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Tannin chemistry in relation to digestion

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Abstract

Tannins are a diverse group of compounds which precipitate protein. The impact of tannins on herbivory has been difficult to assess because of diversity in tannin chemistry and in animal physiology. We have evaluated the effects of tannin on large ruminants (deer, sheep) using artificial diets containing well-defined tannins, and have compared the results to those obtained with natural forages. The different effects of condensed tannins and gallotannins on herbivores are related to the chemical stability of the tannins. Commercial tannic acid does not have the same effects on herbivores as gallotannins in natural forages. Molecular weight apparently determines the metabolic fate of gallotannins from various sources.

Tannins are a chemically diverse group of water soluble phenolics which bind proteins to form soluble or insoluble complexes (Bate-Smith and Swain 1962, Hagerman 1989). Tannins are widespread among dicotyledenous forbs, shrubs, and trees (Haslam 1979) and are thus ingested by many herbivorous mammals. Dietary tannin diminishes protein and dry matter digestibility in some mammals (Robbins et al. 1987a, 1987b) but does not decrease digestion in others (Driedger and Hatfield 1972). Tannin sometimes acts as a toxin rather than a digestion inhibitor (Mehansho et al. 1987a). The diversity of effects of tannin on digestion is due in part to differences in the physiological capabilities of animals to handle tannins and in part to differences in the chemical reactivity of various types of tannins.

Recent work has demonstrated that several mechanisms are used by animals to counteract the effects of ingested tannins on digestibility. For example, tannin has little effect on digestibility in some insects because gut adaptations, such as elevated pH or detergency, inhibit interaction of tannin with protein (Martin et al. 1985). In some mammals salivary tannin-binding proteins apparently protect other, more valuable proteins from tannin (Mehansho et al. 1987b, Austin et al. 1989, Robbins et al. 1991). Hamsters, which do not produce salivary tannin binding proteins, cannot be maintained on a tannin-containing diet, while rats and mice accommodate dietary tannin (Mehansho et al. 1987a, b). The tannin-binding proteins from rats and mice have been characterized, and are proline-rich glycoproteins with lower molecular weights (Mehansho et al. 1987b). A similar protein with high affinity for tannin has been isolated from the saliva of deer but is not found in the saliva of sheep (Austin et al. 1989). The production of salivary tannin binding proteins may correlate with feeding niche; deer are browsers and often ingest tannin, and sheep are grazers which only occasionally consume tannins (Robbins et al. 1991).

Tannins are divided into 2 classes, condensed and hydrolyzable, based on their chemical structures (Hagerman and Butler 1989). Condensed tannins (proanthocyanidins) are flavonoid polymers (**1**, Fig. 1). Although condensed tannins can be oxidatively degraded in acid to yield anthocyanidins (**2**, Fig. 1) (Porter et al. 1986), under mild or anaerobic conditions the polymer is stable. Hydrolyzable tannins (**3**, **4**, Fig. 1) are gallic acid (**5**, Fig. 1) or hexahydroxydiphenic acid (**6**, Fig. 1) esters of glucose or other polyols (Haslam 1979). The gallotannins (**3**, Fig. 1) are simple esters of gallic acid, and may contain up to 5 galloyl groups esterified directly to the polyol (mono-, di-, ...pentagalloyl glucose), and additional galloyl groups esterified to the core galloyl groups (hexa-, heptagalloyl glucose, ...). Since they are esters, the hydrolyzable tannins are easily hydrolyzed, yielding gallic acid or hexahydroxydiphenic acid and the parent polyol.

Although the structural diversity of tannins is well documented, there have been only a few attempts to evaluate how structure

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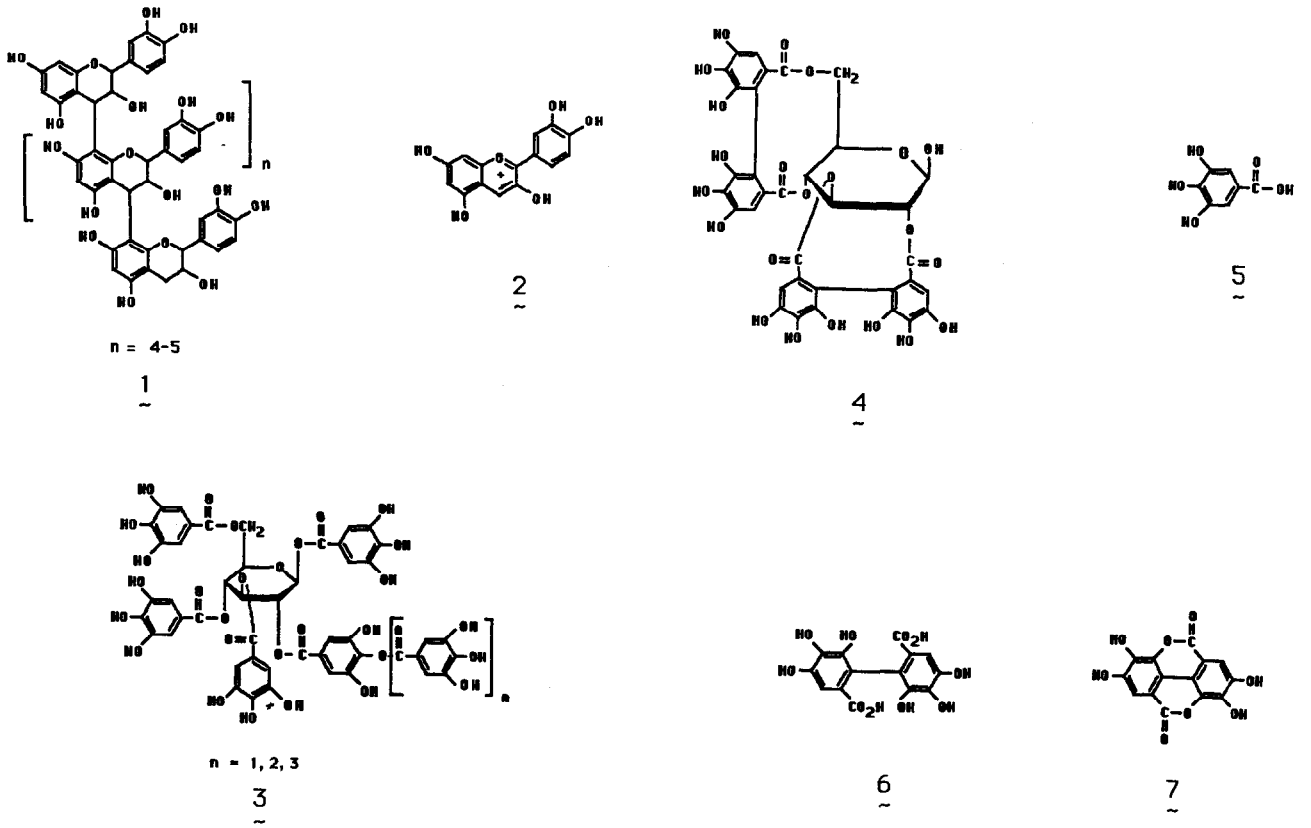

Fig. 1. Structures of tannins and their degradation products. 1 Condensed tannin from *Sorghum* grain. 2 The anthocyanidin cyanidin. 3 A gallotannin. 4 An ellagitannin. 5 Gallic acid. 6 Hexahydroxydiphenic acid. 7 Ellagic acid, which spontaneously forms from 6 in solution.

influences the biological activity of tannin (Clausen et al. 1990). We hypothesized that major structural differences between condensed and hydrolyzable tannins would have a substantial effect on activity of the tannin. We anticipated that condensed tannins would decrease protein and dry matter digestibility, but that hydrolyzable tannins would not affect digestibility. Instead, hydrolyzable tannins would be degraded in the gut to small phenolics which would not interact with protein. We tested that hypothesis by conducting complete digestion trials with 2 ruminants and 2 diets, one containing commercial condensed tannin (quebracho tannin) and the other containing commercial hydrolyzable tannin (tannic acid). Furthermore, we hypothesized that within a single group of tannins (hyudrolyzable or condensed) subtle structural differences should not alter the activity of the tannin. We tested that idea by comparing the structure and in vivo effects of the hydrolyzable tannin found in fireweed flowers to the structure and effects of tannic acid.

Methods

Tannic acid, a gallotannin, (technical grade) was obtained from Mallinckrodt Chemical Co. (St. Louis, Mo.). Quebracho tannin, a condensed tannin, was obtained from Tannin Corp. (Peabody, Mass.). Tannin was extracted from fireweed flowers with 70% acetone and purified by adsorption on Sephadex LH20 (Wilson 1989).

Gallotannins were separated by high performance liquid chromatography (HPLC) in a system which separates galloyl esters according to molecular weight (Wilson 1989). A silica (normal phase) column (Alltech Econosphere, 150 mm × 4.6 mm, 5 um particles; Alltech, Deerfield, Ill.) and a 25 mm precolumn containing Perisorb A (Anspec Co., Inc., Ann Arbor, Mich.) was used. The mobile phase, 58% (v/v) hexane and 42% (v/v) solvent A, was run at a flow rate of 1.0 mL/min. Solvent A contained methanol/-tetrahydrofuran (3/1, v/v) and citric acid (0.25%, w/v). Samples were dissolved in the mobile phase and introduced with a 20 ul sample loop. Components were detected at 280 nm as they eluted from the HPLC. The detector was interfaced with an Apple IIe computer for peak integration with Chromatochart 2.0 software (Interactive Microware, State College, Penn.).

Total collection digestion trials were conducted with 6 mule deer *(Odocoileus hemionus hemionus)* and 5 Suffolk sheep *(Ovis aries)* as previously described (Robbins et al. 1991). Commercial tannin was mixed with coarsely ground alfalfa pellets (tannin-free) containing 14.8% crude protein to yield diets with crude tannin contents of either 3% or 6%. The trials were conducted with a 10-day pretrial followed by a 7-day total collection of feces while animals were in metabolism crates. The amount of feed offered during the early pretrial was reduced until all feed was consumed. This level of feeding was then used during the remainder of the pretrial and the trial. Fresh undried feces for analysis were frozen, ground with dry ice, and stored at –40° C. Other feces were dried at 100° C, weighed and discarded.

No attempt was made to hold dietary protein content constant as tannin was added. Comparisons of protein digestibility between diets thus require a correction for the reduction in dietary protein because of dilution. Digestible protein content is a linear function of dietary protein (Robbins 1983, Van Soest 1982) and can be calculated if the true digestibility of the dietary protein is known. The true digestibility of dietary protein in mule deer and domestic sheep is 0.93 (National Research Council 1971, Robbins et al. 1987a). The reduction in digestible protein was compared to the reduction expected from dilution using analysis of variance (F test, $p<0.05$).

Crude protein in the feces was determined by macro-Kjeldahl analysis. Gallotannin in plants and feces was quantitated by measuring gallic acid released upon hydrolysis. Plants were extracted with agitation at room temperature 4 times with acetone:water, 70:30 (v/v), and the extracts were combined (10 mL solvent/g dry matter). Samples of dry feces (50 mg) or plant extracts (equivalent to 20 mg dry matter) were hydrolyzed at 100° C for 26 hours with 1 ml of 2N sulfuric acid. The hydrolysate was diluted to 10 ml with water, and a 1-ml aliquot was analyzed with the rhodanine assay (Inoue and Hagerman 1988). Condensed tannin in the plant extracts was quantitated with the acid butanol assay (Porter et al. 1986) using polyvinylpyrrolidone (Watterson and Butler 1983). A modified acid butanol assay was used to determine condensed tannin in feces (Robbins et al. 1991).

The ability of tannin to precipitate protein was determined with a modification of the blue dye labeled bovine serum albumin (BSA) precipitation assay (Asquith and Butler 1985). Mixtures containing 5.12 mg protein and 0.1–0.5 mg tannin were incubated for 2 hours at 4° C, and were then centrifuged at 2,000 g for 10 min to remove precipitated tannin-protein complexes. The supernatants were partitioned against ethyl acetate (4 ml) 3 times, and the ethyl acetate phases, which contained tannic acid that had not interacted with protein, were combined. The ethyl acetate was removed by evaporation under reduced pressure, and the residue was redissolved in the mobile phase for HPLC and was chromatographed. The area of each peak was normalized for comparison to the control sample, which was not treated with protein. The area of the peak decreased relative to the control if the component was precipitated by protein. Peak areas were compared using the t-test for comparison of means (95% C.L.).

Results

In deer, protein digestibility is reduced when plants containing condensed tannin, gallotannin, or a mixture of tannins are ingested (Table 1). Commercial condensed tannin added to a tannin-free diet similarly reduces protein digestibility in a dose-dependent fashion in deer or sheep (Fig. 2, Robbins et al. 1991). The reductions in protein digestibility were significantly greater in domestic sheep than in mule deer. When tannic acid, a commercial gallotannin, was added to a tannin-free diet, protein digestibility did not decrease in deer or sheep (Fig. 2) and gallic acid did not appear in the feces of either species. When deer were fed fireweed flowers, which contain gallotannin and only trace amounts of condensed tannin, protein digestibility was reduced (Table 1) and 27% of the ingested gallic acid was recovered in the feces.

Table 1. Effect of tannin in several forages on protein digestibility in deer.

Plant	Reduction in protein digestibility	Proantho-cyanidins	Gallotannins
	(g/100 g feed[1])	(mg/g[2])	(mg/g[3])
Alder	6.30	9.4	39
Fireweed plants	6.34	0.9	27
Dogwood	6.42	1.5	42
Fireweed flower	7.07	1.3	39

[1]Data from Robbins et al. 1987 (Figure 6).
[2]mg *Sorghum* tannin equivalents/g dry matter
[3]mg esterified gallic acid/g dry matter

The commercial tannic acid used in the feeding trial (Mallinckrodt technical) was largely comprised of low molecular weight galloyl esters such as tri- and tetragalloylglucose (Fig. 3a), and the average molecular weight of this material was 789 g/mol. There were substantial differences in the components present in 8 preparations of tannic acid (Fig. 4a, b; Wilson, 1989). The samples

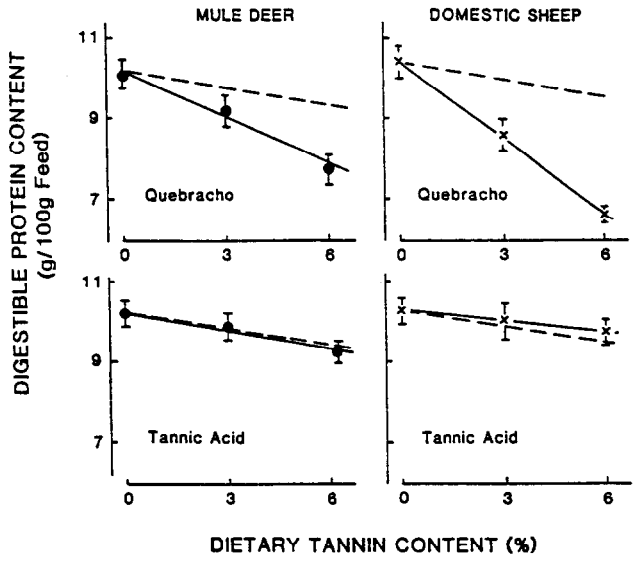

Fig. 2. Protein digestibility as a function of tannin added to the diet. Protein digestibility was determined with 6 deer or 5 sheep fed alfalfa pellets supplemented with quebracho tannin or tannic acid. The points show the means and standard deviations, and the dotted lines show the expected change in digestibility due to dilution of the diet with the tannin.

contained between 5 and 10 components and had average molecular weights which ranged from 789 to 1,027 g/mol. The gallotannin extracted from fireweed flowers elutes as a single broad peak, with a mobility intermediate that of octa- and nonagalloyl glucose (Fig. 3b), and has an average molecular weight of 1,475 g/mol.

The interaction of individual components of the commercial tannic acids with protein was examined. The areas of peak A–E (Fig. 5) were not substantially decreased when a representative sample of tannic acid was treated with protein, indicating that gallic acid (peak A) and mono, di-, tri-, and tetragalloylglucose (peaks B–E) do not precipitate protein. Moderate sized galloyl esters including pentagalloylglucose (peak F) do not precipitate when small amounts of tannin are mixed with protein but do precipitate when larger amounts of tannin are used (Fig. 5). The higher molecular weight components, such as nonagalloylglucose (peak J), were precipitated even when a small amount of tannin was mixed with protein (Fig. 5). With all the preparations of tannic acid that were tested the higher molecular weight gallotannins were preferentially precipitated by protein.

Discussion

The reactions between tannin and protein have been extensively studied, and in general, are similar for condensed tannin and gallotannins (Hagerman and Klucher 1986). Tannins bind proteins selectively and have especially high affinity for large proteins, conformationally open proteins, and proline-rich proteins (Hagerman 1989). Tannins do not precipitate proteins at pH values above the pKa of the phenolic group (pH 9) and precipitate proteins most effectively at pH values near the isoelectric point of the protein (Hagerman and Klucher 1986). Modifiers of solvent polarity, including organic solvents in vitro and detergents in vivo, influence tannin-protein interactions (Hagerman and Klucher 1986). For either gallotannins or condensed tannins, the tendency to interact with proteins increases as the molecular weight of the tannin increases (Verzele et al. 1986, Hagerman 1989). Purified preparations of condensed tannins and gallotannins with similar molecular weights precipitate similar amounts of protein (Hager-

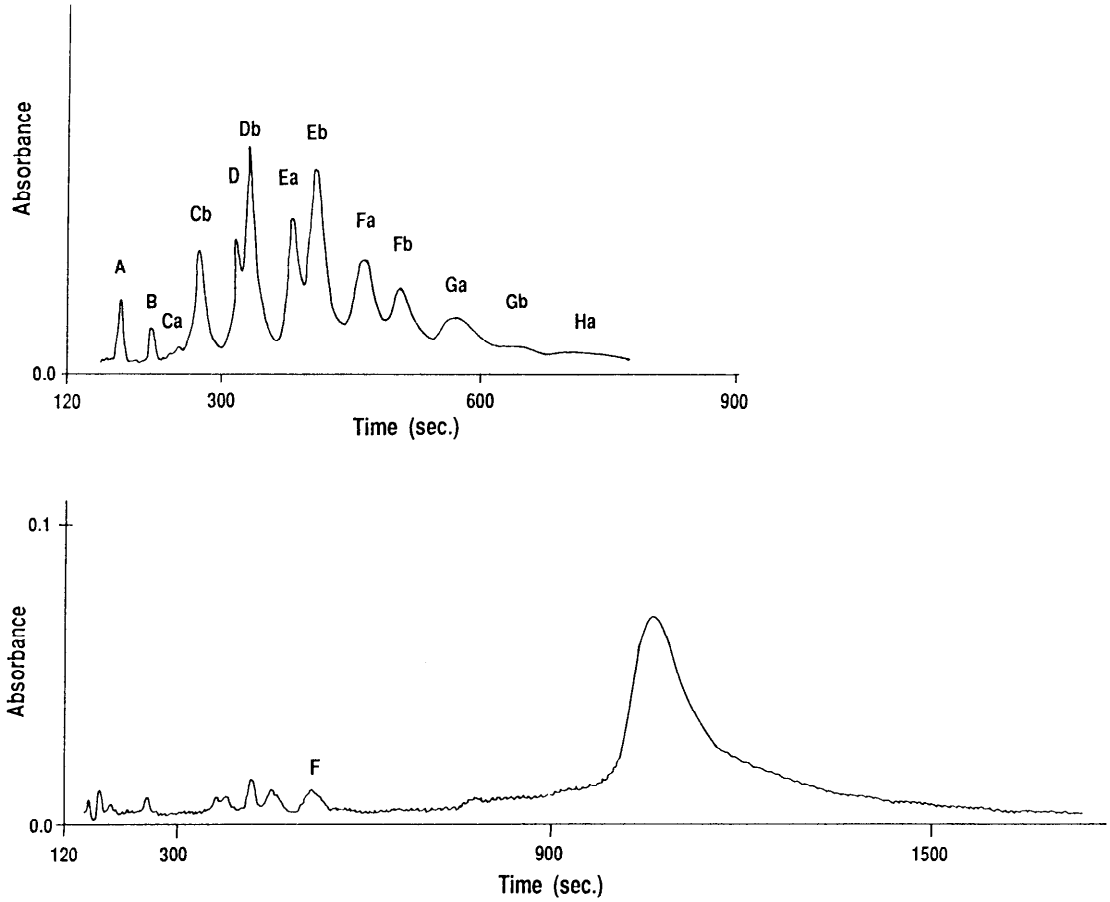

Fig. 3. Chromatograms of gallotannins. Hydrolyzable tannins were chromatographed on a silica column with hexane/methanol/tetrahydrofuran as described in the text. No peaks were eluted before 120 seconds. Letters indicate peaks with retention times that are the same, and subscripts (a, b) indicate shoulders of the major peaks. a) Mallinckrodt technical tannic acid. b) Fireweed flower tannin.

man and Klucher 1986). Since in vitro of interaction with protein suggest that condensed tannin and gallotannins are similar, generalizations about the effects of dietary tannin have often been based on the results of feeding trials performed with tannic acid.

However, because hydrolyzable tannins are more easily degraded than condensed tannins, we predicted that hydrolyzable tannins would not affect protein digestibility. Consistent with our hypothesis, we found that dietary quebracho tannin, a condensed tannin, diminished protein digestibility in deer and in sheep; dietary tannic, a hydrolyzable tannin, did not affect protein digestibility. Furthermore, none of the ingested tannic acid is excreted in the feces of deer or sheep because the tannin is hydrolysed soon after ingestion and the gallic acid that is produced is absorbed and excreted in the urine (Booth et al. 1959). The metabolic fate of the condensed tannins is more complex. Deer excrete 100% of the ingested quebracho tannin in the feces (Robbins et al. 1991), consistent with formation of indigestible complexes with protein. Sheep excrete only about 60% of the ingested quebracho tannin in the feces (Robbins et al. 1991), suggesting that some of the tannin may be absorbed (Mehansho et al. 1987b).

We postulated that structural differences between gallotannins from different sources would not influence the biological activities of the tannins. This hypothesis was not supported by our data. In deer, the effects of dietary tannic acid are not like the effects of gallotannins found in plants such as fireweed flowers, perhaps because commercial tannic acid is low molecular weight and heterogeneous. The interaction of gallotannins with proteins preferentially involves higher molecular weight galloyl esters. The high molecular weight species found in fireweed flowers may bind protein and be protected from hydrolysis. The low molecular weight species found in tannic acid do not interact strongly with protein, and are not protected from hydrolysis in the gut.

Both gross chemical differences, such as those distinguishing condensed tannins from gallotannins, and subtle differences, such a molecular weight or stereochemical configuration (Clausen et al. 1990) can influence the biological activity of tannins. Generalizations about the effects and function of tannins should not be based on studies with tannin from a single source, but should be drawn from studies that address the diversity of tannin chemistry. The physiological capabilities of the herbivore also influence the activity of the ingested tannin and must be considered when drawing conclusions about the effect of tannins (Robbins et al. 1991, McArthur et al. 1991).

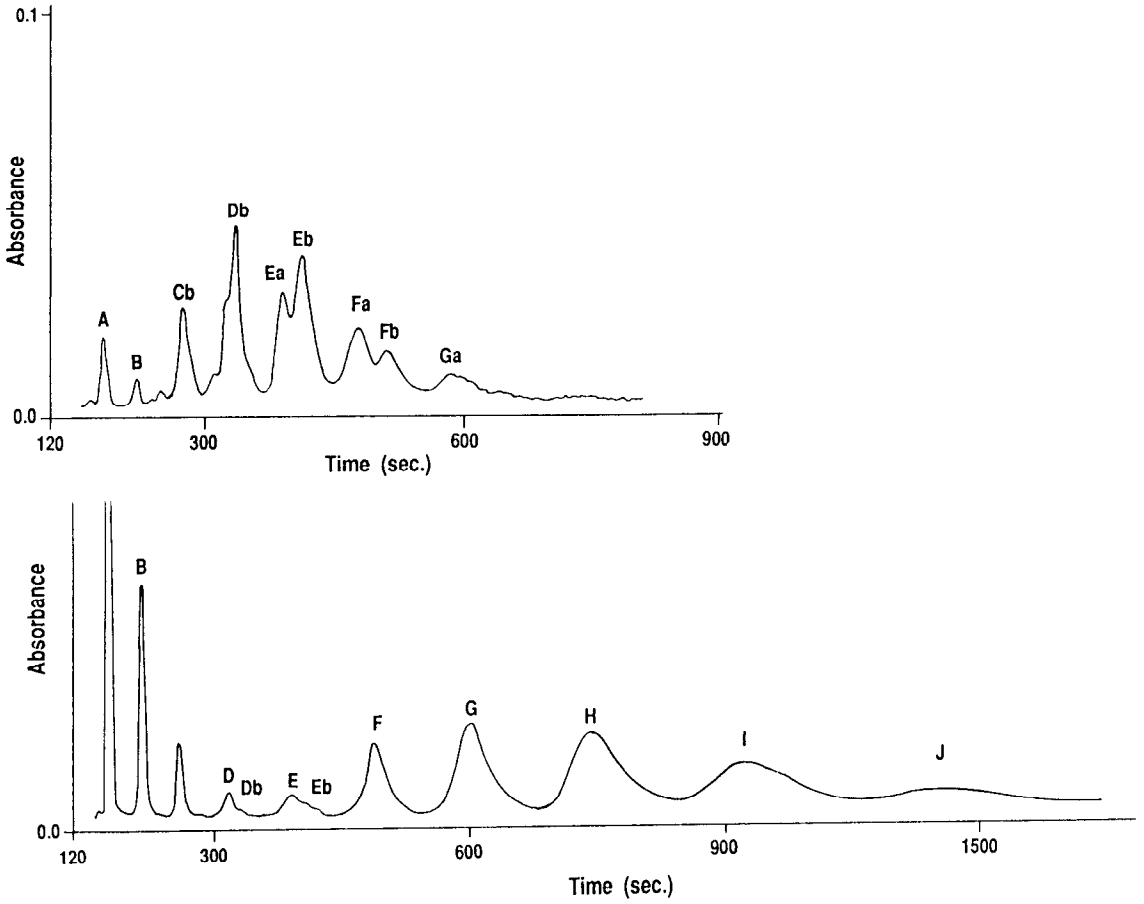

Fig. 4. Chromatograms of commercial gallotannins. Commercial gallotannis were chromatographed on a silica column with hexane/methanol/tetrahydrofuran as described in the text. No peaks were eluted before 120 seconds. Letters indicate peaks with retention times that are the same, and subscripts (a, b) indicate shoulders of the major peaks. a) Mallinckrodt reagent grade tannic acid (lot KCAZ, 1988). b) Mallinckrodt reagent grade tannic acid (lot BJN, 1974).

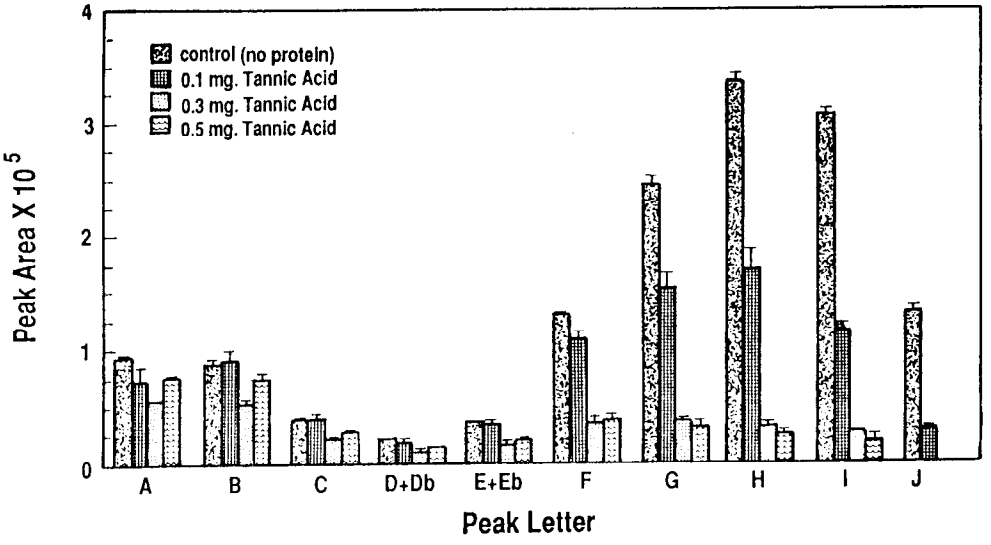

Fig. 5. Precipitation of components of tannic acid. Various amounts of tannic acid (Mallinckrodt reagent grade, lot BJN) were mixed with blue dye labeled bovine serum albumin or with buffer (control). The precipitated tannin-protein complex was removed, the the supernatant was extracted with ethyl acetate and was chromatographed. Peaks were identified by retention time, and labeled with letters as in Figures 3 and 4. Peak areas were normalized so that the recovery of each component in samples containing various amounts of tannic acid can be directly compared. Peak J was completely precipitated in the mixtures containing 0.3 or 0.5 mg tannic acid.

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