weight and esters of gallic acid and their derivatives are found with molecular weights in the region of 3,000 daltons. Bate-Smith and Metcalfe[24] and Bate-Smith and Swain[25] first drew attention to the very close similarity in the recorded systematic distribution between leucoanthocyanins (as proanthocyanidins were generally referred to at that particular time) and the diverse class of substance known in the botanical literature as "tannins". These authors suggested that proanthocyanidins were most commonly responsible for the range of reactions which, up until that point, had been attributed to the presence of tannins in plants. Subsequent detailed work in which polyphenolic metabolites have been isolated from plant materials and identified has given credence to that view. It is, however, certainly incorrect to state, as did Joslyn and Goldstein,[1] that "only members of the condensed tannin class occur in fruits". For many fruits this is true, but it is not true for all. Some examples of authentic polyphenol (tannin)-containing plant materials which are used widely in different areas of the world as foods, forage crops, livestock feeds, in beverages, and in herbal preparations are shown in Table 1. In many cases, the observed astringency (and related properties) of plant tissues is due directly to the polyphenolic metabolites themselves. In other cases, these phenomena are related to the presence of postharvest transformation products of the original polyphenols. Black tea is just such a case.[26]

Tea is one of the most widely consumed beverages in the world. It is the immature vegetative portions of the tea plant (the "tea flush") which are suitable for use in the

manufacture of black tea. The crucial fermentation is initiated by leaf withering and rolling which causes the phenolic flavan-3-ol metabolites (1 to 3) in the green leaf and the nascent catechol oxidase to come into contact. The profound biochemical changes which ensue are in large part responsible for the development and flavor of the finished tea product. The "tea flush" contains caffeine (3 to 4%) and up to 30% (in some recorded cases) of polyphenols of which the major proportion are the flavan-3-ols-(−)-epicatechin gallate (1), (−)-epigallocatechin (2), and (−)-epigallocatechin gallate (3). The most distinctive change that occurs during the fermentation is the enzymic oxidation of these flavan-3-ol substrates which is responsible for the formation of the characteristic pigments which give color and flavor (including astringency) to black tea. Two major groups of pigments are formed: the theaflavins (and its associated gallate esters, 4) and the thearubigins.[26] Present evidence suggests that the various theaflavins may be regarded as primary oxidation products and that the heterogeneous polymeric thearubigins (whose structures are, as yet, chemically undefined) may be regarded as secondary oxidation products derived in part from the theaflavins.



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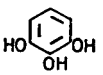

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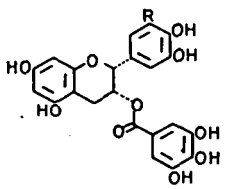
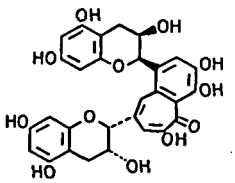


(1, R=H)
(3, R=OH)



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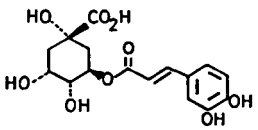


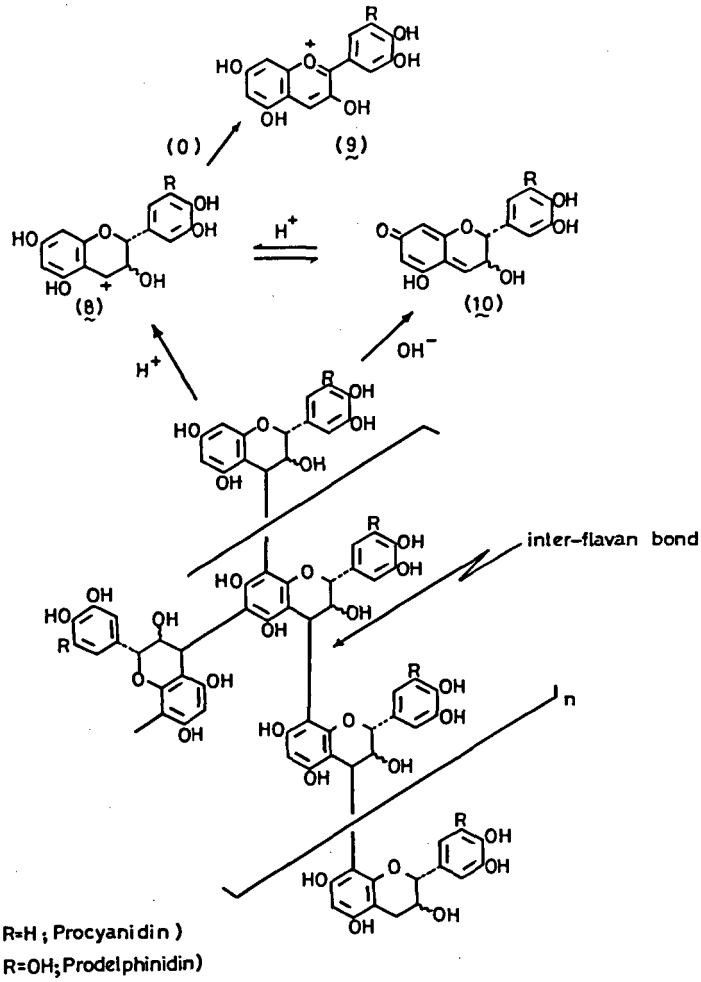
(6)

Following Bate-Smith and Swain's[20] definition of vegetable tannins, it has been customary in the intervening years, too readily perhaps, to ignore or even disregard the contribution that simple phenols (~200 or less mol wt) may make to phenomena such as astringency. This is certainly an unwarranted assumption based on an erroneous understanding of the mechanism of phenol binding to proteins. While polyphenols of the vegetable tannin class not only have the propensity to bind simultaneously to different points of the protein but also to cross-link separate protein molecules — properties that give them such a distinctive character[27] — the capacity to bind to protein is one which derives from the chemical and physical nature of the phenolic nucleus itself. Thus, the simplest of phenols such as catechol, resorcinol, and pyrogallol (5) bind, albeit weakly, to proteins. Concentrated (1 to 2 molal) solutions of both resorcinol and pyrogallol (5) may indeed precipitate proteins from aqueous media.[27] In these circumstances, it is quite possible that a significant astringent response may derive from plant tissues that contain (as defined above) no authentic vegetable tannins whatsoever, but are nevertheless rich in simple phenolic metabolites. Green tea, for example, contains substantial quantities of the flavan-3-ols (1 to 3), and a cup of coffee is said[28] to contain some 250 mg of the ubiquitous plant phenol chlorogenic acid (6). Neither green tea

nor coffee contains polyphenolic metabolites of the vegetable tannin class. Presumably, the distinctive effects that these beverages have on the palate derive, in part, from the presence of the relatively high concentrations of the simple phenols noted above. Thus, although this review concentrates on an interpretation at the molecular level of the role of polyphenols (vegetable tannins) in the development of the astringent response, the consequences of the presence of particularly high levels of simple phenols may, in certain plant tissues, be of much greater significance in the determination of acceptability and taste.

1. Proanthocyanidins

Plant proanthocyanidins[22] are based structurally on a polyflavan-3-ol structure (7), and form a phylogenetic viewpoint they first appeared as plants developed a vascular character — in ferns, for example. The nomenclature proanthocyanidin does not imply any biogenetic relationship, but it represents a terminology that is chemically derived.[29] Thus, when heated with acid, the interflavan carbon-carbon bonds of proanthocyanidins are ruptured and the flavan units, released initially as a carbocation species (8), are converted by aerial oxidation to characteristic anthocyanidins (9). The interflavan bonds are also susceptible to base-catalyzed cleavage and give initially the quinone-methide (10) intermediate.[30] This liability of the proanthocyanidins to break down under both acidic and basic conditions is of undoubted significance when changes in astringency of plant products (particularly fruits and wines) are considered. They are likewise probably of similar importance in relation to the adverse nutritional effects which these polyphenolic substances have[15,16] in certain foodstuffs.



(7, R=H; Procyanidin)
(7, R=OH; Prodelphinidin)

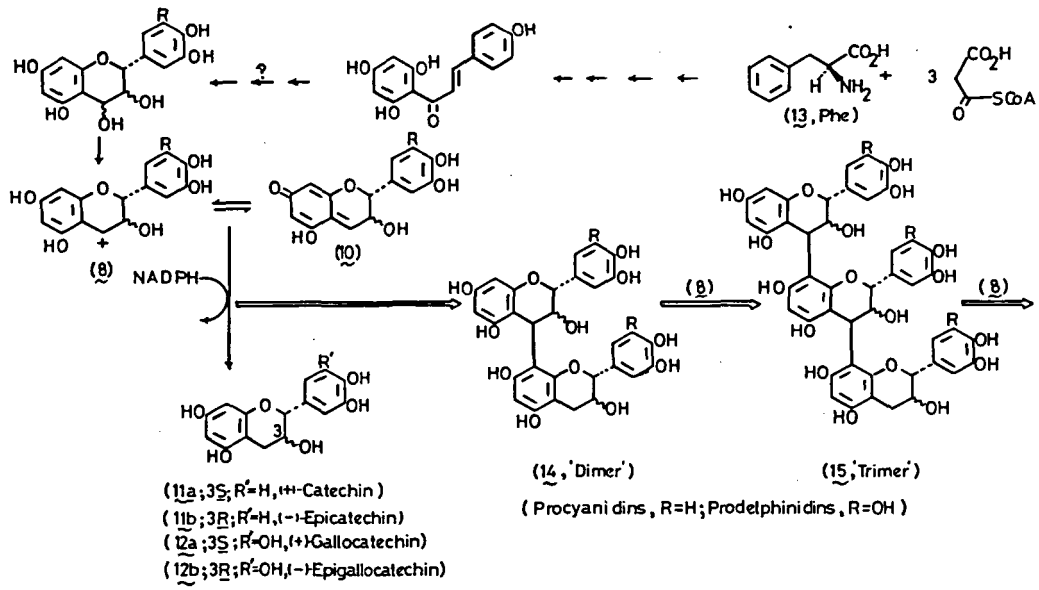


FIGURE 1. Biosynthesis of proanthocyanidins — an outline.

Current thought suggests that the proanthocyanidins are formed as byproducts of the processes in which the parent flavan-3-ols (11, 12) are biosynthesized in the plant tissue.[31,32] A key step visualized in this synthesis is the formation of the carbocation intermediate (8) or its protonated quinone-methide equivalent (10); cf. (8) and (10) intermediates of proanthocyanidin breakdown. Soluble dimeric, trimeric, tetrameric.... oligomeric proanthocyanidins are thought to derive in circumstances in which the supply of biological reductant (say NADPH) required to convert (8) to the flavan-3-ol is limited. If the supply of reductant is rate limiting in these circumstances, the transient carbocation is envisaged to escape from the active site of the enzyme and to react with the end product flavan-3-ol (11, 12) to produce first dimers and then the spectrum of higher oligomeric forms (Figure 1). In any plant tissue where proanthocyanidin synthesis occurs, there invariably is found this range of molecular species — from the monomeric flavan-3-ols (the catechins and the gallocatechins) to the polymeric forms. For each plant tissue, the balance between these various kinds of molecules is probably determined by the corresponding balance between the metabolic flux to the carbocation (8 or 10, Figure 1) and the rate of supply of the biological reductant (say NADPH). Tissues in which the flux is low and the NADPH supply is high will contain a range of proanthocyanidins of all molecular sizes. Conversely, those tissues in which the supply of NADPH is limited and the metabolic flux is high will contain predominantly the higher oligomeric forms. With increasing degrees of polymerization, the proanthocyanidins become more difficult to solubilize in aqueous and alcoholic media. It has been suggested that those that can be coaxed into solution may have molecular weights up to ~7000, corresponding to an accumulation of up to 20 flavan-3-ol units in the polymer.

In addition to these various "soluble" forms of proanthocyanidins, plant tissues invariably (in the authors' experiences) contain polymeric forms that are entirely resistant to all forms of ready solubilization.[33,34] Proanthocyanidins of this class often predominate[35] over the more freely soluble forms, typically by as much as 5 or 20:1. Indeed in the tissues of some plants — e.g., the persimmon fruit — they overwhelmingly predominate. It is pertinent in this context to recall the original observation of Sir Robert and Lady Robinson[33] — pioneers in this field — made in the 1930s. They drew attention to various categories of proanthocyanidins (then referred to as leucoanthocyanins) and in particular to a group (a) — "those

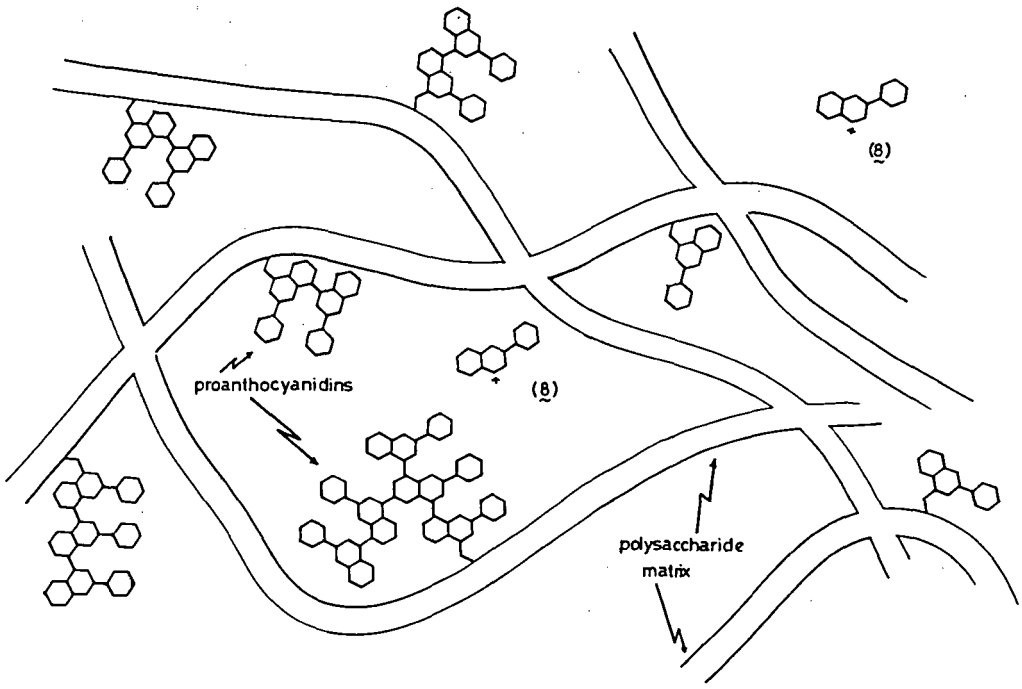


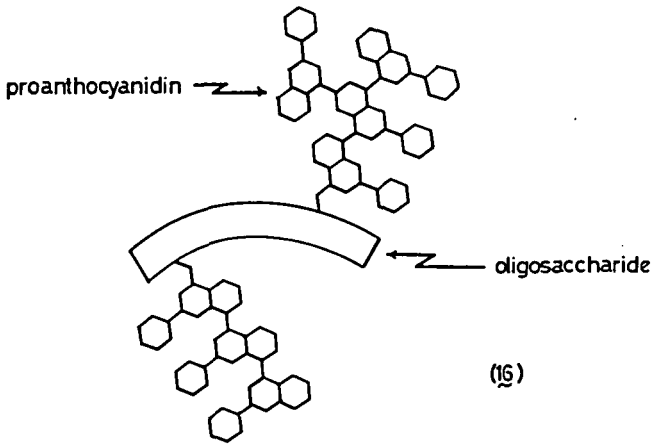
FIGURE 2. Proanthocyanidins bound within a cellular matrix.

which are insoluble in water and the usual organic solvents''. While noncovalent forces may be involved in the complexation of polyphenols with polysaccharides, present evidence strongly supports the view that polymeric proanthocyanidins that fall within the category (a) defined by the Robinsons are covalently bound to a carbohydrate matrix within the plant cell. Such proanthocyanidins it has been suggested may well result from the capture of the putative carbocation intermediate (8), or its quinone-methide equivalent (10), during biosyntnhesis by hydroxy groups of saccharide structures in the plant cell (Figure 2).

Procyanidins (7, R=H) are the most commonly found group of proanthocyanidins in plants,[36] and their occurrence invariably is (*vide supra*) associated with the metabolism of (+)-catechin (11a) and (−)-epicatechin (11b) in the plant tissue. In contrast to the ubiquitous procyanidins, the same detail of the chemistry and biochemistry of the prodelphinidins (7, R=OH) is not yet available.[32] They are restricted in distribution, but in an analogous fashion they occur alongside the corresponding flavan-3-ols (+)-gallocatechin (12a) and/or (−)-epigallocatechin (12b). However, their chemistry is complicated very frequently by the fact that in plant tissues where prodelphinidins are found, almost invariably procyanidins are cometabolized.

Despite the remarkable advances in knowledge made in this area in recent years several important, and maybe crucial, questions remain unanswered. Proanthocyanidins are essentially polymers based on a small number of closely related flavan-3-ol ''monomer'' units and, as the foregoing discussion has highlighted, the proanthocyanidin content of a particular plant tissue may comprise a range of molecular forms from the soluble dimeric and oligomeric species (generally the tip of the iceberg) to various polymers, many of which are rendered insoluble because of their molecular size or because they are bound to an insoluble polysaccharide matrix. When such a plant tissue is sampled or tasted it has been tacitly assumed heretofore that, according to the definition of Bate-Smith and Swain,[20] it is the soluble proanthocyanidins that are important in the development of astringency. The role of the

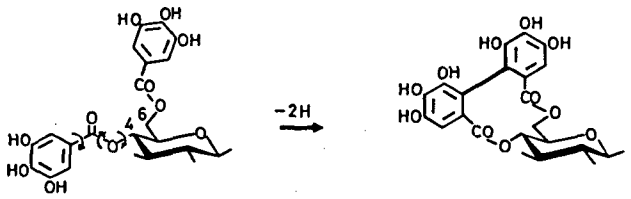
insoluble forms is far from clear. Circumstantial evidence (e.g., the persimmon fruit) suggests that these forms may be of considerable significance. Where the plant tissue is extracted in aqueous media over a long period of time (e.g., the production of red wines, the manufacture of beers, etc.) it seems probably that partial breakdown of the insoluble proanthocyanidins may take place bringing them into solution either as lower oligomeric forms or as fragments (16) cleaved from the polysaccharide matrix but retaining a saccharide structural element. These are all questions that warrant further investigation.



(16)

2. Esters of Gallic Acid and Hexahydroxydiphenic Acid

Although C_6–C_1 phenolic acids are found in plants and microorganisms, their occurrence is nevertheless sporadic and often constitutes something of a taxonomic specialty.[37] In this respect, one of the most familiar examples is the willow family (Salicaceae) in which derivatives of salicyclic acid are found. Hydroxybenzoic acids and benzoic acid itself are consistently located throughout the plant kingdom esterified to both terpenoid and alkaloid structures. Likewise, although the various hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, sinapic) are ubiquitous in higher plants,[38] they are normally only found as mono- and occasionally as bis-esters with polyols (e.g., chlorogenic acid, 6). In direct contrast to the hydroxybenzoic acids and the hydroxycinnamic acids, gallic acid and the biosynthetically derived hexahydroxydiphenic acid[39] are both widely distributed and found in a range of esterified forms. The occurrence of gallic acid[40] has been noted in some 20 or so plant families and 1 freshwater alga in ester forms that vary from the very simple mono-esters such as β-D-glucogallin (17), theogallin (18), and the flavan-3-ol gallates (1, 3) — all found, for example, in young green tea shoots (*Camellia sinensis*) — to the complex polyesters with D-glucose whose molecular weights span the range from 500 to 3000. These polyphenols constitute the second major class of vegetable tannin — the hydrolyzable tannins[21,22] — and they are almost uniquely confined to dicotyledons. They are found (Table 1) in forage crops, livestock feeds, some fruits, beverages, and herbal remedies. Unlike some proanthocyanidins, they are in general freely soluble plant metabolites and as such may be expected to contribute to taste and palatability of plant materials in which they occur.



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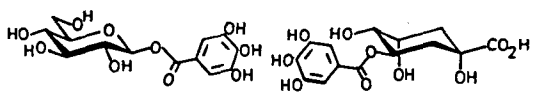


(17) (18)

A routine feature of gallic acid metabolism in a great many plants is the oxidative coupling of appropriately oriented galloyl ester groups in a particular metabolite to give derivatives of hexahydroxydiphenic acid (19) — usually referred to generically as ellagitannins.[41,42] Intramolecular covalent bonding of this type produces, from a conformationally flexible and mobile galloyl ester precursor, a more rigid and compact molecular structure. The apotheosis of this effect is seen in the derivation of the diastereoisomeric phenolic metabolites vescalagin[43] and castalagin,[44] in *Quercus* and *Castanea* spp., by oxidative transformation of the pivotal biosynthetic intermediate β-pentagalloyl-D-glucose (20).[23] The latter has a flexible disc-like structure in which the galloyl ester groups are displayed on the periphery of the molecular surface; vescalagin (21) and castalagin (22) have propeller-shaped conformations, compact and rigid. Parenthetically, it is, however, noteworthy that β-penta-*O*-galloyl-D-glucose (20) is only poorly soluble in water and at certain critical concentrations its solutions gel, while vescalagin and castalagin, although both crystallize from water, have a significantly higher water solubility. Where *inter*molecular oxidative coupling of galloyl ester groups occurs, this also produces phenolic metabolites of increased molecular size ("dimers", "trimers", etc.). It is perhaps significant that the major biosynthetic thrust in plants is towards the formation of these highly convoluted structures (e.g., vescalagin, castalagin, geraniin; Figure 3) *or* the "dimers" and "trimers".[40] Thus, in the Rosaceae plant family, for example, the leaves of *Rosa* sp. and *Filipendula ulmaria* (meadowsweet) contain as the principle phenolic metabolite rugosin-D (23). Similarly, in *Rubus* sp. (blackberry and raspberry), *Geum* sp., and *Potentilla* sp., the "dimer" (24) predominates. Likewise, the familiar polygalloyl ester tannic acid (25), in which additional galloyl ester groups are linked as depsides to β-pentagalloyl-D-glucose (20), dominates the metabolic profile of *Rhus* sp., *Cotinus* sp., and some *Acer* sp. The astringency of all these plant tissues, it is assumed, is attributable in very large part to these polyphenolic galloyl esters.

Because of the distinctive properties which derive from the combination of moderate molecular size and polyphenolic character in the proanthocyanidins and the polygalloyl esters it is tempting, but doubtless misleading, to suggest similar metabolic functions. Comparative studies show that the two forms of metabolism are not mutually exclusive, but often where tissues are rich in proanthocyanidins the synthesis of gallic acid and its derivatives is minimal, a suggestion perhaps of a metabolic balance. Conversely, in some plants such as *Camellia sinensis*, the leaves of which are rich in flavan-3-ols, but principally as their 3-*O*-gallate esters (1, 3) the level of proanthocyanidin synthesis is vanishingly small.[22]

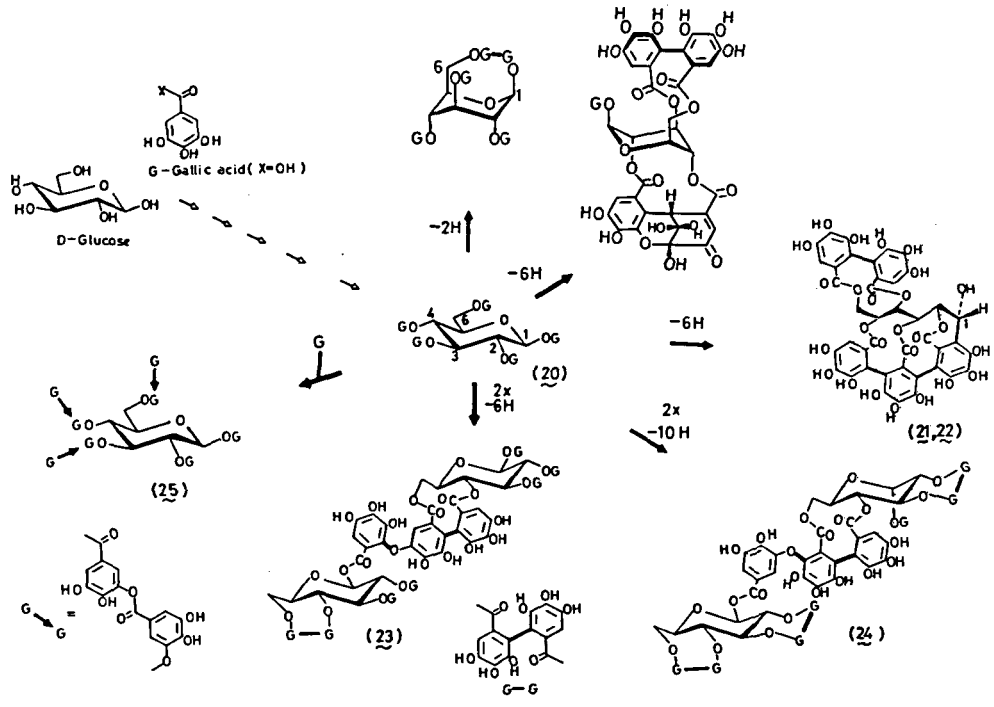


FIGURE 3. Pathways of metabolism of gallic acid — galloyl esters biosynthetically related to β-pentagalloyl-D-glucose.

II. PHYSICOCHEMICAL STUDIES OF POLYPHENOL COMPLEXATION

A. Proteins

The interaction between polyphenols and proteins which is generally believed to underly the phenomenon of astringency may be either reversible or irreversible. Studies of the reversible association of polyphenols with proteins have a long history; one of the first scientific papers on this subject is that of Sir Humphry Davy[45] in 1803. Among the many observations described by Davy, there is not one that has not stood the wear and test of time. Davy's work was undertaken at the instigation of the Directors of the Royal Institution and was directed towards an understanding of the age-old process of vegetable tannage whereby "astringent vegetable matter" (Davy's description) converts animal hides and skins to leather. The ability of tannins to combine with proteins is the basis of this process and this relationship has, until comparatively recently, been responsible for the sustained interest in this area. During the last 30 years, the increasing recognition of the important contributions that vegetable tannins make to the taste and palatability of foodstuffs and beverages,[1,11,12] to adverse nutritional effects in forage crops,[15,16] and to strategies of plant defense[13,14] and herbal remedies[3,4,8] has broadened considerably the scope of interest in protein-polyphenol interactions. Attention is principally directed here to processes of reversible complexation, but it should be noted that in relation to nutritional effects there is considerable evidence to suggest that irreversible association with protein is of great importance.

The early work in this field, such as that of Davy,[45] demonstrated some of the macroscopic features of reversible complexation and it enabled several wholly empirical definitions of the term "vegetable tannin" such as that of Nierenstein in his book (1934) *The Natural Organic Tannins* to be advanced. However, until such times as structurally defined plant polyphenols became available, the molecular mechanisms underlying the development of

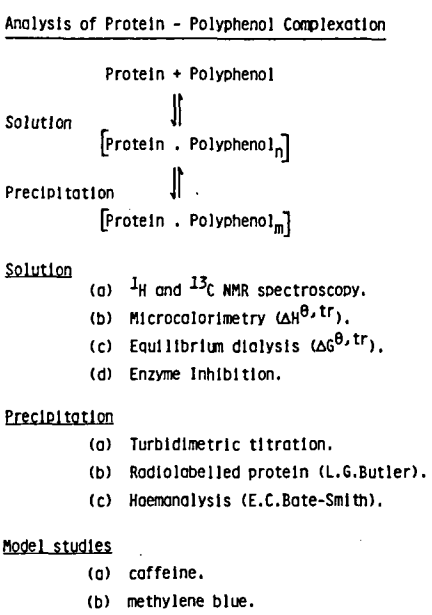


FIGURE 4. Protein-polyphenol association.

astringency and the loss of astringency in foodstuffs and beverages remained poorly understood. Various studies of polyphenol complexation and in particular the delineation of structure-activity relationships have now been carried out in recent years. Although as a subgroup the proanthocyanidins are most commonly responsible for the range of reactions generally attributed to tannins in plants, many of these particular studies have been most conveniently pursued with a series of biosynthetically interrelated esters of gallic acid (Figure 3). They are accessible in homogeneous forms and differ systematically in phenolic content, solubility, molecular size, and conformation.

Polyphenol complexation with proteins may be studied in solution or by an investigation of the precipitation process which ultimately ensues after extensive complexation (Figure 4). Various physicochemical techniques are appropriate to such analyses.

1. Equilibrium Dialysis and Microcalorimetry[46]

Quantitative measurements of the binding of various simple phenols and polyphenols to proteins may be obtained by equilibrium dialysis. The technique is, in principle, a very useful one to gain information about the extent of binding of ligands to macromolecules. The normal Scatchard analysis assumes that the protein has a fixed number of independent binding sites each of which has the same propensity to bind ligands. The validity of this assumption is questionable for polyphenols, which are multidentate ligands and in which cooperativity of binding generally appears to occur. An alternative method of analysis of the data leads to the evaluation of the free energy of transfer of the protein from an aqueous solution to an aqueous solution containing ligand (polyphenol), $\Delta G^{\theta,tr}$. The free energy of transfer thus gives a useful, direct, model-independent, and quantitative measure of the net interaction between two associating species; the more negative the value, the greater is the mutual interaction of ligand (polyphenol) for protein. Table 2 shows some typical values of $\Delta G^{\theta,tr}$ for the interaction between bovine serum albumin (BSA) and various galloyl esters. Molar enthalpies of transfer, $\Delta H^{\theta,tr}$, may analogously be determined using microcalorimetric methods. Plots of $\Delta H^{\theta,tr}$ vs. $T\Delta S^{\theta,tr}$ for a range of galloyl esters interacting with BSA gives linear plots (slope ~1.0) indicative of enthalpy-entropy compensation. This proportionality

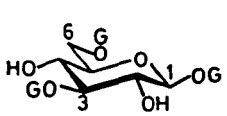
Table 2
REVERSIBLE COMPLEXATION OF POLYPHENOLS WITH PROTEINS

Polyphenol	Molecular weight	β-Glucosidase inhibition K_i (10^{-4} *M*)	BSA $-\Delta G^{0,tr}$ (kJ/mol)[a]	Hemoglobin precipitation (relative astringency)[b]
β-1,2,6-Tri-*O*-galloyl-D-glucose (26)	636	10.8	0.9	0.20
β-1,2,3,6-Tetra-*O*-galloyl-D-glucose (27)	788	2.50	9.1	0.58
β-1,2-3,4,6-Penta-*O*-galloyl-D-glucose (20)	940	0.85	26.9	1.0
Casuarictin (28)	936	1.57	—	—
Rugosin-D (23)	1874	0.08	58.7	2.4
Sanguin H-6 (24)	1870	0.40	11.3	

[a] $-\Delta G^{0,tr}$: free energy of transfer of the protein BSA from an aqueous solution to an aqueous solution containing the polyphenol.[46]

[b] Relative astringency by hemanalysis; method employed due to Bate-Smith.[59] Values related to (20) as standard 1.0.

indicates that the trends in the magnitude of protein-polyphenol interactions as the structure of the polyphenol is systematically varied may, with caution, be examined also by reference to $\Delta H^{0,tr}$ (microcalorimetry).



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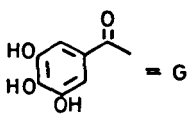

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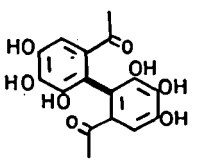


= G—G

2. Protein Precipitation[45,51-61]

The primary reaction whereby astringency develops in the palate is, according to Bate-Smith,[11,12] by precipitation of glycoproteins in the mucous secretions of salivary glands. The efficacy of polyphenol binding to protein derives from the fact that polyphenols are multidentate ligands able to bind simultaneously (via different phenolic groups) at more than one point to the protein surface.[27,47,50] When polyphenols cause precipitation of proteins from solution, two situations may be envisaged.[27] At low protein concentrations, the polyphenol associates at one or more sites on the protein surface to give a monolayer which is less hydrophilic than the protein itself (Figure 5a). Aggregation and precipitation then ensue. Where the protein concentration is high, the relatively hydrophobic surface layer is formed by complexation of the polyphenol onto the protein and by cross-linking of different protein molecules by the multidentate polyphenols (Figure 5b). Precipitation then follows as above. This tendency to cross-link protein molecules at higher protein concentrations explains the changing stoichiometry of the aggregates with changing protein concentrations — an observation first hinted at by Sir Humphry Davy.[45] More polyphenol is thus required to precipitate proteins from dilute solution than from concentrated solutions.

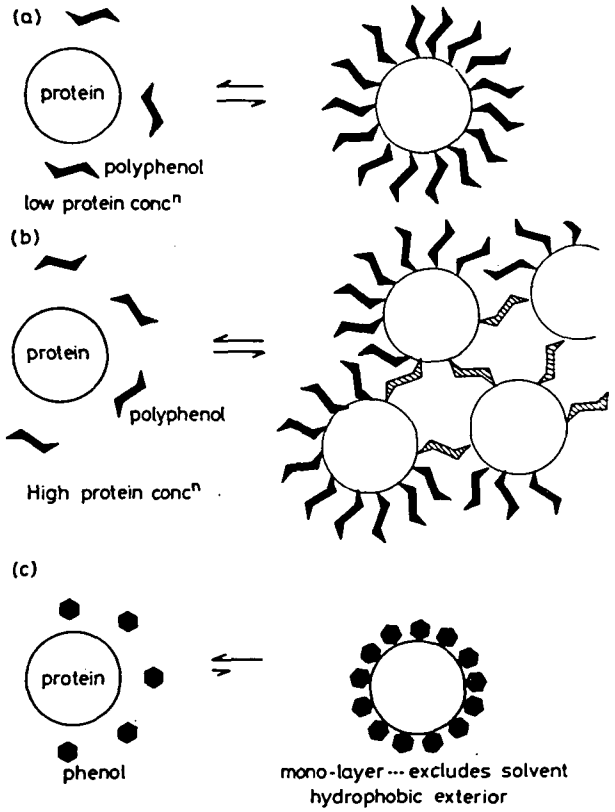


FIGURE 5. Protein-phenol, protein-polyphenol precipitation.

An interesting corollary of this hypothesis is that simple phenols such as pyrogallol should also be capable of precipitating proteins from solution if they can be maintained in solutions at concentrations sufficient to push the equilibrium in favor of the protein-phenol complex and thus form a hydrophobic layer of simple phenol molecules on the protein surface (Figure 5c). For many simple phenols, the limit is provided by their solubility in water, but it can be achieved with, for example, BSA (3×10^{-5} molal) and pyrogallol (1 molal).[27] Clearly, as indicated earlier, such factors are important when assessing the total astringency of some plant extracts and beverages rich in comparatively simple phenolic compounds. The precipitation of protein by polyphenols may be reversed by the addition of further protein solution, and in his observations on the analysis of astringent vegetable infusions using gelatin in 1803, Sir Humphry Davy remarked: "In ascertaining the proportions of tannin in astringent infusions, great care must be taken to prevent the presence of any excess of gelatin; for when this excess exists, I have found that a small portion of the solid compound formed is redissolved . . . "

In a similar manner, protein-polyphenol complexes may be dissociated by treatment with solvents such as acetone,[51] without denaturation of the protein, and with caffeine,[52,53] urea,[54] polyvinylpyrollidone (PVP),[55] polyethylene glycols,[56] and detergents.[56]

Various methods based on protein precipitation have been used to measure the relative affinity of different polyphenols for proteins. Most familiar of these are precipitation by gelatin,[57] by β-glucosidase,[58] the method of hemanalysis due to Bate-Smith,[59] and the procedure of Hagerman and Butler[61] using radioiodinated protein (BSA). The extent of protein precipitation using β-glucosidase is monitored by the change in enzyme activity;[58]

in hemanalysis it is followed colorimetrically (λ_{max} = 578 nm)[59] as the protein is removed from solution; and similarly the procedure of Hagerman and Butler[61] relies on the loss of radioactivity from solution. Results may be expressed in several ways; in hemanalysis the relative affinity of different polyphenols for hemoglobin is expressed as relative astringency (RA) and compared to a standard polyphenolic substrate, tannic acid,[59] or β-pentagalloyl-D-glucose[60] (20, RA = 1.0; Table 2). Results from protein precipitation generally show a broad comparability to those obtained by other methods. The technique is relatively simple and rapid to apply and the concept which underlies it is readily comprehended.

3. Enzyme Inhibition[16,56,62-66]

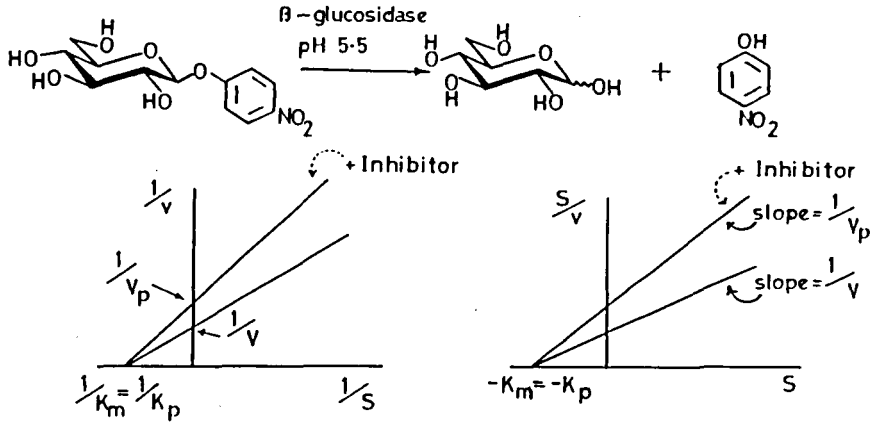
The presence of polyphenols (tannins) in plant materials is of considerable significance in several areas of agriculture.[15,16] Thus, polyphenols are known to decrease the nutritional value of important forage crops, particularly legume pasture species, sorghum, and various categories of fodder tree leaves. Likewise, polyphenols in plant tissues are known to inhibit the rate of microbial decomposition of plant materials in the formation of soils.[57] In all these situations, the adverse role of polyphenols is inevitably mediated in several different ways. However, one of these is certainly that of enzyme inhibition. The reduced digestibility of tannin-rich feeds, for example, may be explained, in part, on the basis of the inhibition of digestive enzymes.[62,63] Thus, trypsin is inhibited[16,62] by tannins from oak leaf, carob, lucerne, field beans, and *Vicia faba*. Phenolic compounds may inhibit enzymes by precipitation of enzyme protein, by the formation of soluble but inactive enzyme-inhibitor complexes, and/or by complexation with the substrate(s) to confer reduced reactivity in the enzyme reaction. In a study of pectinesterase, Hall[64] observed reversible inhibition with tannic acid and a degree of inhibition proportional to the tannic acid concentration, and noncompetitive enzyme reaction kinetics have been reported for β-glucosidase,[56,55] trypsin,[16] amylase,[16] and lipase.[16] For systems in which the natural substrate of the enzyme is another protein, e.g., proteases, inhibition of the enzymic reaction may be due to polyphenol complexation with either the protein substrate (in which case it exerts a protective role) or the enzyme itself.[63] Thus, Feeny[62] noted that the water-soluble fraction of oak leaf tannin has the ability to form a complex with casein and the complex is almost protected from hydrolysis by trypsin at pH 7.6.

Detailed studies of the enzyme β-glucosidase[65] have shown that polyphenols initially inhibit this enzyme in a classical "noncompetitive" manner.[66] Inhibitor (I, polyphenol) and substrate (S) bind simultaneously to the enzyme (E). Most commonly, the dissociation constant of (S) from the (EIS) complex is different from that from the normal enzyme substrate (ES) complex (Figure 6). In this case, both K_M and K_{cat} are altered. In the simplifying case of the Michaelis-Menten mechanism where the dissociation constant of (S) from (EIS) is the same as that from (ES) but (ESI) does not react (Figure 7), $K' = \emptyset$ and $K_M = K_M'$, K_{cat} is lowered by a factor of $(1 + 1/K_i)$. This situation appears to be closely reflected in the initial inhibition of β-glucosidase by polyphenols. The relative affinity of a group of polyphenols for β-glucosidase may be determined by the evaluation of values of K_i from kinetic measurements of enzyme inhibition.

This experimental system is of particular importance since it (of the methods described) most readily permits the assessment of the effect which additional substrates (e.g., other proteins, saccharides, alkaloids) may have on the affinity that particular polyphenols have for a given protein. This in turn is particularly relevant when it is necessary to assess the significance of other substrates such as saccharides and alkaloids on the changing astringency of plant tissues (e.g., ripening fruit).

4. Structure-Activity Relationships

There is a broad, although not exact, comparability in the information derived from the three types of measurement (*1, 2, 3, vide supra*); see Table 2. They show that a given



$E + I \rightleftharpoons E{\cdot}I$, K_i Dissociation constant of E·I

Polyphenols — non-competitive inhibition

$$K_i = I \cdot \frac{V_p}{V - V_p}$$

I = concentration of inhibitor(I)

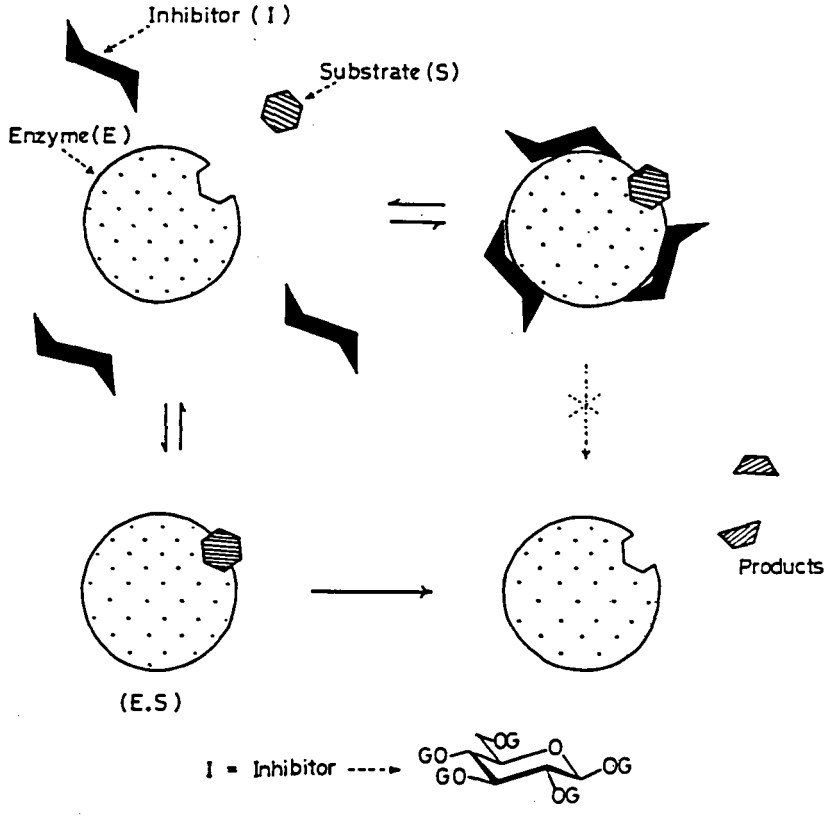


FIGURE 6. Enzyme inhibition by polyphenols.

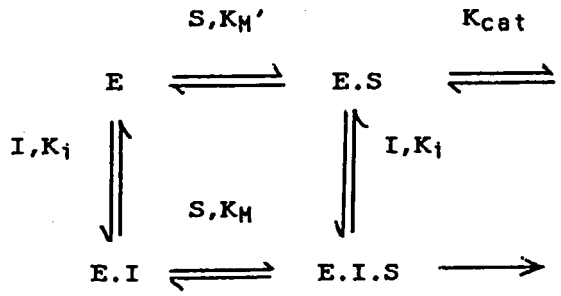


FIGURE 7. Classical noncompetitive enzyme inhibition.[66]

polyphenol may exhibit substantially different affinities for different proteins; Hagerman and Butler[61,67,69] have shown, for example, that flexible "open" proteins and those rich in the amino acid proline bind polyphenols more effectively than compact globular proteins, although for a given series of polyphenols their comparative behavior with one protein generally parallels the behavior with other quite different proteins.[46,70] On the basis of present information, the associative process appears to be a surface phenomenon. Whether there are preferred modes of association and preferred binding sites is not yet entirely clear. Such an observation would obviously be of some significance in respect to the function that these compounds may or may not have in plant metabolism.

The effectiveness of plant polyphenols as complexing agents derives from the fact that they are polydentate ligands with a multiplicity of potential binding sites provided by the numerous phenolic groups and aryl rings on the periphery of the molecule. Because of the propinquity of these groups in the polyphenol, cooperativity of complexation to the protein surface is observed. Because of their molecular size and structure, polyphenols form stable cross-linked structures with different protein molecules (Figure 5). Molecular size is thus critically important as a determinant of the ability of a polyphenol to bind to protein — as inferred in Bate-Smith and Swain's[20] definition of a vegetable tannin. Thus, in the galloyl-D-glucose series, the efficacy of association with protein is enhanced with the addition of each galloyl ester group (di → tri → tetra → penta; Table 2) and reaches a maximum in the flexible disc-like structure of β-penta-*O*-galloyl-D-glucose (20). Further metabolism of this intermediate often substantially lowers its powers of association. Equally significant as molecular size of the polyphenol is conformational mobility and flexibility. In the galloyl D-glucoses when vicinal galloyl ester groups are constrained by the biosynthetic intramolecular formation of biphenyl linkages and the generation of hexahydroxydiphenoyl ester groups (→ 19), the loss in conformational freedom is reflected in a reduced capacity to bind to protein (Table 1). The apotheosis of this effect is seen in the case of the unique open-chain D-glucose derivatives vescalagin and castalagin, metabolites of *Quercus sp.*[43,44] These rigid, virtually inflexible, propeller-shaped molecules are in a sense analogs of β-penta-*O*-galloyl-D-glucose (21, 22; Figure 3), but on a molar basis they are bound much less effectively to protein than (20). In this context, the observed "relatively lower astringency" of the proanthocyanidins (7) compared to other polyphenols may be explicable, in part, in terms of the conformational restraints imposed by restricted rotation about the repeating 4,8- or 4,6-interflavan bonds. Collectively, these results fully complement those of Hagerman and Butler,[61] who showed that proline-rich and conformationally mobile proteins have high affinities for polyphenols. Thus, complimentarity between the polydentate ligand (polyphenol) and the receptor (protein) is maximized by conformational flexibility in both components. What is not yet clear is whether there is specificity in the association with the protein (surface) or whether the binding is entirely random in nature.

One of the key factors in this whole problem is, of course, the solvent water. To date,

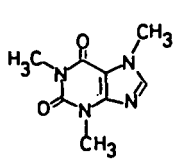
its presence has been tacitly ignored. Biologists have long been intrigued by the concept of structured water solvation shells around molecules and whether there are preferred sites thereon for the association of inorganic ions and water molecules. Thus, while it is pertinent to inquire what is the effect of bound water on the properties of the biological substrate (e.g., protein) and what is the effect of solvation on complexation with polyphenols, present evidence gives no simple answer to such questions.

The principal means whereby proteins and polyphenols are thought to reversibly complex with one another are via (1) hydrogen bonding and (2) hydrophobic interactions.[58,71,72] The relative importance of these two types of interaction remains, however, uncertain. While the tendency has been to intuitively emphasize the part played by intermolecular hydrogen bonding, several workers, including Hoff et al.,[73] have drawn attention to the fact that hydrophobic effects may dominate the interaction. Some insight into this problem has been revealed recently by studies of polyphenol complexation with model systems and in particular the heterocycle caffeine (29)[74,76] and the cyclodextrins (30).[75] It is well known that the latter are able to form inclusion complexes with aromatic substrates, and these molecules have been employed to probe this type of noncovalent interaction as a model for analogous complexations with particular types of polysaccharide.

B. Caffeine[52,56,74-76]

While it is not possible to extrapolate directly to behavior in solution, X-ray crystallographic analysis of various caffeine-phenol complexes confirms the importance of (1) hydrogen bonding and (2) apolar hydrophobic interactions and coordination around a metal ion as the primary intermolecular forces in caffeine-polyphenol complexation. Insofar as caffeine has structural elements which resemble a peptide-like structure, these observations probably also point to the significance of these same noncovalent forces in the association of polyphenols with proteins.

X-ray crystallographic analysis of complexes of caffeine[74,76] with a variety of phenolic substrates shows that in the solid state a layer-lattice structure is frequently present. In this array, caffeine and aromatic molecules are arranged in stacks in alternating layers, approximately parallel, with an interplanar separation of ~3.3 to 3.4 Å (Figure 8). In the case of methyl gallate (31), this stacking structure is complemented (Figure 9) by an extensive in-plane system of hydrogen bonding between the three phenolic hydroxyl groups of methyl gallate and the two keto-amide groups and the basic N-9 of caffeine. The crystal structure of the caffeine complex with potassium chlorogenate (6, potassium salt), first isolated from coffee beans by Gorter,[77] shows similar features to those noted (Figures 10 and 11). However, an additional critical stabilizing factor is the coordination of seven oxygen atoms in an irregular polyhdedral arrangement around the central potassium ion (K^+ . . . O distances between 2.67 and 2.85 Å; Figure 12). A further feature of interest in these crystalline phenol-caffeine complexes is the relative orientation of the planar caffeine and phenolic partner in the layer lattice. The term "polarization bonding" has been used[78] to describe both charge-transfer bonding and the generally weaker interactions between polar groups of one component and a polarizable second component. For weak noncovalent bonding of this type, the principal feature to be expected is the juxtaposition of the polarizable groups of one component and the polarizable regions of the second. It is therefore interesting to note that in the various phenol-caffeine complexes, the phenolic groups and associated nuclei are generally stacked above the six-membered ring of the caffeine molecule. This suggests that



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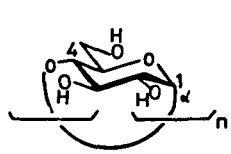
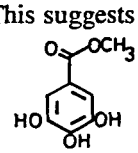


(30, n=6,7,8.....)



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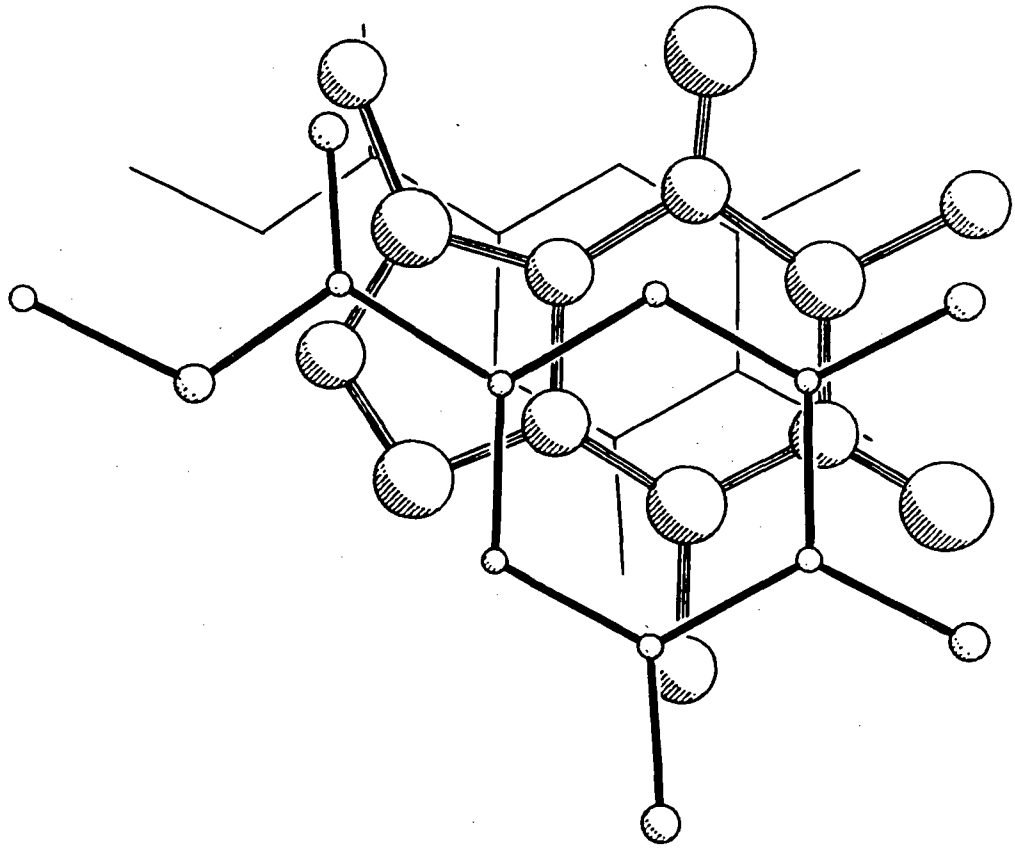


FIGURE 8. Crystal structure of the caffeine-methyl gallate complex — apolar hydrophobic interactions, vertical stacking.

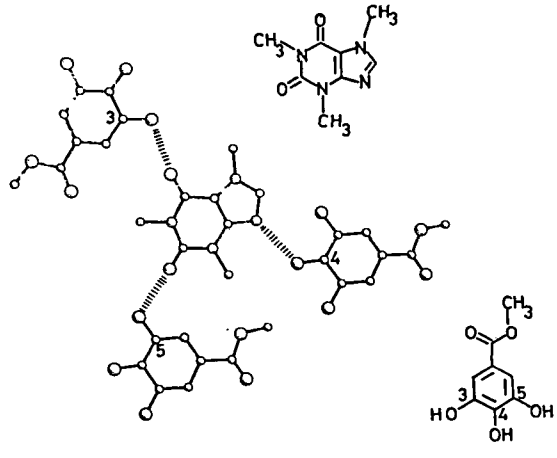


FIGURE 9. Crystal structure of the caffeine-methyl gallate complex — in plane hydrogen bonding.

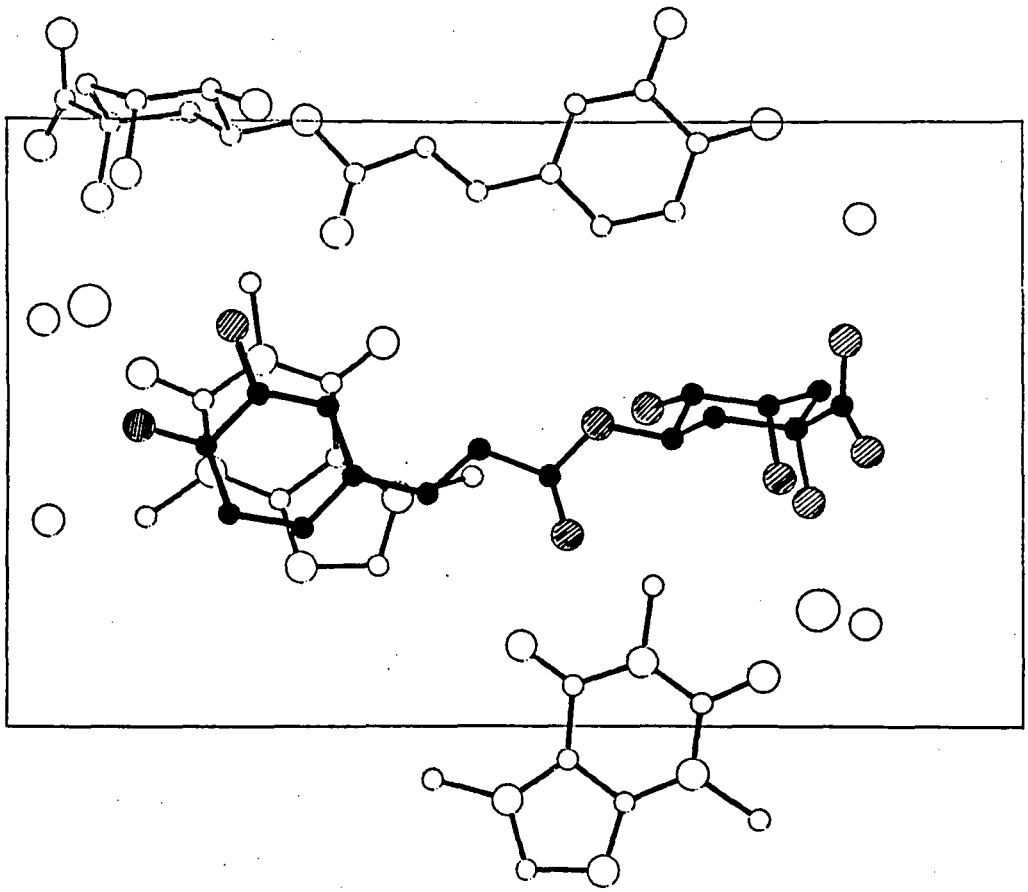
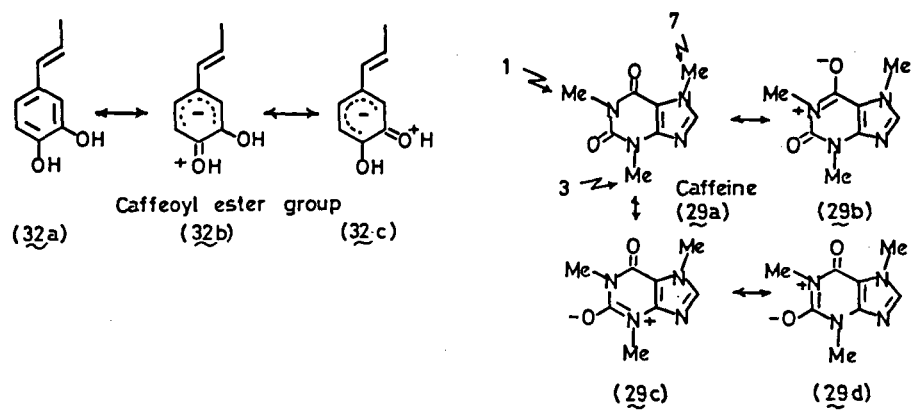


FIGURE 10. Crystal structure of the caffeine-potassium chlorogenate complex — apolar hydrophobic interactions, vertical stacking.

in this form of association, the two components (e.g., caffeoylester and caffeine) develop polar characteristics of the type shown (29, 32, a to c, etc.).



Previous work has shown that the heterocycle caffeine will reversibly associate with a range of substrates to form molecular complexes. The research of Mejbaum-Katzenellenbogen et al.[52] further demonstrates that caffeine competes effectively with proteins for polyphenolic

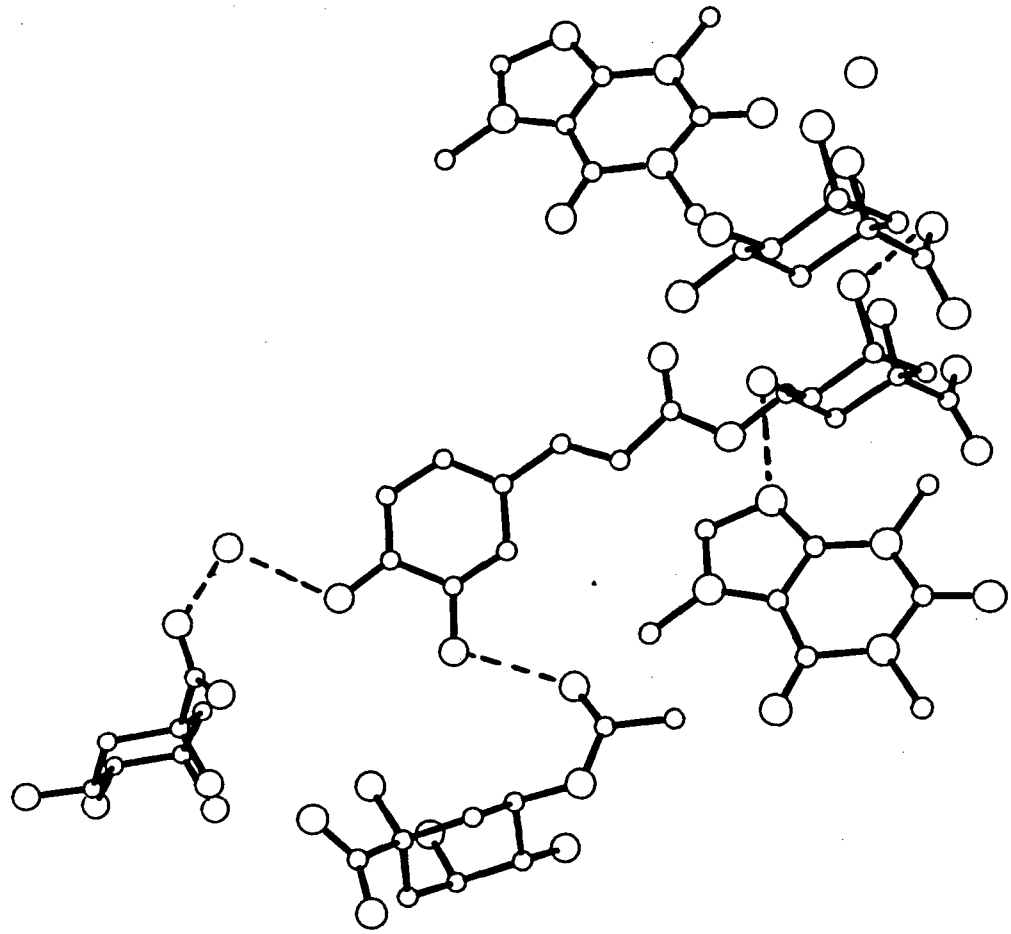


FIGURE 11. Crystal structure of the caffeine-potassium chlorogenate complex — intermolecular hydrogen bonding.

substrates and that it is possible to regenerate a wide variety of proteins, in a biologically active state, from insoluble protein-tannin complexes by treatment with caffeine. The heterocycle precipitates polyphenols from aqueous media by complexation and the ratio of caffeine to polyphenolic substrate in the complex is *higher* with *lower* initial concentrations of the polyphenolic substrate — an observation which, in a sense, is directly analogous to the stoichiometry of protein-polyphenol complexes formed by precipitation. Studies with two polyphenolic substrates, at equivalent molar concentrations — β-penta-*O*-galloyl-D-glucose (20) and β-1,3,6-tri-*O*-galloyl-D-glucose (26) — show, rather interestingly, that the latter forms complexes with a higher caffeine-polyphenol ratio before precipitation ensues. The association of caffeine with various polyphenols has been studied by microcalorimetry and by ^{1}H nuclear magnetic resonance (NMR) spectroscopy.[75] The latter technique exploits the tendency of phenolic molecules and caffeine to "stack" (Figures 8 and 10) in solution. This in turn results in a deshielding of the aromatic protons of the phenol and of the three methyl groups and the single proton of the caffeine molecule, and this causes upfield shifts in the respective ^{1}H NMR signals. Figure 13 shows the relationship between ln (K_{obs}) and ln (N), where N is the number of galloyl ester groups and K_{obs} is the heterotactic association constant for the binding of caffeine to a series of galloyl glucose derivatives in D_2O at 25°C. If the galloyl groups in each polyphenol bound caffeine independently and to the same

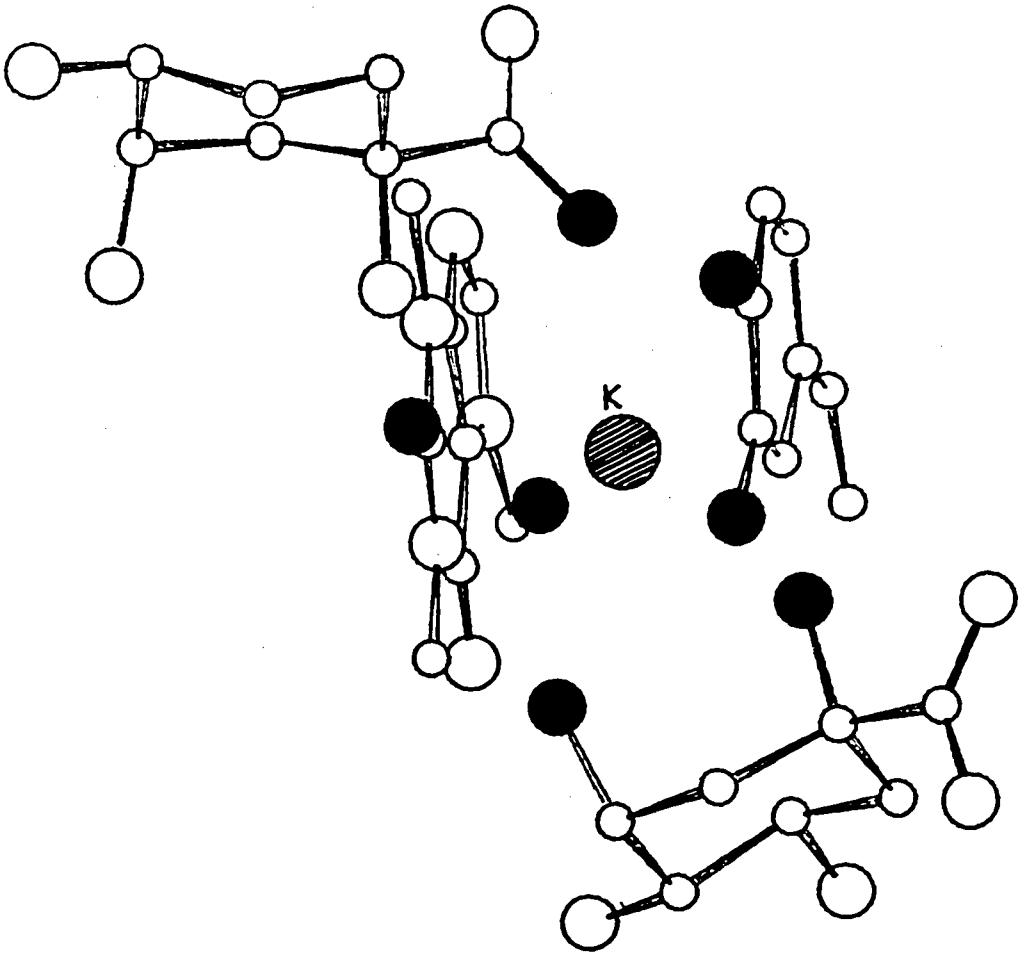


FIGURE 12. Crystal structure of the caffeine-potassium chlorogenate complex — coordination around a central metal ion (K^+).

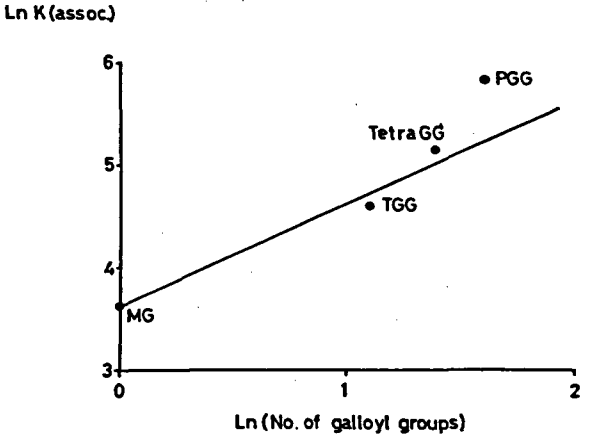


FIGURE 13. Caffeine-galloyl glucose complexation. Relationship between the association constant and N, the number of galloyl groups.

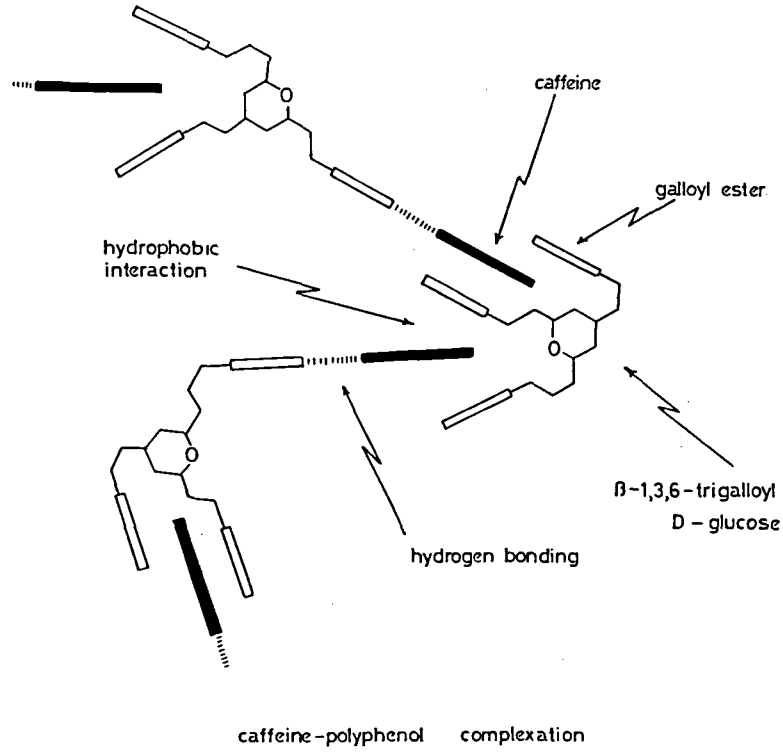
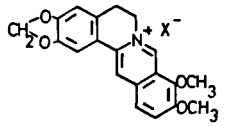
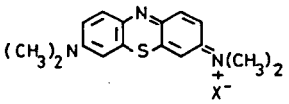


FIGURE 14. Caffeine-polyphenol precipitation.

degree, then the observed association constants would fall on a line of gradient 1 passing through ln K (methyl gallate) — the ideal (independent) binding model. For β-1,3,6-tri-galloyl-D-glucose, this is broadly true; on the other hand, for the polyphenols β-1,2,3,6-tetra-*O*-galloyl-D-glucose and β-penta-*O*-galloyl-D-glucose, these substrates show a binding reaction with caffeine which increasingly deviates from that of the independent binding model-statistical line. The nature of the deviation indicates that these polyphenols bind to caffeine to a much *greater* extent than would be predicted — probably indicative of co-operative binding or a chelating effect of vicinal galloyl ester groups on the caffeine molecule. Extrapolating from the crystal data described earlier, the aggregates which form in solution, sufficient to cause precipitation, probably therefore take the general form shown in Figure 14. In this model, it is postulated that the caffeine links separate polyphenol molecules, rather as the latter cross-link proteins in the analogous protein-polyphenol precipitation process (Figure 5). In the context of polyphenol complexation with caffeine, it is finally interesting to note that polyphenols may also be precipitated from solution by other substrates such as berberine (33), cinchonine, papaverine, quinine and strychnine, and the dyestuff methylene blue (34). It is perhaps significant to note that molecules such as berberine and methylene blue[79] are planar and bear a positive charge (formally located on nitrogen, but extensively delocalized around the ring system) and their interaction with polyphenols may be interpreted, in principle, in terms that are very similar to those of caffeine with the caffeoyl group of chlorogenic acid (*vide supra*, 32 a to c). Okuda and collaborators[79] have conducted a survey of natural polyphenols comparing their ability to precipitate hemoglobin (cf. relative astringency) with their propensity to precipitate methylene blue from solution (RMB). A close correlation between the two sets of data was observed.



(33)



(34)

C. Polysaccharides

Quantitative measurements of the affinity of natural polyphenols for polysaccharides have generally been hampered by the lack of water-soluble polysaccharides with precisely defined structures and molecular weights.[80] The most satisfactory data, to date, have been obtained using polysaccharides in the solid state, e.g., cellulose triacetate (in membrane form) and chromatography on cellulose and the Sephadex gels (G-25, G-50, and LH-20). These observations suggest a remarkably similar pattern of affinities to those noted above for proteins. The gradations in strength of binding are thus strongly influenced by both molecular size and conformational flexibility. However, molecular shape and mobility is seen as a rather more critical characteristic in the association with cellulose triacetate and cellulose. Thus, in the galloyl ester series, the formation of the intramolecular hexahydroxydiphenoyl ester group (→ 19) with its consequent freezing of elements of mobility has a rather more dramatic diminution on complexation than is observed with proteins.

The affinity of aromatic compounds for dextran gels is well documented, but its origins are uncertain. It is certainly not possible on present evidence to fully rationalize this phenomenon, but hydrogen bonding is presumably involved and a further significant factor is thought to be the sequestration of aromatic groups, or indeed whole molecular species within the cavities and pores of the gel. In this context it is certainly noteworthy that open-chain filamentous 1-α-6-dextrans bind polyphenols very weakly but those polysaccharides, such as amylose, which can develop secondary structure with hydrophobic cavities, e.g., the familiar helical forms, have an enhanced affinity for polyphenolic substrates.

This capacity of such polysaccharides to bind polyphenols more effectively has been examined at the molecular level using the cycloamyloses as model polysaccharides.[76,80] The cyclodextrins, sometimes referred to as Schardinger dextrans, are a series of cyclic oligosaccharides produced by the action of the amylase of *Bacillus macerans* on starch. They have the shape of a doughnut with all the D-glucopyranose units in substantially undistorted ($^4C_1$, C-1) chain conformations. The cavities are slightly "v" shaped: the secondary hydroxyl groups (at C-2 and C-3) on the upper side of the torus and the primary hydroxyl groups (at C-6) on the lower face. The interior of the torus consists of the glycosidic oxygen atoms (1-α-4) and two concentric rings of C–H groups (at C-3 and C-5; Figure 15). Compared to an aqueous environment, this cavity is relatively apolar. Because the primary hydroxyl groups (C-6) are free to rotate so as to block partially the entrance to the cavity while the secondary hydroxyl groups (C-2 and C-3) are constrained to fixed positions, the cavity is more accessible from the face bearing the secondary hydroxyl groups.

One of the most significant characteristics of the cyclodextrins is the formation of inclusion complexes in which guest substrates are sequestered in the cavity.[81,82] The nature of the binding and the driving force which lead to inclusion remain uncertain although several proposals have been canvassed. These include (1) van der Waals interactions, (2) hydrogen bonding, (3) release of solvent water molecule(s) from the cavity during complexation, and (4) the release of strain energy in the macrocycle.[81,82] Inclusion complex formation may be monitored by a number of spectroscopic techniques and guest substances range from small inorganic ions (ClO_4^-, SCN^-, and Br^-) to rare gases and aromatic compounds. ^1H NMR spectroscopy suggests that *p*-disubstituted aromatic substrates penetrate the cyclodextrin ring from the secondary hydroxyl group face (C-2 and C-3) with their axis parallel to the axis of the cyclodextrin cavity (Figure 15). Microcalorimetric and ^1H and ^{13}C NMR studies[76,80]

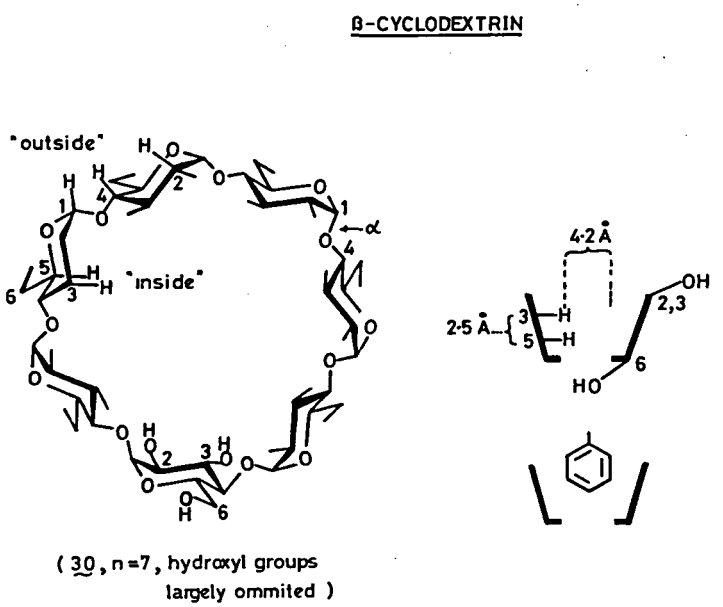


FIGURE 15. β-Cyclodextrin.

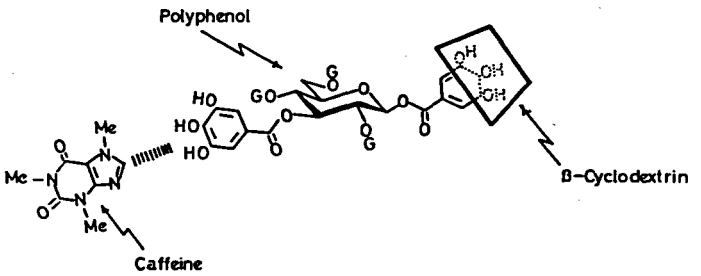


FIGURE 16. Cyclodextrin-galloyl glucose complexation. Encapsulation of the aromatic ring.

strongly support the view that galloyl esters, e.g., β-1,2,3,4,6-penta-*O*-galloyl-D-glucose (20), bond to β-cyclodextrin by encapsulation of the "phenolic end" of the galloyl ester group in the saccharide cavity (Figure 16).

The ternary system caffeine-β-cyclodextrin-polyphenol has been exploited to determine the manner in which polyphenols partition between the pseudopeptide (caffeine) and the model saccharide (β-cyclodextrin) in aqueous media (Figure 16). The results confirm the prediction that the extent of association of polyphenols with substrates such as proteins is likely to be substantially modified by the presence of polysaccharides, particularly those which, in aqueous media, develop secondary structures containing hydrophobic cavities. Similar conclusions were derived from other experimental investigations, most notably the relief of polyphenol-induced inhibition of the enzyme β-glucosidase by the cyclodextrins[65] and sodium polygalacturonate[65] and the effect of the cyclodextrins on the line width (^{1}H NMR) of the aromatic protons of galloyl ester groups measured in the presence of protein. The ^{1}H NMR signal from the aroyl protons of a galloyl ester group undergoes considerable line broadening in the presence of added protein due to polyphenol-protein association and the concomitant reduced rotational diffusion coefficient of the bound phenol. When β-

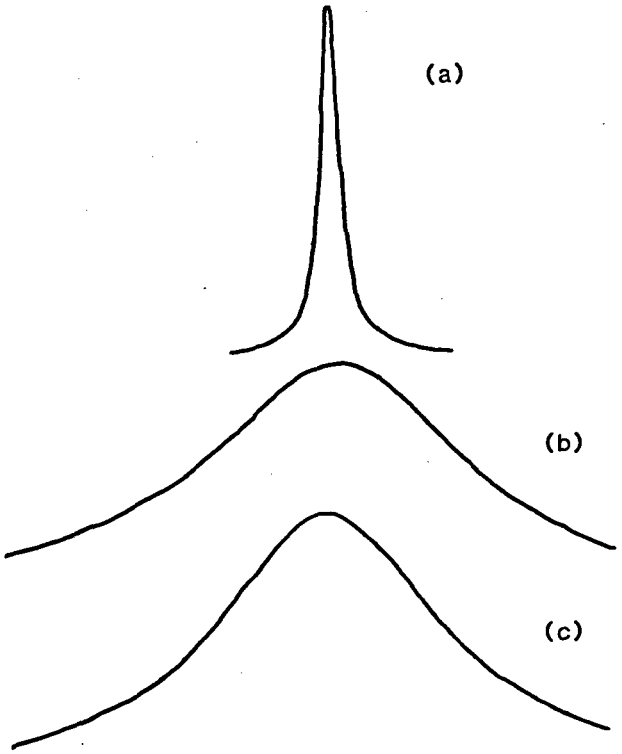


FIGURE 17. ^{1}H-NMR line broadening of the aromatic protons on (a) methyl gallate, (b) methyl gallate + BSA, and (c) solution b + β-cyclodextrin.

cyclodextrin is added to the system, the signal from the aroyl protons sharpens significantly once again and this certainly reflects the detachment of the phenolic substrate from the macromolecular protein surface by the competitive complexation process which takes place between the phenol and the cyclodextrin. This is illustrated in Figure 17 in the system methyl gallate-bovine serum albumin (BSA) and β-cyclodextrin.

III. ASTRINGENCY: FOOD AND FOOD PRODUCTS

The perception of astringency in the mouth is not instantaneous but rather requires time to develop and, if one accepts Bate-Smith's[11,12] view of its causes (*vide supra*), then the primary reaction is the precipitation of proteins and glycoproteins in the mucous secretions induced by complexation with the astringent principles. Insofar as fruit, wines, and teas are concerned, these principles are natural polyphenols and secondary products derived therefrom. The last decade has seen a considerable extension of our knowledge of the ways in which these substances complex with proteins and polysaccharides and contribute to the quality of astringency. Equally important from both a fundamental point of view and its practical implications is an understanding of the various ways in which the astringent response may be modified and ultimately lost, e.g., the loss of astringency in fruits upon ripening, the loss of astringency in wines and beers on storage and maturation, the formation of nonbiological hazes, and the development of fugal astringency in beverages.

A. Aging of Red Wines[83-97]

"The making of red wine, which involves the skins and pips as well as the juice of grapes,

leaves extra substances dissolved, above all tannin. This gives the wine the special quality of hardness of drying up the mouth. These extras need time to resolve themselves to carry out slow and obscure chemical changes which make all the difference in the world to the eventual glass of red wine." So wrote Hugh Johnson[83] in his famous book *Wine*. In fact, few subjects are more likely to cause a more heated discussion among the wine cogniscenti and the connoisseurs of claret than the subjective comparisons made between the great vintages. While a certain amount of tannin is both desirable and essential in any claret to give the wine body, longevity, and backbone, it is nevertheless possible, according to various authorities, to have too much. Where vintages have an apparent excess of tannin, the reason is invariably the weather, particularly when there are extreme variations. Thus, apart from fully ripening the grapes, a spell of intense summer heat thickens their skins — a major source of both tannin and pigments (anthocyanidins). If these factors are combined with, say, a relatively small concentrated yield, then this produces a claret that starts life with, among other attributes, a deep color and a high tannin content. The question as the wine matures over the next decade or so is whether the claret will retain sufficient "fruit and flavor" to balance the tannin content or whether it will retain a hard unyielding backbone of tannin throughout.

The processes involved in the aging of red wines have been of an unending fascination to man for centuries. There is no doubt that during this transformation the polyphenols (tannins) and pigments (anthocyanidins) which are present are of great importance.[84,90] However, progress toward defining their precise roles in molecular terms has been slow. In an admirable review of the state of the art written in 1969, Singleton and Esau[91] commented: "Unfortunately, what we would really like to know — the specific amount of each individual substance and how it participates and changes in wine in each type of storage reaction — requires much more detailed knowledge than is now available." However, now, almost two decades later, it is possible to suggest, at least in broad outline, the part that oligomeric and polymeric procyanidins (condensed tannins) probably play in the aging process.

In the stalks, skins, seeds, and, to a lesser extent, the leaves of *Vitis vinifera*, in addition to the various associated flavonols and hydroxystilbenes, the balance of polyphenols is present as proanthocyanidins of largely oligomeric and polymeric forms, some of which are soluble and others (*vide supra*) which are not. (+)-Catechin (11a) is the principle flavan-3-ol present and (−)-epicatechin (11b) is present in lesser amount; procyanidins B-1 (cat-4-β-8-epicat, 35) and B-2 (epicat-4-β-8-epicat, 36) are the principal diastereoisomeric procyanidin dimers present. Treatment of the polymeric procyanidins with acid gives both cyanidin (37) and delphinidin (38) in the approximate ratio 4:1. This evidence clearly implies that in the vinification of red wines, the expressed juices in the fermentation will contain the flavan-3-ols (11a, b), some procyanidin dimers, and, depending on their solubility, various oligomeric proanthocyanidins of differing molecular size and constitution.[88,89] On the basis of present evidence, these are likely to include species such as (7) composed entirely of flavan-3-ol structural units and those such as (16) in which the flavan-3-ol oligomers are bound to saccharide structures (*vide supra*). In order to fully comprehend the processes that occur during the aging of red wines, it is essential to mention briefly the two characteristic chemical reactions of pro(antho)cyanidins: (1) the acid-catalyzed rupture of the interflavan bonds of pro(antho)cyanidins[92] and (2) the facile electrophilic substitution of the phloroglucinol "A" ring of the flavan-3-ol units which comprise the oligomeric structures. The first of these reactions has been referred to previously (*vide supra*, 7 → 8), and two examples of the second type of chemical reactivity are shown in Figure 18: (1) the reaction with aldehydes, under acid catalysis, which leads to condensation products [the reaction shown is that of vanillin with a flavan-3-ol and is the basis of the familiar color reaction for flavan-3-ol metabolites in which a red color, probably due to the quinonoid intermediate (39), is formed[93]], and (2) the reaction with the flavan carbocation which leads to procyanidin dimers and

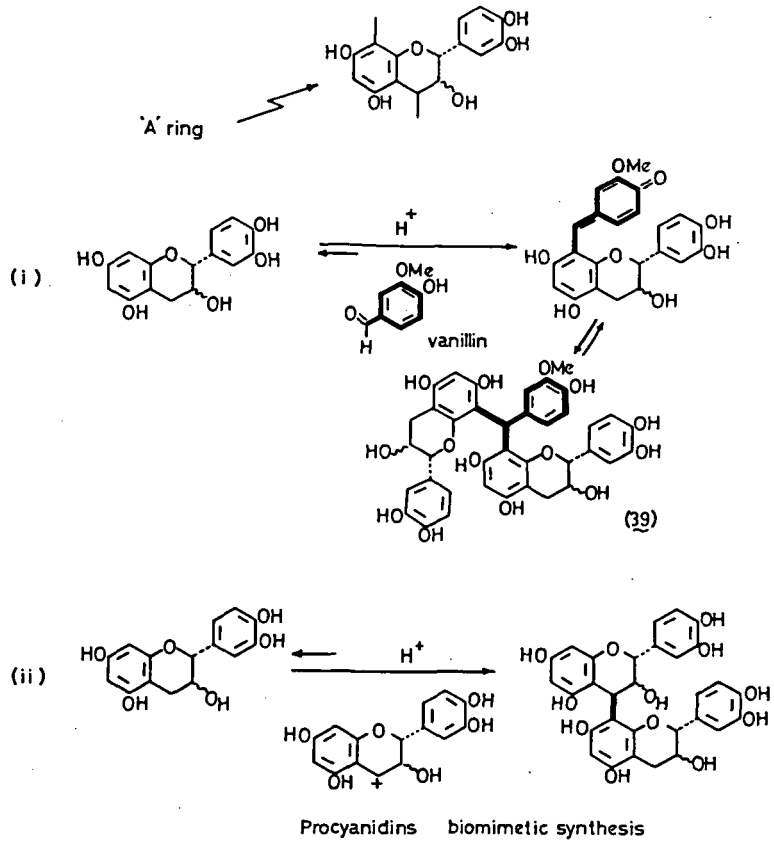
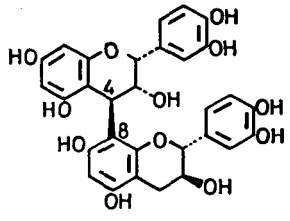
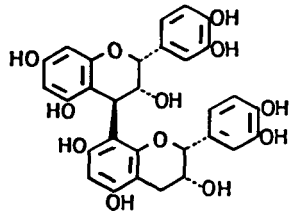


FIGURE 18. Electrophilic substitution of flavan-3-ols.

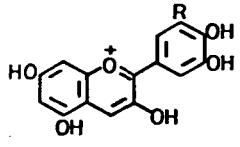
higher oligomers — biomimetic synthesis.[94] Under the mildly acidic (pH 3 to 4) conditions which appertain in wines, both of these familiar and characteristic types of carbon-carbon bond-breaking and bond-making processes are believed to occur.



(Procyanidin B-1, 35)
epicatechin 4β→8 catechin



(Procyanidin B-2, 36)
epicatechin 4β→8 epicatechin



(37, R=H, cyanidin)
(38, R=OH, delphinidin)

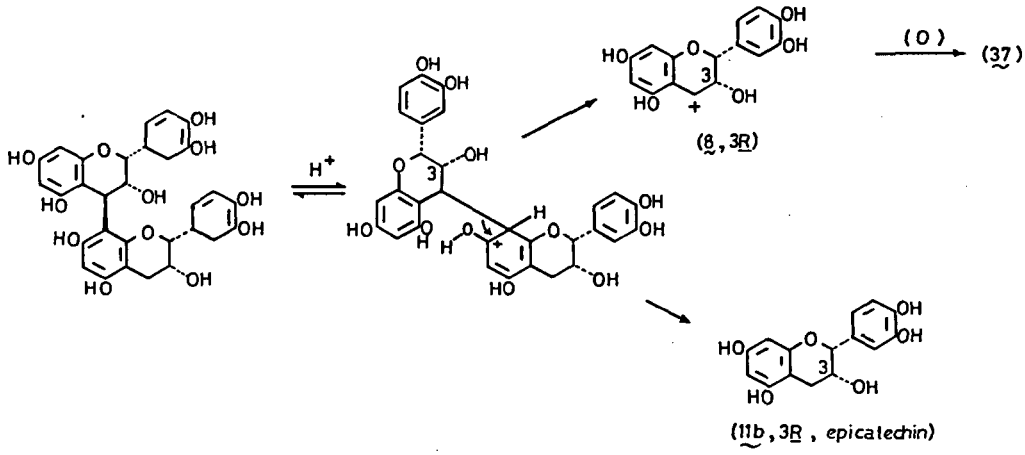


FIGURE 19. Procyanidin B-2 — acid-catalyzed breakdown.

The hydrolytic decomposition of various procyanidins has been subject to a detailed kinetic analysis;[95] the reaction is a specific acid-catalyzed one which is first order in hydrogen ion concentration. Mechanistically, the rate-determining step is the protonation of the phloroglucinol type "A" ring in the flavan-3-ol oligomer (Figure 19). Extrapolation of the detailed kinetic data obtained for procyanidin B-2 (Figure 19) has permitted a rough estimate for the hydrolytic rate constant at pH 4.0 (such as appertain in wines and beers) and at 25°C to be made — $K_{obs} = 6 \times 10^{-6}\ hr^{-1}$. For example, in acetate buffer solutions at pH 4.1 at 25°C both procyanidin B-2 (36) and a soluble oligomeric procyanidin fraction from hawthorn (*Crataegus monogyna*) at concentrations at 50 to 100 ppm gave steadily over 7 weeks a fine brick-red precipitate (phlobaphen). This heterogeneous polymer results presumably from the interplay of the two characteristic carbon-carbon bond-breaking (7 → 8) and bond-making (Figure 18) processes referred to above and is depicted schematically in Figure 20. In the case of red wines, as high molecular weight oligomers are formed and precipitate from solution, then the equilibrium moves away from the various soluble oligomeric forms (7 and 16) and the associated astringency is lost from the beverage.

Aging of red wines first in oak barrels and then in corked glass bottles produces other desirable effects on quality, taste, and flavor which may also lead to the loss of the astringent polyphenols from the wine. The color, for example, shifts from a purplish-red in the young wine to the more fiery amber of the mature wine, and ultimately to the tawny hues of a long-aged wine. This is, at one and the same time, one of the most readily appreciated visual aspects of wine aging and yet one of the least well understood.[86] Somers[90] has studied this problem in great detail. During the aging of red wines, the grape anthocyanins responsible for the initial color of the wine are displaced progressively and irreversibly by more stable polymeric pigments which for a wine within the first year may account for up to 50% of the observed color density. These new pigment forms are, moreover, much less sensitive to changes in pH. Somers has suggested, on the basis of the model reactions of flavylium salts discovered by Jurd and Waiss, that the anthocyanins, such as occur in wines, are susceptible to attack by nucleophilic reagents including phenolic substrates such as (+)-catechin. The sequence of reactions by which the color of young wines may be altered and stabilized during aging is thought therefore to be of the type shown in Figure 21. In this case, electrophilic substitution into the oligomeric flavan-3-ol structure is carried out by the anthocyanin molecule leading by involvement of a red-ox reaction to the condensed anthocyanin species (40). (Somers suggested the more stable deprotonated quinonoid structure for this oligomeric pigment.) The enhanced stability of these pigments is believed to be due

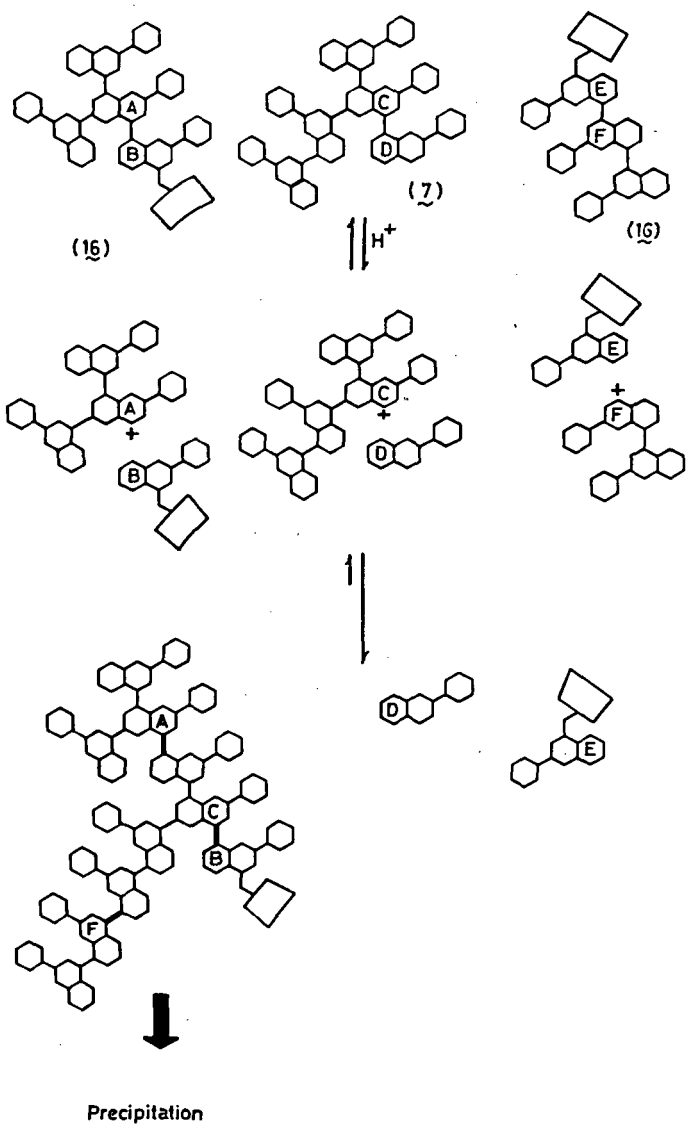


FIGURE 20. Proanthocyanidin oligomers — acid-catalyzed polymerization.

to the aryl substitution of the anthocyanin nucleus at the 4-position. Clearly, further substitution of the anthocyanin is also possible by reaction with an additional oligomeric procyanidin (16). Alternatively, polymerization initiated by the acid-catalyzed bond-making or bond-breaking processes discussed above (Figure 20) would lead ultimately to precipitation of the polymeric pigmented species and at the same time to loss of astringency in the wine in an entirely analogous manner to that described previously.

Finally, another electrophilic condensation reaction that is probably of significance in the aging of red wines with the associated loss of astringency is that with acetaldehyde.[96] This reaction is entirely analogous to that described above with vanillin (Figure 18), and the cross-linking of separate oligomeric flavan-3-ol molecules in solution would eventually lead to molecular species of sufficient size to precipitate from solution (Figure 22). Oxidation and its control have been primary considerations in both wine making and aging for more than a century. Riberau-Gayon[97] showed that a wine under barrel storage would be expected

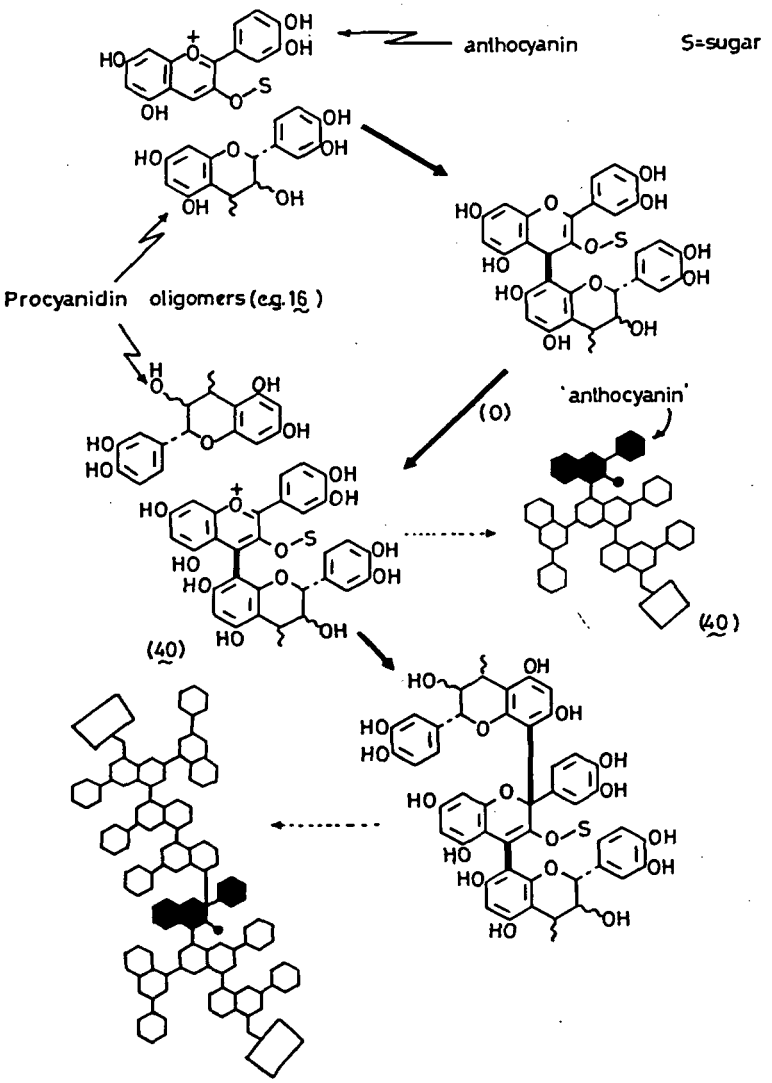


FIGURE 21. Proanthocyanidins — electrophilic substitution and polymerization. Reaction with anthocyanins in red wines.

to adsorb about 30 mg O_2 per litre per year and even in the corked bottle there is evidence for oxygen penetration. Acetaldehyde formation in wine aging presumably derives from the oxidation of ethanol and in a very early series of papers it was clearly implied that it subsequently combined with and precipitated the tannins and pigments of red wine. The typical cross-linking reactions suggested in Figure 22 are almost certainly the manner in which this final mode for the loss of the astringent principles (oligomeric procyanidins) of red wine takes place. A complete understanding, particularly at the quantitative level, of the manner in which each particular substrate in red wine participates and changes during maturation is not yet possible. However, the evidence presently available strongly suggests that each of the processes outlined in Figures 20 to 22 is strongly implicated in the polymerization of polyphenols which undoubtedly underlies the processes involved in the loss of astringency from red wines on aging.

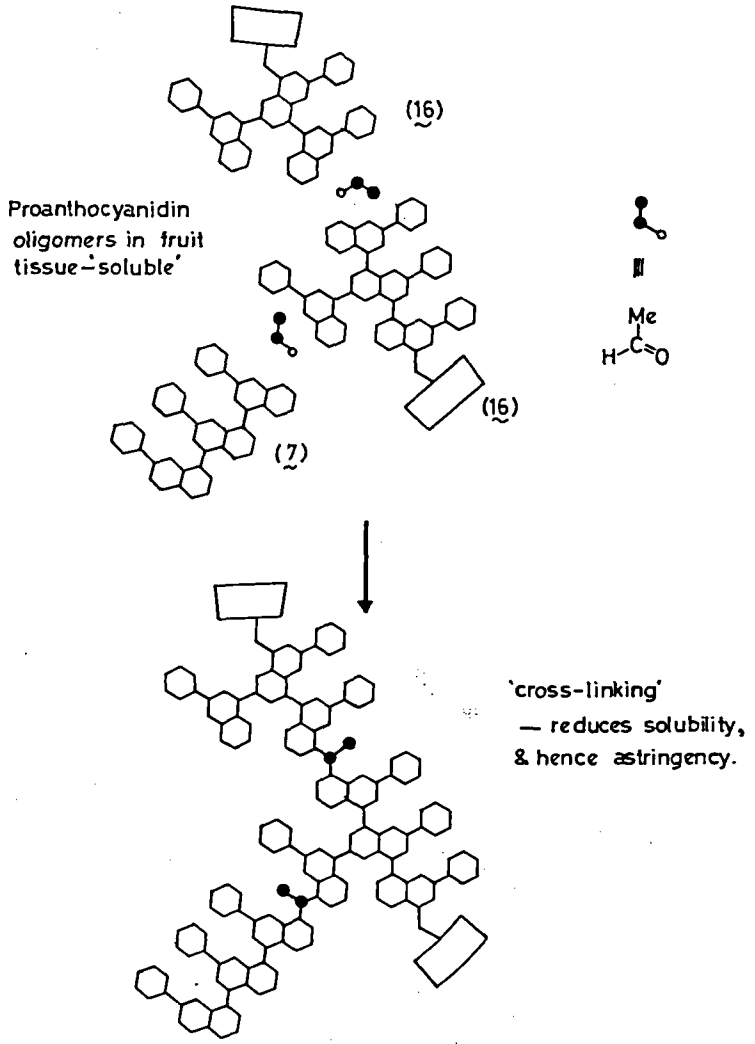


FIGURE 22. Proanthocyanidins — electrophilic substitution and polymerization. Reaction with acetaldehyde in red wines and fruit.

B. Nonbiological Hazes in Beers

The importance of polyphenols in brewing of beers and lagers has long been recognized particularly in relation to the development of chill and permanent haze in such beverages. Chill hazes can appear on strong cooling, but redissolve on warming. Permanent hazes, as the name suggests, appear but do not redissolve. Analysis shows that the principal components of such hazes are proteins, polyphenols, and carbohydrates along with traces of inorganic matter.[95] The polyphenols are of the proanthocyanidin type since the haze, if collected by centrifugation, gives with acidic butanol an initial reaction (producing cyanidin) typical of this class of substance.[95] Beers and lagers contain significant quantities of proteins and barley gives rise to some soluble oligomeric proanthocyanidins.[98] Together the polyphenols and proteins associate to give a series of soluble complexes (Figures 4 and 5). Chilling the beer lowers their solubility and aggregation, and the formation of a haze ensues. Warming the solution increases the solubility of the protein-polyphenol complexes and the haze redissolves. The formation of the permanent hazes takes place over a period of time

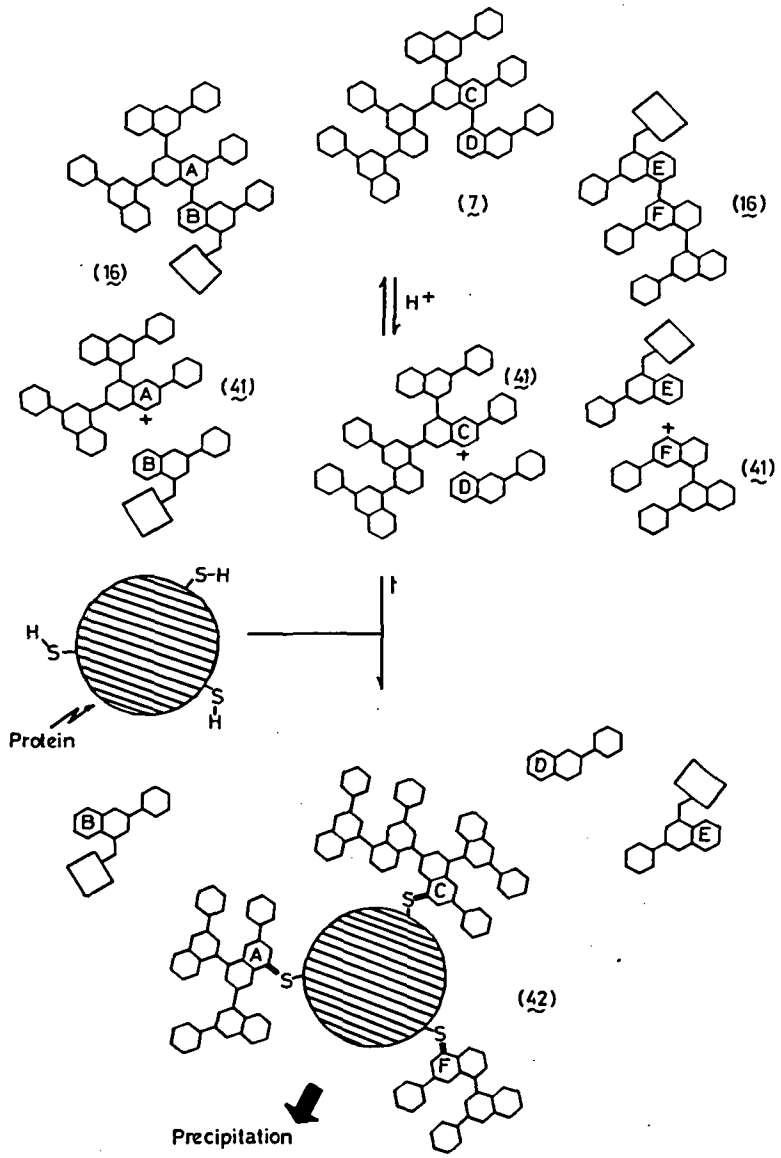


FIGURE 23. Proanthocyanidins — acid-catalyzed reaction with − SH groups in protein. Permanent haze in beers.

and involves the slow acid-catalyzed (pH ~4.0) bond-breaking and bond-forming reactions so characteristic of the proanthocyanidins (*vide supra;* Figure 20). Under the weakly acidic conditions that prevail, decomposition of the proanthocyanidin structure occurs within the preformed protein-polyphenol complexes to generate the characteristic flavan-3-ol-based carbocation (41). However, instead of polymerization, the reactive species, it is postulated, is captured by nucleophilic (−SH) groups on the protein structure (Figure 23; 42).[95] Kinetic studies show[95] that thioether adducts such as (42), although themselves acid labile, have much greater kinetic stability in acidic media than do the parent proanthocyanidins. This process lends the surface of the protein a much greater hydrophobic character. This ultimately results in aggregation and finally separation of an insoluble haze.

C. Fruit Ripening[1,99-107]

Writing some 25 years ago, Goldstein and Swain[99] noted that "Loss of astringency is one of the major changes which takes place during the ripening of many edible fruits. It is generally agreed that this property is due to the presence of tannins, but although some astringent fruits show a reduction in tannins on ripening, others do not. Even in those fruits where both tannins and astringency are reduced on ripening, the biochemistry underlying these changes has not been fully studied, and it is not at all obvious how the two are interrelated." They suggested that, in several fruits, the loss of astringency which occurs on ripening is most probably connected with the increased polymerization (and hence decreased solubility) of tannins. The thesis was later modified in view of certain inconsistencies, and the question remains a fundamental and intriguing one. Variety, maturity, and climate are known to influence the astringency of fruit, and present evidence suggests that it is at present preferable to regard each fruit as an individual case rather than attempting an all-embracing explanation for the phenomenon. Two examples best illustrate the question.

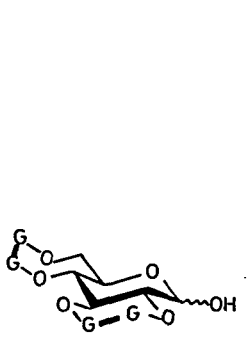
1. Persimmon

Japanese persimmon fruit (*Diospyros kaki*) such as cv. "Hiratanenashi" and "Yokono" are remarkably astringent often even at a mature stage.[100-102] This astringency invariably is assumed to be due to tannins present in the fruit of the proanthocyanidin type. The structures of the soluble oligomeric proanthocyanidins are based on a flavan-3-ol monomer units (+)-catechin, (+)-catechin-3-gallate, (+)-gallocatechin, and (+)-gallocatechin-3-gallate, and on acid treatment they release both cyanidin and delphinidin.[101] The tannin (kaki tannin) has a very strong affinity for proteins and this property has, for example, been utilized successfully in the brewing of sake (Japanese rice wine) and the manufacture of fishing nets. Its presence in the fruit may also lead to medical problems. When an excess of fruit is consumed, this often leads to the formation of insoluble conglomerates in the stomach which may require surgery to effect their removal. As early as 1911, it was found that the astringency of persimmon fruits was removed substantially by treatment of the harvested fruit with a carbon dioxide-enriched atmosphere. Later reports have indicated that treatment for 1 to 2 days with a high concentration of carbon dioxide (80%) gives deastringent fruit which retain their quality and firmness.[103,104] Another, though less efficacious method, is an analogous treatment with nitrogen atmospheres.[105] Nakamura showed that in naturally nonastringent varieties of persimmon fruit, the level of acetaldehyde (ethanol) was some ten times greater than in astringent varieties.[105] It has been demonstrated subsequently that the carbon dioxide treatment of immature fruit similarly enhances the acetaldehyde content.[106] Matsuo and Ito[103] have proposed that the loss of astringency is due to the immobilization of the kaki tannin within the fruit and is caused by reaction with acetaldehyde. In model experiments they showed that at pH 6 to 8 acetaldehyde reacts in a relatively short time with kaki tannin to produce a gel.[103] Quite clearly in this instance the evidence points unmistakably to the conclusion that, in the presence of acetaldehyde, cross-linking of proanthocyanidin oligomers occurs with concomitant loss of solubility and loss of astringency in an entirely analogous manner to that suggested earlier (Figure 22) for the insolubilization of proanthocyanidins which occurs in red wines on aging. Whether similar changes occur upon ripening in other fruit which contain proanthocyanidins, such as apple and cranberry, is still far from clear. Experimental data, for example, suggest that in many proanthocyanidin-containing fruits, the ripening process is *not* accompanied by a substantial diminution of soluble polyphenolic content of the fruit.

2. Raspberry and Blackberry

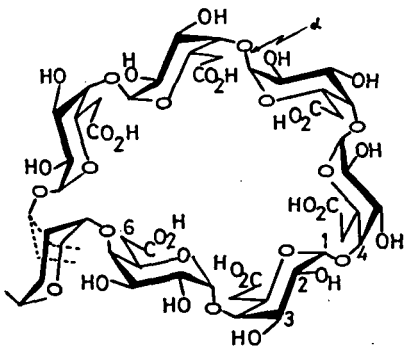
The principal polyphenols in fruit of *Rubus* sp., such as raspberry and blackberry, are based on gallic acid and hexahydroxydiphenic acid and the major phenolic metabolites are

pedunculagin (43), casuarictin (28), and the dimer (24; Figure 3). During the ripening process and in the ripened fruit there is again no substantial or significant quantitative or qualitative changes in the phenolic content of the fruit. This evidence presages an alternative explanation for the loss of astringency which demonstrably occurs upon ripening.



(43)

(1→4) POLY-α-D-GALACTURONIC ACID



(44, $-CO_2H$ as $-CO_2Na$)

An interesting possibility that has not been investigated in some detail is that other "macromolecular species" become available (soluble) in the ripened fruit and subsequently modify and disrupt the ability of polyphenols to bind to glycoproteins in the mouth when the fruit is tasted. Thus, it is well known and fully documented that in the ripening process the cellular structure of many fruits is changed, softening occurs, and this is accompanied by the release of smaller pectin fragments in which some deesterification of the galacturonic methyl ester units has occurred.[107] The ability of molecules such as alkaloids, soluble polysaccharides, and α- and β-cyclodextrins to disrupt the complexation of polyphenols with proteins has been examined using the enzyme inhibition assay (*vide supra*).[65] Polyphenols inhibit the enzyme β-glucosidase by virtue of classical noncompetitive means, and characteristic inhibition constants K_i may be determined. Addition of other proteins (lysozyme, BSA), alkaloids such as caffeine, α- and β-cyclodextrins, and, significantly, sodium polygalacturonate (44) substrates, all of which complex in particular ways with polyphenols (*vide supra*), relieve the induced polyphenol inhibition of the enzyme β-glucosidase. They all thus act as competitors for the polyphenolic inhibitor (Figure 24). It thus seems distinctly possible, if not highly probable, that in many fruit the cause of the loss of astringency which occurs on ripening is not any great change in soluble polyphenolic content (quantitative or qualitative), but rather is the formation of significant amounts of water-soluble fragments of the pectin structure[107] (Figure 25), quantities sufficient to effectively compete with the mucal glycoproteins for the polyphenol substrates when the fruit is tasted.

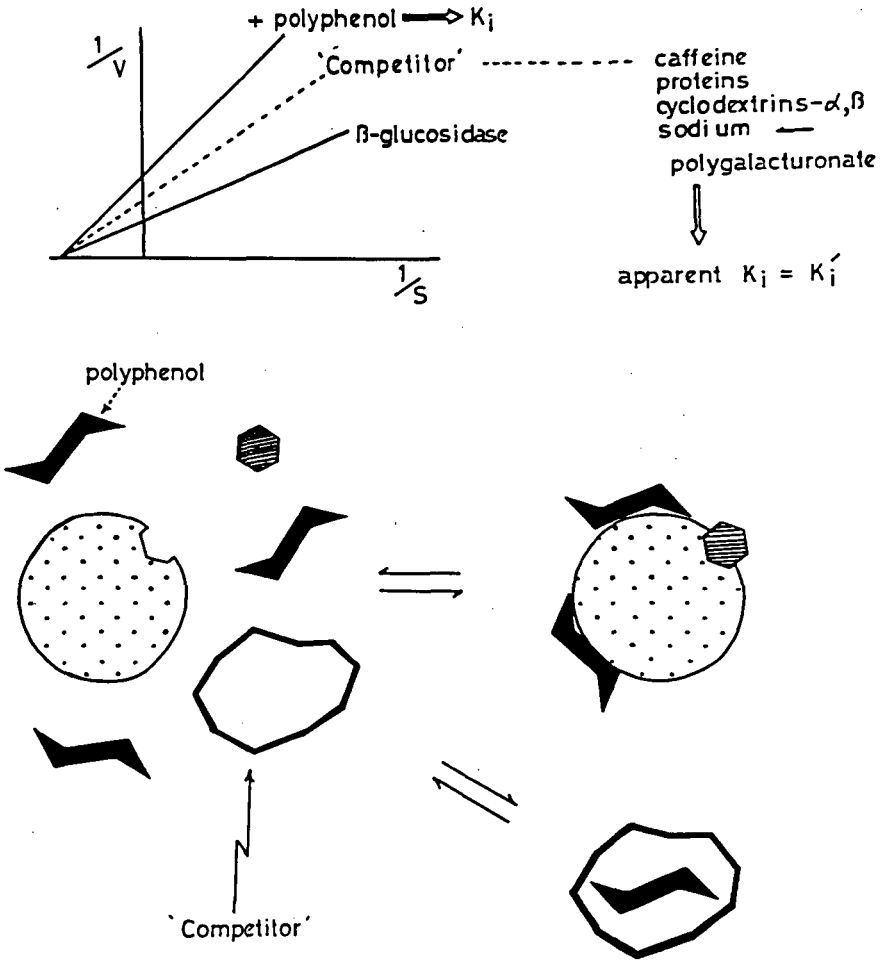


FIGURE 24. Competitors and the relief of enzyme inhibition by polyphenols.

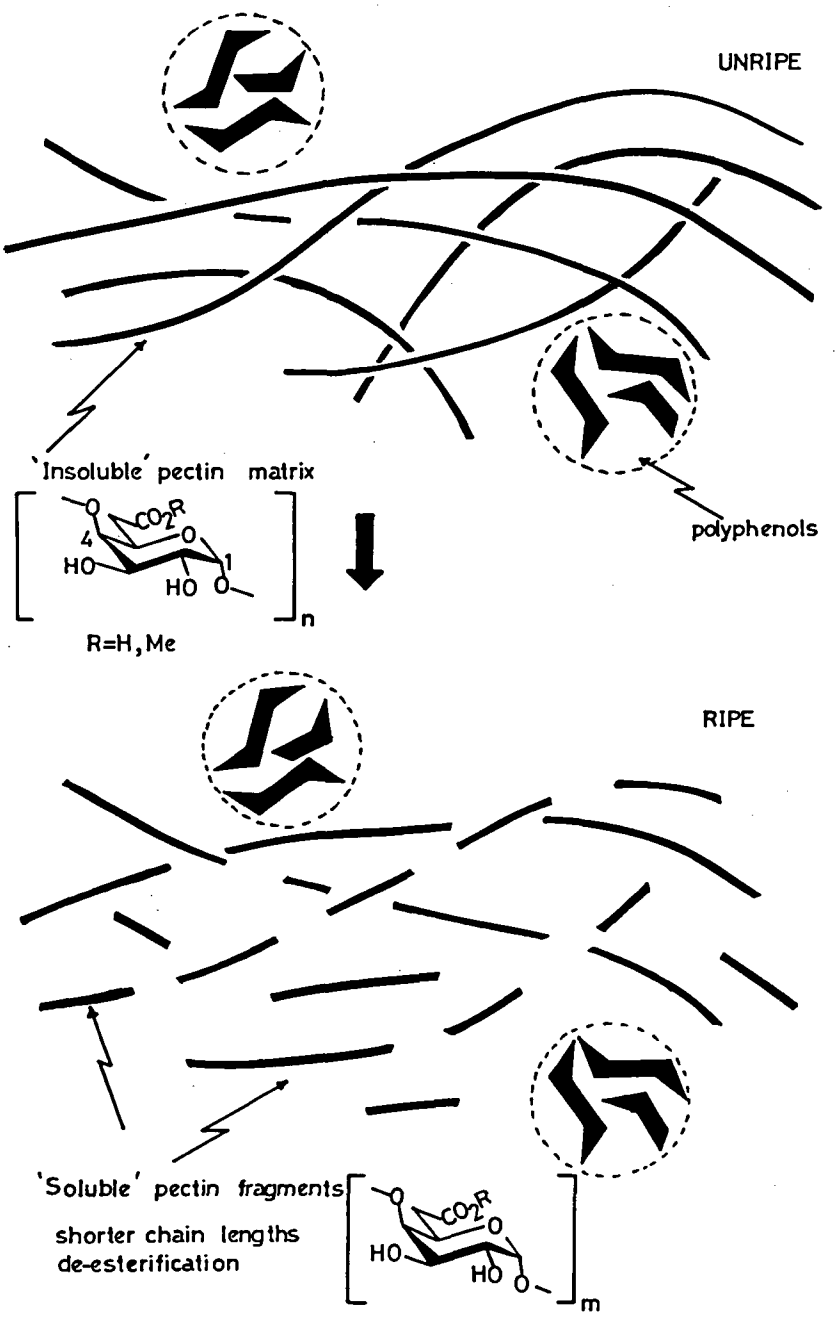


FIGURE 25. Fruit ripening — loss of astringency in blackberry (*Rubus* sp.).

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