Interaction of Plant Polyphenols with Salivary Proteins

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ABSTRACT. Tannins are polyphenols that occur widespread in plant-based food. They are considered to be part of the plant defense system against environmental stressors. Tannins have a number of effects on animals, including growth-rate depression and inhibition of digestive enzymes. Tannins also have an effect on humans: They are, for example, the cause of byssinosis, a condition that is due to exposure to airborne tannin. Their biological effect is related to the great efficiency by which tannins precipitate proteins, an interaction that occurs by hydrophobic forces and hydrogen bonding. Two groups of salivary proteins, proline-rich proteins and histatins, are highly effective precipitators of tannin, and there is evidence that at least proline-rich proteins act as a first line of defense against tannins, perhaps by precipitating tannins in food and preventing their absorption from the alimentary canal. Proline plays an important role in the interaction of proline-rich proteins with tannins. In contrast, it is primarily basic residues that are responsible for the binding of histatins to tannin. The high concentration of tannin-binding proteins in human saliva may be related to the fruit and vegetable diet of human ancestors.

Key words. Plant polyphenols, tannin, salivary proteins, proline-rich proteins, histatin.

(I) Introduction

In recent years, there has been an increased interest in polyphenolic compounds found in plant foods. This is partly due to the mounting evidence that some of these, the flavonoids, may have beneficial effects on humans. In contrast, other polyphenols, including the so-called tannins, may interfere with the growth and well-being of livestock and could potentially also be harmful to humans. It is generally believed that tannins are synthesized by plants to act as deterrents to animals because of their bitter, astringent properties. The challenge to animals is then to be able to eat plant foods without suffering ill effects from tannins, and recent studies indicate that saliva may play an important role in a defense against tannins. This review concerns the interaction of polyphenols and salivary proteins and the biological consequences of such interaction. Other aspects of polyphenols will be covered to the extent that they are necessary to the understanding of polyphenol-salivary protein interaction. A more extensive description of dietary tannins can be found in the monograph by Salunkhe *et al*. (1990).

(II) Plant Polyphenols

Polyphenols constitute one of the most common and widespread groups of substances in plants. They are considered to be secondary metabolites and have no specific metabolic function in plant cells. However, it is becoming increasingly clear that many of these phenolic compounds are essential to plant life—for example, by providing defense against microbial attacks and by making food unpalatable to predators. Several thousand plant polyphenols are known. These are substances that contain at least one aromatic ring with one or more hydroxyl groups in addition to other substituents, and they can be divided into 15 major classes according to their chemical structure (Harborne and Simmonds, 1964). Among these classes are compounds with a C_6 aromatic ring such as phenol, those with a C_6-C_1 structure such as gallic acid (Fig. 1A), and others with more complex skeletons such as the C_6-C_3-C_6 structure of flavonoids (Fig. 1B).

(A) Flavonoids

Over 4000 flavonoids have been described (Middleton and Kandaswami, 1994), and they constitute the largest and most diverse family of polyphenols. The common structure consists of two aromatic rings linked by 3 carbons, most often forming a heterocyclic ring as shown in Fig. 1B. There are two branches of the flavonoid family: 3-desoxyflavonoids and 3-hydroxyflavonoids. Variations in position, number, and nature of substituents give rise to a huge number of different flavonoids. Flavones, which are desoxyflavonoids (Fig. 1C), and flavonols (3-hydroxyflavonoids) (Fig. 1D) are the most common flavonoids, and they can occur either as aglycones or as glycosides (Herrmann, 1988). An example is quercetin, which constitutes the major part of flavonols. Flavan-3-ols, or catechins (Fig. 1E), also occur in polymerized forms as proanthocyanidins or condensed tannins, and epigallocatechin-gallate, a derivative of a flavan-3-ol, is a major component of tea (Fig. 1F). Flavonoids have anti-oxidant and free-radical scavenging properties, and these activities may protect plants against ultraviolet light, insects, fungi, viruses, and bacteria.

(B) Tannins

Tannins constitute a complex group of naturally occurring polymers, and a rigorous chemical definition is difficult. The term was originally used to describe vegetable components that are responsible for converting animal hides into leather in the process of tanning by forming stable complexes with skin collagen. Thus, tannins are considered to be polyphenolic metabolites of plants with a molecular weight larger than 500 and with the ability to precipitate gelatin and other proteins from solution (Mehansho *et al*., 1987a), but it should be noted that other

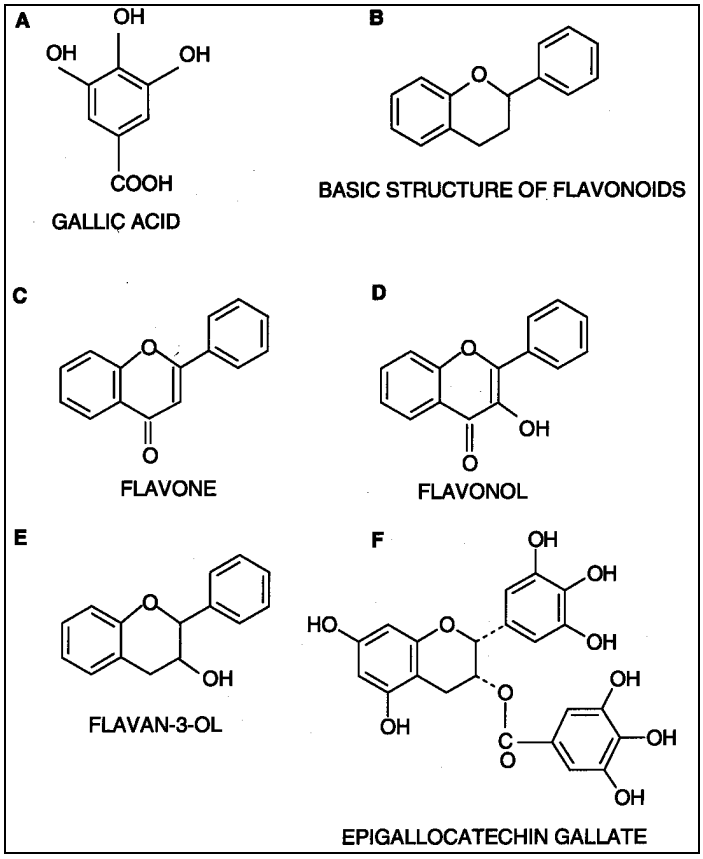

Figure 1. Structure of representative polyphenols.

phenolics may bind strongly to protein without causing precipitation. Based on their structure, tannins can conveniently be divided into two groups, hydrolyzable and condensed tannins.

(A) Hydrolyzable tannins

These tannins consist of a polyhydric alcohol, such as glucose, to which is linked gallic acid or its dimer hexahydrodiphenic acid in ester linkages. Considerable structural variation is introduced by additional molecules of gallic acid linked depsidically to other gallic acid moieties. As the name implies, these compounds are easily hydrolyzed in alkali, giving rise to a polyhydric alcohol and gallic acid, in the case of gallotannins, or ellagic acid, the condensation product of hexahydrodiphenic acid, in the case of ellagitannins. An example of a hydrolyzable tannin is the tannic acid pentagalloyl glucose, shown in Fig. 2A.

(B) Condensed tannins

The monomeric unit is a flavan-3-ol such as catechin or epicatechin that is linked through C-C bonds (Fig. 2B). They can be depolymerized in hot strong acid, giving rise to anthocyanidin pigments and other products. Hence, proanthocyanidin is an alternative name for condensed tannin. The degree of polymerization varies considerably from a few to more than 50 flavanol molecules, and condensed tannins with molecular weights larger than 30,000 have been described (Wünsch *et al.*, 1984). While small molecules of condensed tannins are soluble in aqueous or organic solvents, the large polymers are insoluble, and this has hampered their analysis, including determination of the amounts of condensed tannins in plant foods.

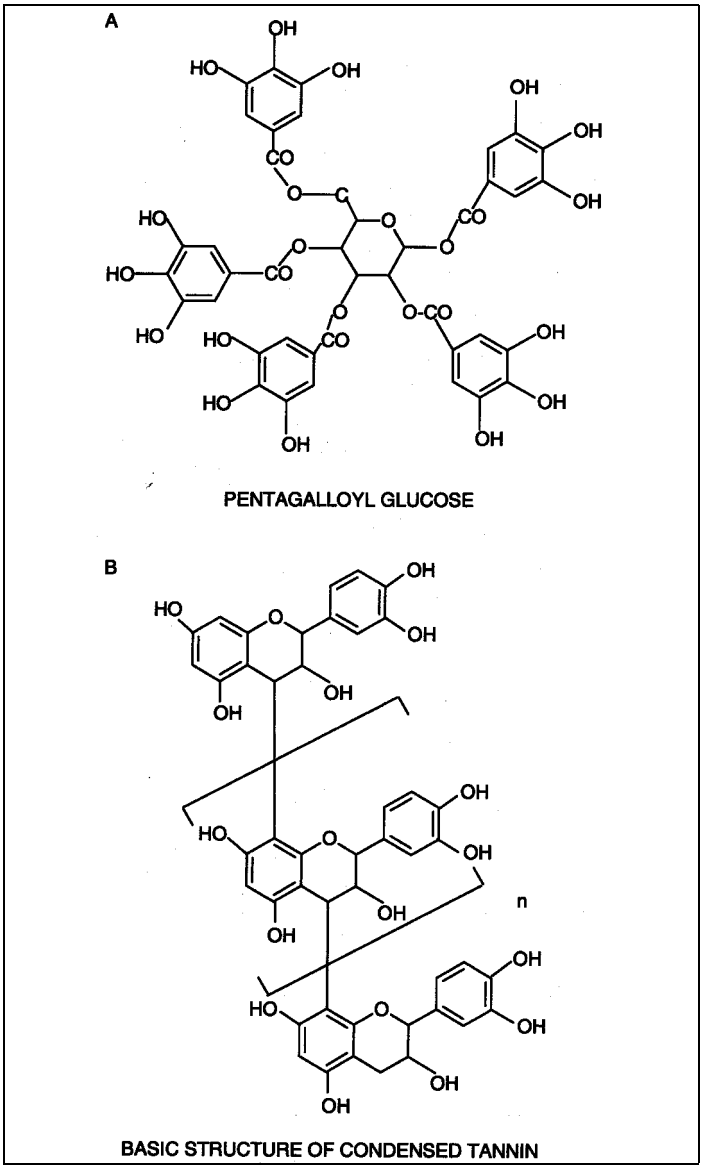

Figure 2. Structure of representative tannins.

(III) Biological Roles of Tannins in Plants

Although phenolic plant compounds are secondary metabolites without any known specific metabolic function, it is becoming increasingly clear that tannins have several roles that are important for plant biology. The astringent properties of tannin may protect plant leaves by making them unpalatable to browsing animals, and the astringency that tannins contribute to unripe fruit results in their avoidance by herbivores until the seeds are ready to disperse. The chemical basis for the defensive role of tannins has been attributed to their ability to precipitate plant protein and to inhibit gastrointestinal enzymes, thereby reducing the digestibility of plant proteins (Zucker, 1983). This suggestion is supported by the observation that reduction in plant protein digestibility by deer, moose, and elk was proportional to the protein-precipitating capacity of tannins in the feed (Robbins *et al.*, 1987). Moreover, the tannin content of sorghum, an important food grain in many African and Asiatic countries, has been correlated positively with its resistance to depredation by birds (Tipton *et al.*, 1970). The content

of tannin in seeds has also been linked to the inhibition of preharvest seed germination (Harris and Burns, 1970) and seed molding (Harris and Burns, 1973). These observations indicate that tannins in plants serve as a defense against environmental stressors, and, taken together, they show that the presence of tannins is beneficial to plants in several ways.

Interestingly, tannins in decaying plant materials inhibit their degradation (Benoit and Starkey, 1968), and in this manner they may also have an ecological effect by controlling rates of nitrogen release and the build-up of organic matter in the soil. This in turn will affect plant growth and, indirectly, the dependent animal and microbial population.

(IV) Effects of Tannins on Animals

The selection of food by animals appears to some extent to be based on its tannin content (McArthur *et al*., 1993), perhaps to minimize the unpalatable astringent properties of tannin. However, besides their astringent effects, tannins can also influence animal metabolism in several ways by interfering with normal growth and metabolism. Consequently, there has been a great interest in the effect of tannin content in animal feed on livestock, but it has also become clear that, under various circumstances, tannins may affect humans adversely. It is noteworthy that tannin administered to experimental animals *per rectum* may be considerably more toxic than when it is given by mouth (Boyd *et al*., 1965), pointing to the existence of mechanisms in the upper part of the alimentary canal that neutralize the harmful effects of tannin.

Several studies have reported that tannin in the feed of experimental animals leads to decreased growth and body weight gain (Joslyn and Glick, 1969; Jambunathan and Mertz, 1973; Featherstone and Rogler, 1975), and tannin has also been reported to impair calcium absorption (Mitjavila *et al*., 1977; Chang *et al*., 1994), which potentially could also affect bone metabolism. Tannins can also interfere with iron absorption, an effect that may be related to the presence of galloyl groups (Brune *et al*., 1989). In view of the ability of tannins to precipitate proteins, it is not surprising that they can affect the activity of digestive enzymes. Inhibition of trypsin, amylase, and lipase by tannin *in vitro* has been reported (Griffiths, 1986; Horigome *et al*., 1988), but the situation *in vivo* is more complex. Inclusion of tannin in the diet of rats caused decreased activity of trypsin and amylase in the digestive tract but an increase in lipase activity (Griffiths and Moseley, 1980). Ahmed *et al*. (1991) found that feeding tannin to cockerels caused an increase in pancreatic trypsin and amylase activities which was associated with an enlargement of the pancreas, but a decrease in the levels of intestinal trypsin and amylase. Thus, it appears that tannin, in an unknown manner, causes an enlargement of the pancreas and, consequently, secretion of an increased amount of amylase and trypsin that to some extent may counteract the inhibition of the enzymes in the intestines. Although the mechanism of inhibition is not well-understood, it is generally assumed that it is due to a non-specific interaction leading to precipitation of tannin-enzyme complexes.

Workers in an environment where there is a high concentration of airborne plant-originated dust, such as cotton mill and grain elevator workers, suffer from an acute inflammatory condition of the lungs known as byssinosis (Cotes *et al*., 1987). It is characterized by an influx of neutrophils in the airways and accumulation of polymorphonuclear leukocytes in the bronchoalveolar lavage fluid of exposed subjects. This condition has been linked to condensed tannins in cotton bracts, which are the source of dust in cotton mills. Purified condensed tannin from cotton bracts has been shown to cause, in rabbits, a condition similar to byssinosis (Lauque *et al*., 1988). Condensed tannin has also been shown to be responsible for various reactions associated with the acute inflammatory reaction, such as inhibition of phagocytosis by macrophages (Rohrbach *et al*., 1992) and stimulation of secretion of a neutrophil chemotactic factor and arachidonic acid from macrophages (Mundie *et al*., 1985; Laque *et al*., 1988; Kreofsky *et al*., 1990; Rohrbach *et al*., 1992). Interestingly, byssinosis is often associated with an acute irritation of the eyes (Becklake, 1980). These effects of airborne tannins on the lungs and eyes point to the need for a tannin defense mechanism in these organs. Byssinosis results from an exposure to environmental dust, but the potentially harmful effect of tannin on humans is also illustrated by the therapeutic and diagnostic use of tannins. From 1925 to 1942, tannic acid was widely use topically for the treatment of severely burnt skin. While this was found to be an effective treatment, it was realized that it also caused necrosis of the liver (Erb *et al*., 1943), and the practice was therefore discontinued. Tannic acid has also been used for diagnostic purposes. Adding it to barium enemas improves the definition of the mucosal pattern on x-ray films. Unfortunately, this led to several deaths due to acute liver failure, and the practice was therefore discontinued (Lucke *et al*., 1963). The use of tannin in treatment of burns and its radiological diagnostic application are unusual types of exposures to tannin that would not normally occur. Nevertheless, they illustrate the potential danger of tannin and suggest the existence of defense mechanisms against these compounds.

The mechanism of absorption of tannins and how they exert their toxic effect is not well-understood. Following the oral administration of ^{14}C-labeled condensed tannin to experimental animals, it was possible to demonstrate the label in various tissues, including plasma and liver, but it is not clear if this reflects the uptake of intact tannin or tannin degradation products or the uptake of low-molecular-weight components associated with the tannin preparation (Jiminez-Ramsay *et al*., 1994).

(V) Consumption of Tannin by Humans

Tannins are widely found in plant foods. Their tannin content varies from nearly zero to several grams *per* kilogram. Common tannin-containing foods are listed in the Table. Widely consumed foods with high tannin content include tea, red wine, beans, and sorghum. Polyphenols constitute from 37% to 56% of solids in green tea beverages, and unoxidized catechins may account for as much as 10% of solids in black tea (Graham, 1992). There are appreciable levels of tannin in wine, particularly red wine, in the order of 750 mg/L. Among legumes, the tannin content of Fava beans varies from 7 to 38 mg/g of seed coat (Martin-Tanguy *et al*., 1977), and skins of red peanuts contain about 17% tannin by weight (Karchesy and Hemingway, 1986). Sorghum, millets, and food legumes are important food crops of several countries in Asia, the Near and Middle East, and Africa. Sorghum has a condensed tannin content that varies from 0.13% to 7.2% and a tannic acid content varying from 0.37% to 1.57% (Rostango *et al*., 1973; Subramanian *et al*., 1983). Other foods with high tannin content include apple juice (48 mg/L) and cranberry juice (248 mg/L).

There is only limited information available on the daily intake of tannin. Values of 1.5 to 2.5 g in different regions of India have been reported (Rao and Prabhavati, 1982), and

Kuhnau (1976) estimated the daily intake of total flavonoids in the USA to be about 1 g. A considerable amount of flavonoids is consumed in beverages, and coffee, tea, fruit juices, wine, and beer are important sources of flavonoids, accounting for 25-30% of total flavonoid intake. In the Netherlands, the daily intake of flavonoids was found to be 23 mg (Hertog *et al*., 1993). It is possible that the intake of tannin may be even higher in regions where crops with high tannin content, such as bird-resistant sorghum, are grown, but although the total amount of tannin consumed may not be known because the tannin content of food cannot be measured exactly, it is clear that the tannin content of human food is of nutritional interest.

(VI) Tannin-Protein Interaction, General Characteristics

One of the characteristic properties of tannins is their ability to precipitate proteins from aqueous solutions. Since most of the biological activities of tannins are believed to be related to their protein binding, the interaction between tannin and protein has been extensively studied.

At present, it is generally accepted that there is a reversible interaction between polyphenol and protein in solution, leading to an equilibrium between the soluble protein/tannin complexes and the reactants. These soluble complexes may reach a size where they are no longer soluble, or they may aggregate or undergo changes resulting in precipitation. The formation of these insoluble complexes is usually reversible, and they may redissolve, for example, by further addition of one of the reactants, unless other processes—such as oxidation, complex formation with metal ions, or changes in pH—make the precipitation process irreversible (Luck *et al*., 1994).

The ability of tannin to precipitate different proteins varies considerably. Using a competitive assay, Hagerman and Butler (1981) found that the relative affinity of proteins for condensed tannin varied more than 1000-fold. The highest affinities were found for proteins, polypeptides, and polymers with high proline content, such as a rat parotid proline-rich protein, and the lowest affinities were obtained for small globular proteins such as lysozyme. In general, it has been found that proteins which are readily precipitated by tannin are large, have high proline content, and lack secondary or tertiary structure, although some of them may possess a polyproline helix. Interestingly, proteins with high affinity for tannin share a high proline content with several plant proteins, such as tannin-associated proteins from sorghum grains, but little is known about the interaction of tannin with these proline-rich proteins. This variability in protein affinity for tannin has important biological consequences; thus, binding of tannin to proline-rich proteins might prevent inactivation of other metabolically active proteins.

It is also interesting to note that different tannins show variations in interaction with a given protein. McManus *et al*. (1985) observed that molecular size as well as flexibility affected the binding of tannin to protein. Thus, in the galloyl-D-glucose series, the presence of additional galloyl ester groups increased binding to bovine serum albumin, reaching a maximum with pentagalloyl glucose. Moreover, a comparison of various tannins showed that increased flexibility of the polyphenols increased the interaction with bovine serum albumin. In agreement with these results, Asquith and Butler (1986) found that tannin isolated from four different sources showed noticeable differences in their relative affinities for a salivary proline-rich protein and soybean trypsin inhibitor. From a biological point of view, it may be an advantage for an organism to have a series of tannin-binding proteins available to ensure the effective precipitation of the various tannins that occur in food.

TABLE
Typical Tannin-containing Foods*

Beverages	Fruits
Red wine	Banana
Tea	Persimmon
Cider	Apple
Coffee	
Cocoa	Cereals
Beer	Sorghum
	Barley
Legumes	
Fava beans	Berries
Pinto beans	Strawberries
Common beans	Red currants
Cowpeas	Blueberries
	Raspberries

* Adapted from Mehansho *et al*. (1987a).

The nature of tannin-protein interaction has been the subject of many studies. Potentially, such interaction could occur *via* covalent or ionic bonds, hydrophobic interaction, or hydrogen bonding. While polyphenols are prone to oxidation and give rise to ortho-quinones which are highly reactive intermediates that potentially could result in tannin-protein covalent crosslinks (Haslam *et al*., 1991), there is at present little evidence for covalent binding of tannin to protein. No interaction has been observed of tannin and protein at pH values where the phenolic hydroxyl groups in tannin would be ionized, indicating that ionic interaction with protein is of little or no importance (Hagerman and Butler, 1978).

Several studies have presented evidence for a hydrophobic effect in tannin-protein interactions. Using condensed tannin, Oh *et al*. (1980) found that the complex formation of tannin with gelatin increased with increasing temperature and ionic strength, indicating hydrophobic interaction, and Hagerman *et al*. (1998) concluded that pentagalloyl glucose precipitated bovine serum albumin by forming a hydrophobic coat around the protein. At the molecular level, Murray *et al*. (1994) have presented evidence that the hydrophobic interaction between a peptide derived from a rat salivary proline-rich protein and pentagalloyl glucose is due to the stacking of phenol groups of the tannic acid against the pyrrolidone ring of proline in the protein.

Hydrogen bonds have also been shown to play a role in the formation of tannin-protein complexes (Hagerman and Butler, 1980), and it has been suggested that proline has an important role because the carbonyl oxygen next to the secondary amine in proline residues is a strong hydrogen bond acceptor (Hagerman and Klucher, 1986). In agreement with this concept, Hagerman *et al*. (1998) found little evidence for hydrophobic interaction between a purified condensed tannin and bovine serum albumin, suggesting that in this instance binding occurred primarily by hydrogen bonding. Thus, different mechanisms involving hydrogen bonds and hydrophobic interactions may be responsible for precipitation of condensed and hydrolyzable tannins.

In general, it has been found that precipitation of tannin-protein complexes is pH-dependent, with the greatest precipitation occurring near the isoelectric point of the protein. This

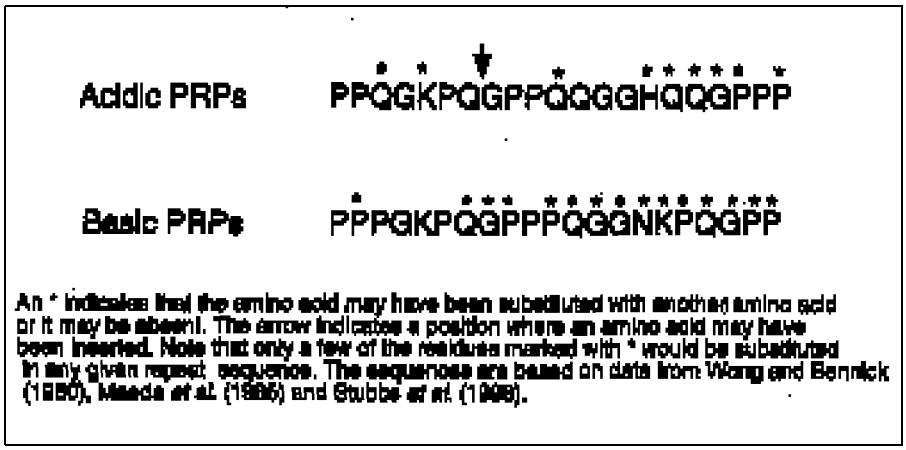

Figure 3. Representative repetitive sequences in proline-rich proteins.

has been ascribed to the decreased electrostatic repulsion of protein molecules at this pH (Hagerman and Butler, 1978).

Studies on the interaction of bovine serum albumin and polyphenols suggest that polyphenols are multivalent ligands and may form crosslinks between two or more protein molecules. Thus, the stoichiometry and size of the polyphenol-protein complexes depend on the concentrations of the reactants and the protein/polyphenol ratio (McManus *et al*., 1985). The multivalent nature of polyphenol-protein interaction is also supported by the observations on haze formation reported by Siebert *et al*. (1996). Similarly, Hagerman and Robbins (1987) found that by adding increasing amounts of albumin to a fixed amount of tannin, there was an optimal ratio of tannin to protein where maximal precipitation occurred. At higher and lower tannin/protein ratios, the amount of precipitated protein decreased. This model may not be applicable to all proteins. Luck *et al*. (1994) observed that with gelatin there was also an optimal ratio where maximal gelatin precipitation occurred, and the further addition of gelatin led to resolubilization of the tannin-protein complex. In contrast, the addition of increasing amounts of a salivary proline-rich protein to a fixed amount of tannin led to the formation of insoluble tannin-protein complexes that remained insoluble regardless of how much protein was added. This observation has interesting implications for the proposed defensive role of salivary proline-rich proteins.

(VII) Salivary Proteins

(A) General characteristics

Several studies have been undertaken on the binding of tannins to salivary proteins because of the potential importance of these interactions as a defense against tannins. Saliva contains a complex mixture of proteins which varies between species, as exemplified by the studies of Chauncey *et al*. (1963). However, by far, the most studies have been undertaken on human proteins.

Many salivary proteins occur as families of isoforms, and it is characteristic that these isoforms may have more than one function. Moreover, the same function may be shared by different families of proteins, resulting in considerable functional redundancy. The reason for this is not clear. It may help to ensure that a given function is always present, and variations in an activity exhibited by different proteins may guarantee the presence of an active protein under a broader range of physiological conditions than is possible for a single protein. These general characteristics are well-represented by two families of salivary proteins, proline-rich proteins and histatins, which, among other activities, share an ability to precipitate tannins readily.

(B) Proline-rich proteins

Most proteins in human parotid saliva and many proteins in submandibular/sublingual saliva belong to a unique multigene family of proline-rich proteins (PRP) (Azen and Maeda, 1988) that are also found in the saliva or salivary glands of monkeys (Oppenheim *et al*., 1979), rats (Fernandez-Sorenson and Carlson, 1974), mice (Mehansho *et al*., 1985), hamsters (Mehansho *et al*., 1987b), rabbits (Rajan and Bennick, 1983), pigs (Madapallimattam *et al*., 1992), and deer (Austin *et al*., 1989).

Based on the sequences of PRP cDNAs and genomic DNAs encoding PRPs, it is apparent that the translation products of PRP genes from at least human, monkey, mouse, rat, and hamster share a common structure consisting of a signal peptide, a transition region, a repeat region, and a carboxyl terminal region. In humans, it has been found that, with the exception of an unusual PRP P-B (Isemura *et al*., 1979), which is encoded by a distinct gene family (Isemura and Saitoh, 1997; Isemura, 2000) that includes the rat VCS gene (Courty *et al*., 1994), the PRP family is encoded by 6 genes: two that encode acidic PRPs (*PRH1* and *PRH2*), and four that encode basic and glycosylated PRPs (*PRB1, PRB2, PRB3,* and *PRB4*) (Maeda, 1985). Many of the proproteins encoded by these genes are subsequently cleaved by proprotein convertases before secretion, giving rise to a large number of secreted PRPs. Further variability in these proteins arises from the fact that several alleles for each gene have been identified (Maeda, 1985; Lyons *et al*., 1988a,b; Minaguchi and Bennick, 1989).

As a result, more than 20 PRPs are present in human saliva, and they are divided into acidic PRPs (APRPs) (Bennick and Connell, 1971; Oppenheim *et al*., 1971; Friedman and Merritt, 1975), and basic and glycosylated PRPs (BPRPs and GPRPs, respectively) (Levine *et al*., 1969; Armstrong, 1971; Friedman *et al*., 1971; Levine and Keller, 1977; Arneberg, 1974; Degand *et al*., 1975). Characteristically, proline, glycine, and glutamine together account for 70% to 88% of all amino acids.

APRPs contain a highly acidic N-terminal end (the transition region). The repetitive region which starts at residue 53 is dominated by pro, gln, and gly (Wong and Bennick, 1980), and a consensus repeat sequence is shown in Fig. 3. APRPs include PRP1 and 3, PIF-s, and PIF-f (Wong *et al*., 1979; Wong and Bennick, 1980; Hay *et al*., 1988). Other APRPs have trivial names such as Db and Pa (Azen and Denniston, 1974; Friedman and Merritt, 1975).

Basic and glycosylated PRPs have many of the characteristics of the acidic PRPs, including a high content of pro, gly, and gln. Their proproteins have the same general structure as APRPs and contain a transition region which is shorter than that of APRPs and a tandem repeat region with a sequence similar to that of APRPs. A consensus repeat sequence is shown in Fig. 3. Following synthesis, the proproteins are cleaved at a characteristic RXXR/S sequence, giving rise to several BPRPs and GPRPs, which are subsequently secreted. In tissue culture cells expressing a PRP proprotein, this cleavage is mediated by

furin, which may also be responsible for cleavage in the salivary glands (Chan and Bennick, 2001). The amino acid sequences of a great many BPRPs and GPRPs have been determined by amino acid sequencing or deduced from their corresponding nucleotide sequences (Saitho *et al*., 1983; Lyons *et al*., 1988a; Stubbs *et al*., 1998). These include, according to the nomenclature of Kauffman and Keller (1979), IB1, IB4, IB5, IB6, IB7, IB8a, IB8b, IB8c, IB9, II-1, II-2, and GPRP. Of particular interest is the determination, in a single person, of both the amino acid sequences of the secreted proteins and nucleotide sequences of the genes encoding BPRPs and GPRPs, since it allows for the unequivocal assignment of the regions encoding the secreted proteins and the determination of the cleavage points in the proproteins (Kauffman *et al*., 1982, 1986, 1991, 1993; Stubbs *et al*., 1998).

PRPs are also present in human tears (Dickinson and Thiesse, 1995) and the respiratory tract (Warner and Azen, 1984). In the latter tissue, it is primarily the BPRPs that are expressed (Sabatini *et al*., 1989), whereas the lachrymal gland contains a mRNA encoding a PRP that has 46% similarity to APRP and a similar domain structure, although it lacks the repetitive domain characteristic of other PRPs (Dickinson and Thiesse, 1996). The activities of these proteins have not been studied, but their presence in these tissues indicates that the function of PRPs is not restricted to the oral cavity.

Despite the high content of proline in PRPs, spectroscopic studies have failed to demonstrate the presence of polyproline structure in APRPs (Wong and Bennick, 1980) as well as BPRPs (Shibata *et al*., 1984). In contrast, circular dichroism provided evidence for polyproline II helix in P-B (Isemura *et al*., 1983), which may be related to the occurrence of $(Pro)_7$, whereas the longest oligoproline in other PRPs is $(Pro)_6$. For rat PRPs, axial ratios exceeding 25 have been calculated, indicating that PRPs in solution have an elongated structure (Muenzer *et al*., 1979). Two-dimensional nuclear magnetic resonance (NMR) studies also failed to detect any recognizable secondary structure, including polyproline (Murray and Williamson, 1994). Present evidence therefore indicates that PRPs exist in solution as extended random coils.

PRPs are constitutively expressed in humans, but in rats, mice, and hamsters, their synthesis is induced by the β-agonist isoproterenol. Synthesis of PRPs in some species can also be induced by tannin. Carlson and co-workers demonstrated that feeding rats and mice high-tannin sorghum led to an initial weight loss, but within 3 days there was a marked stimulation of PRP synthesis, and at the same time weight gain was initiated (Mehansho *et al*., 1983, 1985). Hamsters also showed weight loss when placed on a diet high in tannin, but in contrast to rats and mice, there was no induction of PRP synthesis, and a growth inhibition was observed as long as the animals were kept on this diet (Mehansho *et al*., 1987b). These experiments provided powerful support for a protective role of PRPs against tannins and led to the novel suggestion that salivary PRPs may be important as a first-line defense against dietary tannins (Mehansho *et al*., 1987a). The PRPs induced in rats and mice had high affinity for tannin (Hagerman and Butler, 1981; Mehansho *et al*., 1985; Asquith and Butler, 1986), and the formation of tannin-PRP complexes may prevent tannin from causing damage to the intestinal tract, binding to digestive enzymes and food proteins, and absorption into the organism. The mechanism whereby dietary tannin induces synthesis of PRPs is not known, but it is interesting that feeding tannin to mice causes a stimulation of the β-adrenergic receptor

Histatin 1	NSHEKRHHGYRRKFHEKHHSHREFPFYGDYGSNYLYDN
Histatin 3	DSHAKRHHGYKRKFHEKHHSHRGYRSNYLYDE
Histatin 5	DSHAKRHHGYKRKFHEKHHSHRGY

The asterisk indicates phosphorylation

Figure 4. Amino acid sequences of prominent histatins. (Data from Troxler *et al*., 1990)

and changes in cell cycle proteins, with an increase in the protein p34cdc2 (Waters *et al*., 1998). Moreover, the inducible nuclear orphan receptor NGFI-B has also been implicated in inducible expression of a rat PRP gene (Lin *et al*,. 1996).

Besides tannin-binding ability, PRPs have other biological activities. Human APRPs contain a highly acidic N-terminal region that binds calcium (Bennick *et al*., 1981), inhibits the formation of hydroxyapatite (Hay *et al*., 1979), and mediates hydroxyapatite binding, thereby becoming part of the acquired dental pellicle (Bennick *et al*., 1979, 1983; Moreno *et al*., 1982). Less desirable is the ability of APRPs to mediate binding of bacteria to hydroxyapatite, since it may assist in the formation of dental plaque (Gibbons *et al*., 1988; Kataoka *et al*., 1997). BPRPs have anti-viral activity (Gu *et al*., 1995) and bind to *C. albicans* (O'Sullivan *et al*., 1997), which may affect the colonization of oral surfaces by these micro-organisms.

Salivary GPRP provides a lubricating function in the mouth (Hatton *et al*., 1985), and it binds to micro-organisms (Murray *et al*., 1992) which could facilitate the adherence of bacteria to oral surfaces or clearance of bacteria from the mouth, depending on whether the PRPs are present in pellicle or saliva (Gillece-Castro *et al*., 1991; Azen *et al*., 1993).

(C) Histatins

Histatins or HRPs comprise a group of structurally related, small histidine-rich proteins found only in the saliva of humans and some monkeys. Twelve HRPs, named HRP 1 to 12, have been isolated from human saliva and their primary structures determined (Troxler *et al*., 1990). Additional members of this protein family have been identified (Xu *et al*., 1993). The most prominent members are HRPs 1, 3, and 5, and they account for 85-90% of this family. Their structures are shown in Fig. 4. In contrast to PRPs, they contain no proline except for a single residue in HRPs 1 and 2. Histidine is the prominent amino acid, accounting for about 25% of all residues and, together with basic amino acids, makes up 30% to 75% of total amino acids. In solution, HRPs have a random coil structure, but in a hydrophobic environment they can assume α-helical structure, a phenomenon that has been associated with the antimicrobial activity of the proteins, since it may facilitate interaction of the peptides with the micro-organisms (Raj *et al*., 1990). HRPs 1 and 3 are products of different genes (Sabatini and Azen, 1989), and the remaining HRPs arise by proteolytic cleavage of the 3 major HRPs (Troxler *et al*., 1990); thus, HRP5 consists of the N-terminal 24 residues of HRP3 (Fig. 4). The concentration of the major HRPs in human saliva has been estimated to be 32 μM, and they account for approximately 2.6% of total protein (Sugiyama and Ogata, 1993). HRPs play a role in the non-immune oral defense mechanisms because of their antibacterial and antifungal activities (McKay *et al*., 1984; Pollock *et al*., 1984), an aspect which has been intensely studied

in recent years. Moreover, HRP1 inhibits hydroxyapatite crystal growth and binds strongly to hydroxyapatite, indicating a role in enamel homeostasis and the formation of dental pellicle (Oppenheim *et al*., 1986).

In a survey of human salivary proteins (Yan and Bennick, 1995), it was found that, in addition to PRPs, HRPs were potent precipitators of condensed tannin as well as tannic acid. At neutral pH, HRP5 precipitated up to more than double the amount of tannic acid precipitated by gelatin, which is considered to be a strong binder of tannic acid. Similar results were obtained when tannic acid was replaced by condensed tannin. This was an unexpected finding, because the virtual absence of proline in these proteins and their small size were in sharp contrast to the high proline content and large size of other known tannin-binding proteins. Histatins clearly constitute a novel family of tannin-binding proteins.

(D) Other tannin-binding salivary proteins

The possibility that salivary proteins provide a defense against tannin has spurred an interest in evaluating the presence of tannin-binding proteins in animals whose diet is high in polyphenolic compounds.

Austin *et al*. (1989) compared the saliva of grazing and browsing ruminants for tannin-binding proteins. The diet of grazers is practically free of tannin, and they tolerate phenolics poorly, whereas browsers consume various plants that contain phenolic compounds which they tolerate well. Interestingly, there was no evidence for tannin-binding proteins in domestic grazers such as sheep and cows, but browsers such as deer contained a small tannin-binding glycoprotein which had a high content of proline, glycine, and glutamine/glutamic acid. The tannin-binding ability of deer saliva has also been demonstrated by Fickel *et al*. (1998). Although differences were found in the composition of these proteins and PRPs from other species, analysis of the data suggests that they may belong to the family of proline-rich proteins. The results also suggest that there may be a relationship between the occurrence of salivary tannin-binding components in a given species and its food habits.

The relationship between tannin in the diet and tannin-binding proteins in saliva was investigated by the examination of salivary tannin-binding proteins in moose, beaver, mule deer, and black bear (Hagerman and Robbins, 1993). Moose and beaver produced proteins that bound only linear tannins common in their preferred food (willow, aspen, and birch), whereas mule deer, which have a more varied diet, possessed salivary proteins that bound both linear and branched tannins, but not ellagitannin. In contrast, the omnivorous black bear produced salivary proteins that bound all types of tannin. These results further support a possible relationship between salivary tannin-binding protein and diet, and it would be interesting for the tannin-binding salivary proteins in these species to be characterized, so that we might understand what determines the specificity of interaction of different types of tannin with a protein.

(VIII) Interaction of Salivary Proteins and Tannin

Since the observation by Hagerman and Butler (1981) that a rat salivary protein showed one of the highest affinities for tannin among the proteins and polymers investigated, including gelatin, which is considered to be a strong tannin-binder, there have been many studies investigating the nature of interaction between salivary proteins and tannins. Similar high affinities for rat basic PRP and glycosylated rat and mouse PRPs were shown by Mehansho *et al*. (1985). Estimation of affinities was based on the ability of PRPs to prevent the formation of insoluble complexes of tannin and labeled albumin.

In other studies on human PRPs, precipitation of tannin from solutions containing purified PRPs was used to quantitate and compare the ability of these proteins to form insoluble complexes with tannin (Lu and Bennick, 1998). With the use of various purified PRPs, it could be shown that BPRPs readily precipitated both condensed tannin and tannic acid, but very little of either of these compounds was precipitated by APRPs and GPRPs, suggesting that the presence of tannins in saliva might not affect the biological activities of APRPs and GPRPs. Comparison of different BPRPs showed that variation in size and sequence of these proteins had little effect on their tannin-precipitating abilities, indicating that the considerable phenotypic variations in these proteins may not affect the ability of different individuals to precipitate tannin. APRPs consist of a highly acidic phosphorylated N-terminal region, followed by proline-rich tandemly repeated sequences. While dephosphorylation had no effect on the tannin-binding ability of APRPs, removal of the acidic N-terminal region resulted in proline-rich peptides that had tannin-precipitating ability similar to that of BPRPs. In contrast, the N-terminal acidic peptide did not possess tannin-precipitating ability, thereby indicating that the tannin-binding sites are contained within the proline-rich regions.

In this connection, it is noteworthy that cleavage of APRPs occurs both before and after secretion into the mouth (Wong *et al*., 1983; Madapallimattam and Bennick, 1990), resulting in N-terminal fragments with increased abilities to bind calcium and inhibit hydroxyapatite growth. Consequently, C-terminal peptides with tannin-precipitating activity should be released. Since the ability of APRPs to mediate bacterial attachment to tooth surfaces depends on the N-terminal binding site for hydroxyapatite (Bennick *et al*., 1979) and a C-terminal bacterial binding site, as exemplified by *Strep. gordonii* (Gibbons *et al*., 1991), such cleavages would also prevent APRPs from the undesirable ability to mediate binding of bacteria to the tooth surface. Thus, all the beneficial biological activities of APRPs are in fact enhanced by their post-synthetic cleavage.

The inability of GPRPs to precipitate tannins is due to the presence of carbohydrate sidechains, since deglycosylated GPRPs showed the same ability to precipitate tannin as BPRPs (Lu and Bennick, 1998). In contrast, Asquith *et al*. (1987) found that affinity of a mouse GPRP for tannin decreased upon deglycosylation. These apparently conflicting results may be due to a tendency of native GPRPs to form soluble rather than insoluble complexes with tannin.

Mixtures of tannins are useful for the identification of tannin-binding salivary proteins, since foodstuffs contain many different tannins, but this may result in preferential binding of some tannins. Using condensed tannins from grape seeds, Sarni-Manchado *et al*. (1999) demonstrated that it was the higher polymerized tannins that were preferentially precipitated by human salivary PRPs. The importance of size and structure of procyanidins in their ability to precipitate salivary proteins was also demonstrated by de Freitas and Mateus (2001). Moreover, differences in interaction of various hydrolyzable tannins with human PRPs have also been observed (Bacon and Rhodes, 2000). In contrast to the studies by Lu and Bennick (1998), a given tannin showed similar affinities for APRPs, GPRPs, and BPRPs. The differences in the two studies may be due to the use of different

protein preparations and different assay methods. If salivary proteins serve as a defense mechanism against tannins, the differential binding of tannin to the proteins has interesting implications, since, in a mixture of food tannins, only the highly polymerized tannins might form insoluble complexes which could remain in the alimentary canal, whereas the uncomplexed lower-molecular-weight tannins may be more readily absorbed.

To investigate such possibilities, one must evaluate the stability of tannin-protein complexes. Lu and Bennick (1998) evaluated, *in vitro*, the stability of insoluble complexes of a representative PRP with condensed or hydrolyzable tannin under conditions similar to those found in the alimentary canal and found that most of the complexes with condensed tannin remained insoluble, whereas the PRP-hydrolyzable tannin complexes were somewhat more soluble. These results are in agreement with the notion that PRPs may serve to prevent intestinal uptake of tannins and should encourage further studies on the effects of salivary proteins on intestinal uptake of polyphenols.

The molecular nature of interaction of PRPs with tannins has been the subject of an interesting series of studies by Williamson and co-workers (Murray *et al*., 1994). Using two synthetic peptides, consisting of 19 or 22 residues with sequences that were typical of the mouse PRP consensus sequence, they demonstrated that the main binding sites for pentagalloyl glucose (PGG) were proline residues together with the preceding amide bond and amino acid. It was predominantly a hydrophobic association between the galloyl aromatic ring of PGG and the pyrrolidine ring face containing the Cα proton of proline. Hydrogen bonding also played a role. No changes in the random extended coil structure of the peptides were seen when binding occurred. These studies were extended by Baxter *et al*. (1997) to include polyphenols of different sizes which showed that the binding of polyphenol was dependent upon the amino acid sequence, with Pro-Pro sequences being favored, although interaction with residues other than proline was also observed. Larger and more complex tannins bound more strongly than smaller polyphenols, and the proline-rich peptide was more readily precipitated by larger polyphenols. The polyphenols self-associated when bound to the peptide, and several phenolic rings from the same polyphenol could interact with a given peptide. Thus, polyphenols appear as multivalent binding compounds able to bind other polyphenols as well as peptides or proteins, leading to the formation of large molecular aggregates.

IB5, a 70-residue human BPRP, was selected for evaluation of the interaction of a naturally occurring PRP with polyphenols (Charlton *et al*., 1996). This protein contains three 21-residue tandemly repeated regions, in contrast to the previously used peptide, which contained only one repeat region. Interestingly, the dissociation constants for binding of tri- and pentagalloyl glucose to IB5 were 115- and 1660-fold stronger, respectively, than those found for binding to the single-repeat peptide. The stronger interaction may be due to the increased length of the protein, which allowed it to fold around the polyphenol, resulting in additional co-operative interactions. Lu and Bennick (1998) used human BPRPs of different sizes and found that IB4 (contains 2 tandem repeat sequences) and IB6 (5 tandem repeat sequences) had the same ability to precipitate polyphenol. Thus, increasing the number of tandem repeats beyond a certain number may not necessarily lead to stronger interaction with polyphenol.

Given the lack of polyproline structure in tannin-binding PRPs, it is tempting to speculate that while the presence of proline provides rigidity that favors interaction with polyphenols, the absence of polyproline at the same time provides a flexibility and exposure of proline that facilitates interaction with polyphenols of different structures.

While most proteins depend on well-defined secondary and tertiary structures to perform their functions, the lack of such structures in PRPs may be an important aspect of their ability to form complexes with the many different tannins found in food.

Since it was known that tannin-binding proteins are characterized by high proline content, large molecular weight, and lack of ordered conformation, it was surprising to find, in a survey of salivary proteins, that histatins were effective precipitators of tannin, given that these proteins are small and practically devoid of proline (Yan and Bennick, 1995). Several differences were seen in tannin precipitation by histatins. At a low concentration of condensed tannin or tannic acid, histatins were more readily precipitated from saliva than PRPs, and more condensed tannin and tannic acid were precipitated by histatin than gelatin at neutral pH, demonstrating the effectiveness of histatin in precipitating tannin. Further studies (Naurato *et al*., 1999) indicated that Histatins 3 and 5 apparently share the same condensed tannin-binding region, which is located throughout the shared histatin 5 sequence, and they bound more condensed tannin than histatin 1. Epigallocatechin gallate (EGCG) showed binding characteristics similar to those of condensed tannin, while pentagalloyl glucose (PGG) bound equally well to histatins 1, 3, and 5. Thus, the mechanisms of interaction of condensed tannin and tannic acid with histatins may be different.

Incubation *in vitro* of insoluble complexes of condensed tannin and histatins 1, 3, or 5 under conditions similar to those occurring in the alimentary canal demonstrated that most of these complexes remained insoluble, consistent with the possibility that these proteins may act as defense against tannin (Naurato *et al*., 1999). The latter possibility was also demonstrated by the ability of histatin as well as PRP to prevent the inhibition of salivary amylase by tannic acid (Yan and Bennick, 1995).

Because of the differences in the primary structures of PRPs and histatin, in particular the absence of proline in most histatins, it is of interest to determine the molecular interaction of tannin and histatins. Using two-dimensional NMR and other analytical techniques, Wroblewski *et al*. (2001) found that EGCG interacted with the basic and aromatic residues in histatin 5. While none of these residues was indispensable, the primary structure was important, since a peptide in which the sequence of histatin 5 had been randomized showed diminished interaction with EGCG. Histatin 5 in solution had 5 or 6 binding sites for EGCG, but binding of only 1 or 2 molecules of PGG to histatin 5 led to the formation of insoluble complexes. These differences in behavior have interesting implications, because they may allow small molecules such as EGCG and other flavonoids to remain in solution in the intestines only weakly associated with histatin, thereby making their absorption possible. This may be part of the mechanism that allows for the absorption of flavonoids and the exploitation of their beneficial effects. In contrast, the formation of insoluble complexes of tannic acid, including PGG and condensed tannin, may be part of the defense mechanism against tannins.

The presence of two families of tannin-binding proteins in human saliva may be related to the differential expression of these proteins in salivary glands. The basic PRPs are expressed

only in parotid saliva, where they account for 23% of total protein (Kauffman and Keller, 1979). In contrast, histatins are found in parotid and submandibular/sublingual secretions, where they constitute 2.6% of total protein (Sugiyama and Ogata, 1993). Stimulated saliva which is secreted during meals contains a significantly higher proportion of parotid saliva than does unstimulated saliva which is secreted between meals (Sas and Dawes, 1997). Thus, histatins with their strong tannin-binding ability may be particularly important in neutralizing tannin that enters the mouth between meals—for example, by the inhalation of airborne tannin-containing dust.

Basic PRPs expressed in the lungs may also serve as a defense against the inhalation of tannin-containing dust, a defense that may be overwhelmed in extreme situations, as exemplified by the occurrence of byssinosis in cotton mill and grain elevator workers exposed to high levels of airborne tannin. While the tannin-binding ability of lachrymal proteins has not been demonstrated, it is possible that these proteins serve as a defense against irritation of the eyes by tannins in the air. This notion is supported by the observation that workers suffering from byssinosis can also show acute irritation of the eyes (Becklake, 1980).

(IX) Astringency and Salivary Proteins

The ingestion of certain foods, including polyphenols, is associated with a dry, puckering sensation in the mouth known as astringency. It has been proposed that astringency is associated with the interaction of polyphenols and salivary proteins (Bate-Smith, 1954). The sensation of astringency associated with the drinking of tea can be overcome by the addition of milk to the beverage, and this has been linked to the ability of milk casein, which has a relative high content of proline, to sequestrate tea polyphenols within casein micelles, thereby diminishing the interaction of tea polyphenols with salivary proteins (Luck *et al*., 1994). More recently, attempts have been made to identify the proteins in saliva that are associated with food astringency (Kallithraka *et al*., 2001).

(IX) Evolutionary and Dietary Aspects of Human Salivary Proteins

It is possible that the total capacity of human salivary proteins to bind tannin exceeds that which is necessary to neutralize tannin contained in the present-day diet. In this connection, it is interesting that hominids, which include ancestors of modern humans, in the Miocene Age showed adaptation for a fruit-based diet (Andrews and Martin, 1991) and may therefore have had a greater need for neutralizing food tannin. Biochemical and immunological evidence also indicates that, in anthropoid or apelike primates, the presence of HRPs is related to the diet. Thus, two so-called platyrrhine species, the tamarin and squirrel monkey, are predominantly insect-eaters, but there is a dietary gradient from an insect-based to a predominantly fruit- and plant-based diet through other platyrrhine species to cercopithecoids or "old world" monkeys to hominids. In parallel, no HRPs are present in the insect-eating tamarin and squirrel monkeys, but a multibanded pattern of HRPs is seen in other platyrrhines as well as in cercopithecoids and hominids (Azen *et al*., 1978). It was postulated that the evolution of HRPs might be related to an increased amount of dietary carbohydrate in fruit and leaves, and that HRPs, by adsorbing to the tooth surface, would provide protection against the increased caries risk caused by the enhanced carbohydrate intake (Azen *et al*., 1978). It is equally possible that the appearance of the HRPs allowed for neutralization of the increased amount of tannin in the fruit- and leaf-based diet. PRPs, the other group of tannin-binding salivary proteins, are inducible in mice and rats (Mehansho *et al*., 1987a), but are constitutively expressed at a high level in humans and monkeys (Kauffman and Keller, 1979; Oppenheim *et al*., 1979). It is therefore tempting to speculate that the appearance of a high level of expression of the salivary tannin-binding proteins, HRPs and PRPs, in hominids was an important evolutionary step, which allowed them to eat a much wider range of foods that otherwise would not be accessible because of a high tannin content. The presence of both HRPs and high amounts of PRPs in the saliva of contemporary humans may therefore be related to a much higher level of tannin in the diet of the evolving hominids than in present-day humans.

In this connection, a comparative study of different diets is interesting. One of the diets, which attempted to emulate that of hominids, consisted of 10 pounds of fruits, vegetables, and nuts to provide the necessary daily intake of calories (Jenkins *et al*., 2001). The ingestion of such a large amount of plant food is consistent with a much higher need on the part of our ancestors for tannin-neutralizing proteins and may provide at least part of the explanation for the high amount of tannin-binding proteins in human saliva.

(X) Conclusion

Most of the interest in salivary proteins has centered on their biological roles in the mouth, while their possible functions in other parts of the alimentary tract have received scant attention. As discussed in this review, histatins and proline-rich proteins may provide protection against tannins throughout the digestive system, but at the same time it appears that the closely related flavonoids are absorbed from the intestinal canal. Thus it is possible that salivary proteins serve as a screening mechanism that allows for absorption of flavonoids and exploitation of their beneficial effects but neutralizes the less desirable effects of tannins. If this is correct, clues to a better understanding of the presence of other proteins in human saliva may be obtained by a consideration of the habitat of our hominid ancestors. For example, amylase is the only digestive enzyme that is found in significant amounts in human saliva. Perhaps this is related to a much larger starch intake of our ancestors, necessitating the presence of amylase in saliva as well as in pancreatic juice.

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