Hydrophobic Interaction in Tannin–Protein Complexes

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Involvement of hydrophobic groups in the formation and stabilization of tannin–protein complexes was indicated by several independent lines of investigation: Proteins adsorbed on a column of condensed tannins immobilized on Sepharose 4B were effectively eluted by anionic and nonionic detergents and to a lesser extent by a cationic detergent, while urea and guanidine hydrochloride were ineffective in this regard. A study of the interaction in free solution between condensed tannins and polyamino acids indicated that the number of methylene groups in the amino acid side chain was positively related to magnitude of interaction. Complex formation between condensed tannins and gelatin or poly-L-proline increased with increasing ionic strength and temperature as expected for hydrophobic interactions. The hydrophobic nature of tannins was demonstrated by their effective adsorption on an uncharged polystyrene resin.

Considerable effort has been directed toward elucidating the specific mode of interaction between tannins and proteins, and various mechanisms for this interaction have been proposed. Previous work has been reviewed by Gustavson (1956), Loomis and Battaile (1966), Loomis (1974), and van Sumere et al. (1975).

In consideration of the diversity of molecular structures among the tannins and the many different functional groups present in proteins, all of the following interaction types can in principle take place: hydrogen bonding, ionic bonding, hydrophobic bonding, and covalent bonding (Loomis, 1974). The latter represents an irreversible change that requires molecular oxygen and is favored by high pH or by the presence of polyphenol oxidases.

We have been interested primarily in the interactions that lead to the precipitation of proteins under neutral or acidic conditions without formation of covalent bonds (Hoff and Singleton, 1977; Oh and Hoff, 1979). The complexes formed under such conditions are generally able to dissociate in the presence of suitable agents or solvents.

Tannins were in the past believed to bind proteins primarily through the formation of multiple hydrogen bonds between the phenolic hydroxyl groups of tannins and the carbonyl functions of the peptide linkages of proteins (Gustavson, 1954; Cannon, 1955; Goldstein and Swain, 1963; Morawiecki, 1963; Loomis and Battaile, 1966; van Sumere et al., 1975; Synge, 1975). The emphasis on hydrogen bonding was derived from the fact that tannins were bound by modified collagen and synthetic polymers (nylons), the latter containing –CONH– as the only reactive group.

But evidence that might be interpreted as involvement of hydrophobic bonding in the complex formation is available in the literature. Thus, Goldstein and Swain (1965) observed that the complexes could be dissociated by detergents, and Loomis (1969) has cited several instances where the same was achieved with organic solvents. Gray (1978) observed that low molecular phenolic substances were strongly adsorbed by polystyrene resins.

We are here presenting several lines of evidence indicating that hydrophobic bonding may be the major mode of interaction between condensed tannins and proteins. Ionic bonding is excluded by the absence of charged groups in condensed tannins of the proanthocyanidin type at pH values considerably below the pK_a values of the phenolic groups.

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MATERIALS AND METHODS

Materials. Grape tannins were obtained from Leigh-Williams & Sons, Nr. Chester, England. Partial characterization of this preparation has been previously reported (Oh and Hoff, 1979). It was found to consist of a mixture of proanthocyanidins ranging in apparent molecular weight from 900 to 3000. Quebracho and wattle tannins were obtained from L. H. Lincoln & Son (Coudersport, PA). Low molecular compounds were removed from the tannins by extraction with ethyl acetate–hexane according to van Buren and Robinson (1969). Polyamino acids, bovine serum albumin (BSA, fraction V), and polyvinylpyrrolidone (PVP) were products of Sigma Chemical Co. Polyethylene glycol (PEG) 20 000 was purchased from Fisher Scientific Co. and cytochrome *c* (horse heart) from Nutritional Biochemical Corp. The source of eluting agents were as follows: guanidine hydrochloride, Eastman Kodak Co.; urea, Schwarz-Mann; sodium dodecyl sulfate $NaDodSO_4$, Matheson, Coleman & Bell; Tween-80, ICI United States; Triton X-100, Anheuser Busch; Cetavlon and Manoxal OT, Aldrich Chemical Co. Purified Amberlite XAD-2 (mesh size 20/60, average hydrated pore diameter 90 Å) was obtained from Applied Science Laboratories, Inc. Prior to use the resin, in column configuration, was treated with five bed volumes of methanol, followed by 20 bed volumes of distilled water to ensure complete wettability and maximum pore size.

Chromatography. Tannins were immobilized on Sepharose 4B (Pharmacia) via epoxy activation according to Armstrong (1976). The gel (1 mL) was packed into a 5-mL plastic syringe, in which a disc of coarse filter paper was placed on top and another at the bottom of the gel. Cytochrome *c* solution (2 mL, 1 mg/3 mL in acetate buffer pH 4.0) was applied on the column which was equilibrated with the same buffer. After applying the sample, the column was washed with 5 mL of the acetate buffer and then eluted with various agents. Fractions (3 mL) were collected, and the absorbance was read at 410 nm. Separate columns were used for the individual experiments.

Turbidimetry. An aliquot (100 μL) of tannin solution (15 mg/mL in water) was rapidly injected into a cuvette containing 3 mL of cytochrome *c* solution (0.5 mg/mL in 0.1 M acetate buffer, pH 4.0) through the lid of the spectrophotometer and mixed well. The developing turbidity was monitored by recording the change in adsorbance at 500 nm for 5.5 min. The effect of $NaDodSO_4$ on the tannin–protein complex was studied by injecting 100 μL of tannin (30 mg/mL in water) into a cuvette containing 3 mL of solutions of the individual compounds (1 mg/mL in 0.1 M citrate phosphate buffer of the desired pH) and the turbidity recorded for 5 min at 450 nm.

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Table I. Effect of Various Agents on Elution of Cytochrome *c* from the Tannin Gel at pH 4.0

agent	concn	eluting ability[a]
urea	1 M	±
	6 M	±
guanidine hydrochloride	1 M	±
	6 M	±
Tween-80 (nonionic)	0.01% (v/v)	±
	0.1%	+
	1.0%	++
Triton X-100 (nonionic)	0.01% (v/v)	+
	0.1%	++
$NaDodSO_4$ (anionic)	0.01% (w/v)	+
	0.1%	++
Manoxal OT (anionic)	0.01% (w/v)	−
	0.1%	+
Cetavlon (cationic)	0.01% (w/v)	−
	0.1%	±
	1.0%	++

[a] No release (−); ~5% release (±); gradual release (+); immediate and total release (++).

The effects of variation in ionic strength and temperature on complex formation were carried out as described above using a spectrophotometer with a temperature-controlled sample compartment. Ionic strength was adjusted by adding appropriate amounts of NaCl to 0.01 M citrate phosphate buffer, pH 4.0.

Adsorption on Polystyrene Resin. One milliliter of tannin solutions (1 mg/mL, 0.1 M sodium acetate buffer, pH 4.0) was added to test tubes containing preweighed amounts of XAD-2 resin and the mixture was agitated for 10 min. The amount of tannin remaining in solution was determined by the Folin-Ciocalteu procedure as modified by Singleton and Rossi (1965).

RESULTS

Elution of Cytochrome *c* from Immobilized Tannins. When a solution of cytochrome *c* at pH 4 was added to immobilized tannins, the protein bound strongly to the gel since no protein was eluted upon further washing of the gel with buffer. Urea and guanidine hydrochloride were ineffective eluting agents for cytochrome *c* (Table I). Detergents, on the other hand, both anionic, nonionic, and to some extent cationic, were effective, even at low concentrations, in eluting cytochrome *c* from the gel. Results similar to those obtained with cytochrome c were also demonstrated with myoglobin (not shown).

Dissociation and Association of Tannin–Cytochrome *c* Complexes by Detergents. When a tannin solution was injected into a cuvette containing cytochrome c, tannins rapidly formed insoluble complexes accompanied by an increase in turbidity. When the turbidity reacted its maximum value (5.5 min), a solution of $NaDodSO_4$ was rapidly injected and mixed in the sample cuvette. Injection of $NaDodSO_4$ to yield 0.1% (w/v) final concentration resulted in an abrupt decrease in turbidity (Figure 1). On the other hand, concentrations in the range of 0–0.05% gave a substantial increase in turbidity. When a nonionic detergent, Triton X-100, was injected, qualitatively similar results were obtained (Figure 2).

Interaction of Tannins with Poly-α-amino Acids, PVP, and PEG. The interactions of Tannins with two acidic water-soluble polyamino acids (poly-L-glutamic acid, poly-L-aspartic acid), one basic polyamino acid (poly-L-lysine), one neutral polyamino acid (poly-L-serine), two neutral polyimino acids (poly-L-proline and poly-L-hydroxyproline), PVP, and PEG were studied at various

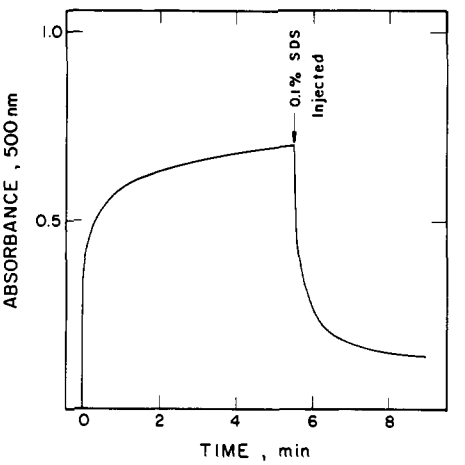

Figure 1. Dissociation of tannin–cytochrome *c* complexes by $NaDodSO_4$ at pH 4.0. One hundred microliters of 3% $NaDodSO_4$ (w/v) was injected into the sample cuvette when the tannin–cytochrome *c* complexes reached their maximum turbidity after 5.5 min.

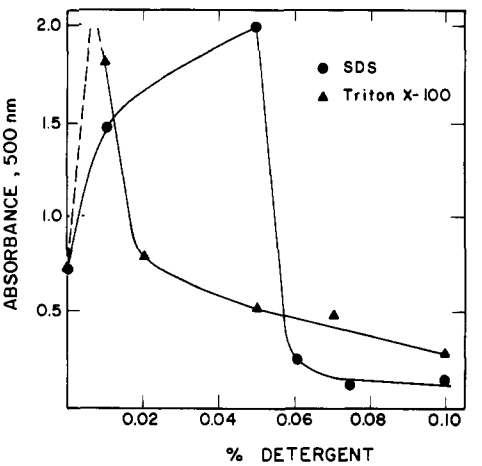

Figure 2. Effect of concentration of $NaDodSO_4$ and Triton X-100 on formation of tannin–cytochrome *c* complexes at pH 4.0. One hundred microliters of tannin solution (15 mg/mL in water) was injected into a cuvette containing 3 mL of cytochrome *c* solution (0.5 mg/mL in 0.1 M acetate buffer, pH 4.0) and mixed well. The developing turbidity was monitored by recording the changing apparent absorbance for 5.5 min at which time the detergents were injected to give final concentrations as indicated.

pH levels. No turbidity could be detected when tannins were mixed with poly-L-aspartic acid or poly-L-glutamic acid under any of the experimental conditions (Table II). Unlike these polymers, poly-L-hydroxyproline, poly-L-proline, poly-L-lysine, PVP, and PEG had a high affinity for tannins, whereas poly-L-serine produced only slight turbidity. In contrast to proteins (Hagerman and Butler, 1978; Oh, 1978), poly-L-hydroxyproline, poly-L-proline, PVP, and PEG gave a relatively constant turbidity independent of pH within the range studied. The extent of turbidity was greatest with PEG, poly-L-lysine, PVP. These were followed in order of decreasing turbidity by poly-L-proline, poly-L-hydroxyproline, and poly-L-serine.

Effects of Ionic Strength and Temperature on Tannin–Protein Interactions. No discernible effect of ionic strength and temperature on complex formation was demonstrated with BSA (Table III). However, in the case of gelatin and poly-L-proline, the turbidity increased with increasing ionic strength. A marked increase in turbidity was also observed with gelatin and poly-L-proline when the temperature was increased from 4 to 25 °C. Interaction of poly-L-proline with tannins at high ionic strength could

Table II. Interaction of Tannins with Poly-α-amino Acids, PVP, and PEG

polymers	approx mol wt[a]	deg of polym[b]	turbidity (A_{450}) pH 3.0	pH 4.0	pH 5.0	pH 6.0
poly-L-aspartic acid	14 000	123	0.00	0.00	0.00	0.00
poly-L-glutamic acid	48 700	377	0.00	0.00	0.00	0.00
poly-L-serine	7 900	91	0.44	0.29	0.22	0.00
poly-L-hydroxyproline	12 000	106	1.30	1.13	1.32	1.34
poly-L-proline	11 000	113	1.83	1.82	1.62	1.64
poly-L-lysine	30 000	234	0.13	1.20	2.68	2.73
polyvinylpyrrolidone	10 000	81	1.85	1.87	1.91	1.91
polyethylene glycol	20 000	455	2.87	2.87	2.87	2.89

[a] Supplier's value. [b] Calculated from molecular weight.

Table III. Turbidity (A_{450}) of Tannin-Protein Mixtures as Affected by Temperature and Ionic Strength[a]

protein	temp, °C	ionic strength, M 0.01	0.1	1.0
BSA	4	1.61	1.64	1.58
	25	1.58	1.54	1.63
	40	1.73	1.78	1.63
gelatin	4	1.34	1.67	1.88
	25	2.95	3.20	3.28
	40	3.29	3.29	3.27
poly-L-proline	4	0.06	1.05	
	25	0.21	1.04	
	40	0.22	1.06	

[a] In citrate-phosphate buffer, pH 4.0.

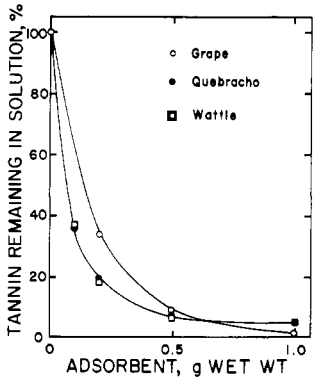

Figure 3. Adsorption of condensed tannins on Amberlite XAD-2. One milligram of tannins was dissolved in 1 mL of 0.1 M sodium acetate buffer, pH 4.0, and preweighed amount of resin was added and agitated for 10 min. Concentrations of tannins remaining in solution were determined by Folin-Ciocalteu method as modified by Singleton and Rossi (1965).

not be studied due to insufficient solubility of poly-L-proline.

Tannins from various sources were effectively adsorbed by a polystyrene resin (Figure 3).

DISCUSSION

In view of the heterogeneous nature of potential attachment sites on the surface of soluble proteins and of the diversity of molecular structures in the family of polyphenolic substances referred to as vegetable tannins, one could reasonably assume that the interaction between them would be one of mixed modes. Thus, the presence of hydrogen donors in the form of phenolic hydroxyl groups in the tannins and of hydrogen acceptors in the form of carbonyl functions of the peptide linkages of the proteins would naturally lead to a possibility of formation of hydrogen bonds. Likewise, since both groups contain hydrophobic regions, the aromatic nuclei of the tannins and the aliphatic and aromatic side chains of the protein amino acids, it would seem equally possible that these would participate in the interaction phenomena. Although charged groups are absent in the condensed tannins (Gustavson, 1956; Haslam, 1966), one cannot completely exclude ionic interaction in preparations of hydrolyzable tannins. Charged groups could arise, particularly in ellagitannins through partial hydrolysis of the depside linkages. But, when discussion is limited to the condensed tannins at acidic to neutral conditions, as we do here, one can confidently eliminate that possibility (Haslam, 1977, 1979). Proanthocyanidins become charged only at high pH by dissociation of the phenolic hydroxyl groups with formation of phenoxide ions.

Among the two attachment modes, hydrogen bonding and hydrophobic bonding, emphasis has been given to the former. Gustavson (1954), Goldstein and Swain (1963), and Synge (1975) among many investigator suggested that tannins bind proteins primarily through formation of multiple hydrogen bonds between the phenolic hydroxyl groups of the tannins and the carbonyl groups of the peptide linkages of the proteins. This concept was derived (Gustavson, 1954) from the fact that large amounts of tannins were fixed by modified polyamides, such as nylons, which contain the peptide linkages as "the only reactive group of any importance". Tannins could be prevented from forming complexes with proteins by addition of large amounts of PVP, which contain linkages of a similar nature (Loomis, 1969). As supporting evidence the work of Flett (1952) and Cannon (1955) have been cited, both of whom found that strong hydrogen bonds were formed in model systems containing such structures.

In discussing the relative merits of the two modes of association in the tannin–protein system, it is important to note that they are both solvent-dependent phenomena. Thus, hydrogen bonding between solutes is favored to occur in hydrophobic solvents such as carbon tetrachloride—which is used by both of the just cited investigators—while hydrophobic bonding between solutes primarily occurs in hydrophilic or polar solvents, such as water. The two functional groups that are assumed to interact according to the hydrogen bonding theory, namely the phenolic hydroxyl and the peptide carbonyl groups, are in aqueous media presumably solvated with hydrogen-bonded water molecules before interaction takes place. The overall reaction therefore involves breaking these solvent bonds as well as establishing the new bond between the solutes and will proceed if the reaction is accompanied by a decrease in free energy. Thermodynamic data are not available for this particular system, but Tanford (1970) found from a study of formation of interpeptide hydrogen bonds between initially solvated solutes that the reaction was not favored thermodynamically. On the other hand, hydrophobic interaction between solvated hydrophobic amino acid residues is strongly favored and will take place if steric factors permit.

By analogy with the system investigated by Tanford, we therefore suspect that the dominant mode of interaction between proteins and tannins might be hydrophobic bonding rather than hydrogen bonding provided tannins express sufficiently strong hydrophobic properties.

We have attempted in this paper to provide evidence that will distinguish between the two association modes and give clues to which mode would be the dominant one under given circumstances. The evidence is of four kinds and was designed to answer the following questions: What agents, hydrogen bond breakers or "hydrophobic bond breakers", will promote dissociation of tannin–protein complexes? What groups in a peptide chain tend to promote association and aggregation of the complexes? Which association mode is indicated by the behavior of the system with respect to temperature and ionic strength? And finally, do tannins express hydrophobic properties?

The first question was approached by means of the tannin–Sepharose 4B gel which was allowed to interact and form complexes with a protein (cytochrome *c* or myoglobin). When this system is operated in the column configuration, it constitutes a convenient way of demonstrating association and dissociation phenomena since the ligand can be physically separated from the adsorbent once dissociation has taken place. When the system was perturbed by the various agents, the results (Table I) were consistent with an interpretation of the complexes as being predominantly based on hydrophobic interaction. However, this interpretation is not rigorous. The mechanistic aspects of the action of the urea and guanidine hydrochloride are not fully understood. Their structures suggest that they may act as proton donors and acceptors (Haschenmeyer and Haschenmeyer, 1973), having the ability to participate in the formation of strong hydrogen bonds (Lumry and Eyring, 1954) and are not intrinsically hydrophobic. Although these agents were ineffective in accomplishing dissociation of the tannin–protein complexes, their failure might be due to steric factors making the putative hydrogen bonds between the tannins and the proteins inaccessible. The detergents, which were effective in accomplishing dissociation, are known to form micellar complexes with proteins through hydrophobic interaction with hydrophobic sites. Their effectiveness in our system suggests competition with inter- and intramolecular hydrophobic bonds involving other groups (Meyer and Kauzman, 1962), and hydrophobic regions of the tannin molecules. But again, alternative interpretations are possible. Thus, the initial perturbing effect of the detergents could result in a change in protein conformation, a "loosening" of its native compact structure, with the effect of rendering hydrogen bonding involving the peptide chain less favorable. It is worth noting that anionic, nonionic, as well as cationic detergents were all effective in dissociating the complexes, thus suggesting that ionic forces are not involved to any appreciable extent.

The experiments conducted with tannin and cytochrome *c* in free solution (Figure 1) yielded results similar to those discussed. The tannin–cytochrome *c* complexes were effectively dissociated by $NaDodSO_4$ evidenced by the disappearance of the turbidity. The concentration effect of $NaDodSO_4$ (Figure 2) indicates that interior hydrophobic groups are increasingly exposed to tannins due to partial unfolding of the protein as the $NaDodSO_4$ concentration increased from zero to 0.05%. At concentrations in excess of 0.05%, which is still well below the critical micelle concentration of $NaDodSO_4$ (0.5%), the turbidity abruptly decreased. At these concentrations protein-bound tannins are apparently completely displaced by $NaDodSO_4$ and the complexes dissociate. An alternative interpretation favoring interference with hydrogen bonds in the tannin–protein complexes, similar to the one discussed in the previous paragraph, can again be suggested, but the initial increase in binding at low $NaDodSO_4$ concentrations is more difficult to rationalize in terms of hydrogen bonding.

In attempting to answer our second question it was necessary to select protein models that differ in the number and size of hydrophobic sites as well as in hydrogen bonding peptide linkages. Gustavson (1954) and later investigators relied on soluble and insoluble PVP as well as on modified nylons as models of collagen. In either case, the presumed only reactive groups were peptide linkages. Binding of tannins to these substances were therefore ascribed to formation of hydrogen bonds. However, hydrophobic sites are available in both nylons and PVP. We have chosen a number of water-soluble substances as models for water-soluble proteins (Table II). This has the advantage of eliminating considerations of diffusional restrictions of tannins into solid receptors due to pore size and tortuosity limitations. The polyamino acids all contain peptide linkages, but differ in the nature of their side chains. Polyethylene glycol contain no peptide linkages, but instead the weakly hydrogen bonding ether linkage. Soluble PVP was included for comparison.

The most striking observation was the absence of interaction between tannins and the acidic polyamino acids. These substances are predominantly negatively charged in the pH region 4–6, but the lack of interaction was evidently not due to the ionic states of the molecules since they are largely neutral at pH 3. An abundance of peptide linkages are present in these molecules and it seems reasonable that interaction should have taken place if hydrogen bonding is the predominant mode of complex formation.

The side chains of these polymers are dominated by the polar carboxyl groups. The hydrophobicity present in the form of the adjacent CH_2 groups would likely be neutralized by a shielding of water molecules surrounding these highly polar groups (Hofstee, 1976; Tanford, 1978). Complex formation would therefore not be expected if the association mode is predominantly hydrophobic bonding. Our results with polyglutamic acid are in general agreement with those of Morawiecki (1963) who obtained a slight precipitate only several hours after adding a large excess of tannic acid.

Considering in sequence the polymers of serine, hydroxyproline, proline, and lysine (Table II), it is noticed that the turbidity increased as the number of aliphatic carbon atoms in the side chains increased. Hydrophobicity is according to Tanford (1973) a linear function of the length of the hydrocarbon chain for a homologous series of *n*-alkanes. The hydrophobicity of amino acid side chains was by that investigator similarly found to increase with the size of the side chain. Furthermore, the $-(CH_2)_4-$ portion of the lysine side chain is also hydrophobic and its hydrophobicity can be realized if the terminal $-NH_3^+$ group remains in contact with water, while the hydrophobic portion associates with other hydrophobic groups.

A relatively low turbidity was realized with polylysine at low pH (Table II). Morawiecki (1963) observed that interaction of tannic acid with polylysine required an ionic strength in excess of 0.1 M and interpreted this phenomenon to indicate that repulsion between the positively charged polymer molecules prevented aggregation at low ionic strengths. The ionic strength was in our system 0.1 M and such repulsion would likely be expressed at the lower pH values.

The strong interaction of tannins with polyethylene glycol (Table II) is particularly interesting. This polymer would seemingly not offer strong proton acceptor sites to the tannins, but is instead known to exhibit relatively strong hydrophobic properties (Bailey et al., 1964). Ikawa et al. (1975) noted the influence of hydrophobic interactions on complexation of PEG with polyacrylic acids.

The validity of homopolymeric polyamino acids as models for proteins interacting with tannins may be questioned on the grounds that their structures are far simpler, and their charge, if present, is quantitatively and qualitatively uniformly distributed. Their response to pH and ionic strength changes can therefore be expected to be quite different from those of proteins. Whatever their limitations, the one property that appears to be consistent with their interactions with tannins is their hydrophobicity.

A characteristic feature of hydrophobic interaction is that its strength increases with an increase in ionic strength and temperature, while the opposite is true for interactions based on hydrogen bonding (Hjerten et al., 1974). These effects could not be unambiguously demonstrated with BSA, but were clearly manifested with gelatin and poly-L-proline (Table III). A similar effect of temperature on complex formation has been reported with gelatin and lysozyme (Mejbaum-Katzenellenbogen, 1955).

In attempting to obtain an answer to our last question, whether tannins express hydrophobicity, we chose to investigate their adsorption behavior with a hydrophobic resin containing no polar groups as used by Gray (1978). This investigator found that a polystyrene resin (Amberlite XAD-2) adsorbed low molecular phenolic substances, while proanthrocyanidins were not affected. He concluded that the former were bound hydrophobically, while the latter, which could be adsorbed by a cross-linked PVP, must use a hydrogen bonding mode. Considering the similarity in structure and functional groups between the low molecular and high molecular compounds used by Gray we found this conclusion unexpected and have reinvestigated some of his results. Amberlite XAD-2 has a nominal pore size of 90 Å when properly hydrated by pretreatment with methanol. This value should provide more than sufficient space for proanthocyanidin to diffuse into the resin, provided sufficient time is made available. Our results (Figure 3) demonstrate that condensed tannins from three diffenent sources are effectively adsorbed by the resin. The adsorption mode must be hydrophobic since no polar groups are present in this resin.

Earlier in this discussion we posed several questions, which when answered were intended to throw light on the mechanism of association between condensed tannins and proteins. The answers provided no unequivocal evidence in favor of hydrophobic bonding, but they are highly suggestive of the concept that this is in fact the predominant mode of interaction. Considering that proteins are very complex structures with a variety of chemical groups of different affinity characteristics, it would, on the other hand, be unreasonable to ascribe the interaction phenomena exclusively to this particular mode. This reservation is accentuated when one further considers environmental solvent effects and the existence of hydrophobic micro domains in the interior of proteins molecules where hydrogen bonding would be favored.

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