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A critical analysis of techniques for measuring tannins in ecological studies

II. Techniques for biochemically defining tannins

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Summary. A series of seventeen taxonomically diverse plant extracts rich in phenolic materials, including condensed and hydrolysable tannins, have been subjected to a series of biochemical analyses in an attempt to gather ecologically significant information about their interaction with proteins and amino acids. Methods employed were (i) protein-precipitation, using bovine serum albumin as substrate, followed by computation of specific activities of the tannins present in the extracts, and (ii) the inhibition of cellulase activity by tannin extracts bound to the cellulose substrate and free in solution. Both techniques revealed that all extracts contained tannin material. However, attempts to relate the results of the two procedures and in turn to relate them to information reported previously on the chemical properties of these extracts revealed that there was little correlation between any of the chemical or biochemical properties examined. From this analysis it would seem that whilst the analytical procedures available for studying tannins may generate ecologically useful information it is at present impossible, at least where plant material that is taxonomically diverse is being examined, to extrapolate from one type of measure to anticipate what would be observed from another type of measure. In addition to the above three other observations arose from this study. First, it appears to be generally true that there is not an absolute positive correlation between the level of protein precipitation and the incorporation of tannin in the tannin-protein precipitate. As relative protein concentration increases the proportion of tannin bound in the precipitate decreases, leading to less stable precipitates. Second, it is confirmed that some basic amino acids will precipitate with tannins, a phenomenon that could potentially influence amino acid balance in the diet. Third, complexation between tannin and protein absorbed on a cellulose substrate is able to interfere with the digestion of that cellulose by cellulase enzymes. Cellulose masking of this type may potentially effect the efficiency of cellulolytic activity in the rumen and if so suggests another subtle variation in the potential antifeedant properties of tannins.

Key words: Tannin – Protein precipitation phenolics – Feeding-deterrents

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The biologically and ecologically important properties of tannins are thought to depend on their complexation with proteins and other components in herbivore diets (Feeny 1976; Rhoades 1977; Zucker 1983). The use of techniques to measure tannins that are dependent upon their operational properties, in contrast to their chemical ones, is long standing. For example, in the leather tanning industry hide powder precipitation has been used extensively to define and assay tannins, although it is recognised that good hide powder precipitants are not necessarily good tannins for leather (Gustavson 1956). A similar lack of correlation may be encountered in ecological studies (Martin and Martin 1982); chemical measures of tannins may not correlate well with the properties of tannins as measured through their interactions with proteins or other biochemicals (e.g. amino acids, cellulose). Similarly, the measure of operational tannin, as exemplified by protein precipitation, may not provide valid information on the effects of dietary tannin in vivo (Martin et al. 1984; Mehansho et al. 1985; Mole and Waterman 1985).

In this paper we examine the operational analysis of tannins using the following methods; (i) tannin precipitation with proteins and amino acids, and (ii) the adsorption of tannins into solid cellulose matrices and their consequent effects on the activity of cellulases. The objective of this work is to assess variation in these "biochemical" characteristics of tannins in relation to each other and as a function of their chemical variation using the series of seventeen tannin extracts whose chemical analyses were reported upon in the previous paper (Mole and Waterman 1987a). This study has been undertaken to try and determine to what degree there is biochemical variation of potential ecological interest between compounds classed as tannins, to what extent measurement of variability is technique dependent, and whether or not simple chemical analyses correlate with variation in activity.

The first technique examined was the measurement of protein precipitating ability. By definition, the precipitation of protein by tannins is the most dependable way of detecting them. However, as will be shown, quantifying tannins by this property has proved difficult. Furthermore, precipitate formation is not in itself a biologically or ecologically useful measurement, it is only the effects of nullifying the function of proteins that are of significance. For instance in digestive systems precipitation is normally assumed to lead to enzyme inhibition which is then taken to be a dele-

terious effect. Yet the gastric enzyme, rennin, is well established as the agent responsible for precipitating and slowing the digestion of milk proteins in young mammals (Orskov 1982), presumably a beneficial process for the animal! In insects the interaction of tannin and protein can be interrupted by surfactants (Martin et al. 1984) and the same appears to hold true for vertebrates (Freeland et al. 1985; Mole and Waterman 1985). In short care has to be taken in interpreting in vitro measures of the effects of protein precipitation by tannins.

Secondly, we examine an alternate assessment of tannin activity by studying their impact on the enzymic degradation of cellulose using a system (Jones and Hayward 1975) which many investigators believe to be a useful one for modelling the in vivo process of digestion in the rumen.

Materials and methods

The sources and method of production of the extracts employed in this study have been reported in the previous paper (Mole and Waterman 1987a).

Protein precipitation analysis

Following Hagerman and Butler (1980) BSA (Sigma fraction V) was prepared as a 5 mg/ml stock solution in a buffer of pH 4.9, which is the protein's isoelectric point. The buffer (170 mM NaCl and 200 mM acetic acid brought to pH 4.9 with NaOH) contained sufficient salt to ensure that ionic strength did not limit precipitation. For each assay, two sets of eleven test tubes containing 0, 0.5, 1.0, 1.5, ...5.0 ml of BSA stock solution were prepared, the tubes being made up to 5.0 ml by the addition of buffer solution. A sample of the tannin extract to be assayed was then dissolved in more buffer solution and any insoluble material was removed by decanting or centrifugation. 0.5 ml of the clear tannin solution was added to each of the twenty-two tubes containing BSA/buffer solution and 15 min allowed for complete precipitate formation, after which the tubes were centrifuged for 5 mins at the top speed of a bench centrifuge to pellet the precipitates. The supernatants were then assayed for phenolics by the method of Hagerman and Butler (1978). The pelleted material was re-dissolved in 4 ml of 4 M NaOH and heated for 4 h in a boiling waterbath. At the end of this period the hydrolysate was neutralised with 4 M HCl and assayed for amino nitrogen by the method of Moore and Stein (1954, 1968). None of the extracts themselves (hydrolysed or unhydrolysed) gave detectable reactions for amino nitrogen when tested at the concentrations employed in the experiments.

Where previously either no precipitation occurred or there was no levelling off in the amount of protein precipitated at the higher BSA concentrations experiments were repeated with tannin solutions of an appropriately altered strength. The 0–5 mg/ml concentration range for BSA employed in the experiments was thus used as a "window" through which maximal levels of precipitation were found by adjusting the concentration of tannin added to the assays. This resulted in a considerable amount of trial and error experimentation in order to obtain the results reported.

Method for amino-acid precipitation by tannin extracts

Amino-acid solutions were made up in water. Following Handley (1954), these were of 20 mg/ml or saturated solutions if the amino acid was less soluble than this. The amino acids tested were lysine, arginine, histidine, leucine, isoleucine, valine, glycine, proline, and alanine. Buffers used were all approximately 50 mM; pH 2 = HCl/KCl, pH 2.5 to 7 = citric acid/disodium phosphate, pH 7.5 to 9 = boric acid/borax, pH 9.5–10 = carbonate/bicarbonate.

Approximately 200 mg of freeze-dried extract was dissolved in 20 ml water, 2 ml of this solution then being combined with 2 ml of each buffer. Amino acid solutions were added dropwise to these solutions and the occurrence of precipitation or cloudiness noted.

Cellulase digestibility assay

A cellulase solution was prepared by dissolving 1.875 g *Trichoderma viride* cellulase (BDH) in 600 ml of a citrate/phosphate buffer (pH 4.6). Pieces of substrate (Whatman 3M chromatography paper) were cut into 15 × 15 cm squares and placed in a shallow tray containing 150 ml of distilled water in which sufficient of the tannin extract had been dissolved to give an assay for phenolics equivalent to 0.1% tannic acid. The paper was exposed to the tannin solution for 10 min with constant agitation from an orbital mixer. For tannin-free controls papers were soaked in distilled water only. Papers were then drained of excess fluid and placed in an oven to dry at 80° C. Those papers receiving a pretreatment with protein were placed in a 1% BSA solution for 10 min prior to the treatment with tannin.

The substrates were cut into strips weighing approximately 1 g and then subjected to a cellulase digestion for 48 h using the procedure of Choo et al. (1981) but omitting the pepsin pretreatment. After the digestion residues of substrate were collected by filtration, oven-dried and weighed to calculate percentage digested.

Results and discussion

Quantification of tannins by protein precipitation

A considerable effort has been made in recent years to quantify tannins by their interaction with proteins. Modern methods have primarily employed either haemoglobin or bovine serum album (BSA) as the protein. Variants on this theme have been attempts to employ leaf protein (Martin and Martin 1983), and β-glucosidase (Becker and Martin 1982). Hoff and Singleton (1977) measured the adsorption of tannin onto BSA immobilised on a solid support. Most recently Hagerman (1987) has developed a method that employs the formation of precipitin lines in protein impregnated agarose gels by diffusion of tannin solutions.

The earliest attempt to use a protein precipitation technique for the routine measurement of tannins in samples collected for ecological or taxonomic purposes was made by Bate-Smith (1973). Haemoglobin from freshly haemolysed blood cells was precipitated by tannin and the loss of haemoglobin from solution, measured spectrophotometrically at 578 nm, was used to gauge the "astringency" of the sample. Schultz et al. (1981) attempted to make this assay more convenient by using other sources of haemoglobin but found the assay to be absolutely dependent on a fresh supply of blood. Furthermore, the threshold level of tannin needed in the assay to initiate the precipitation of haemoglobin was found to be undesirably high (Schultz et al. 1981). It has been observed (Asquith and Butler 1985)

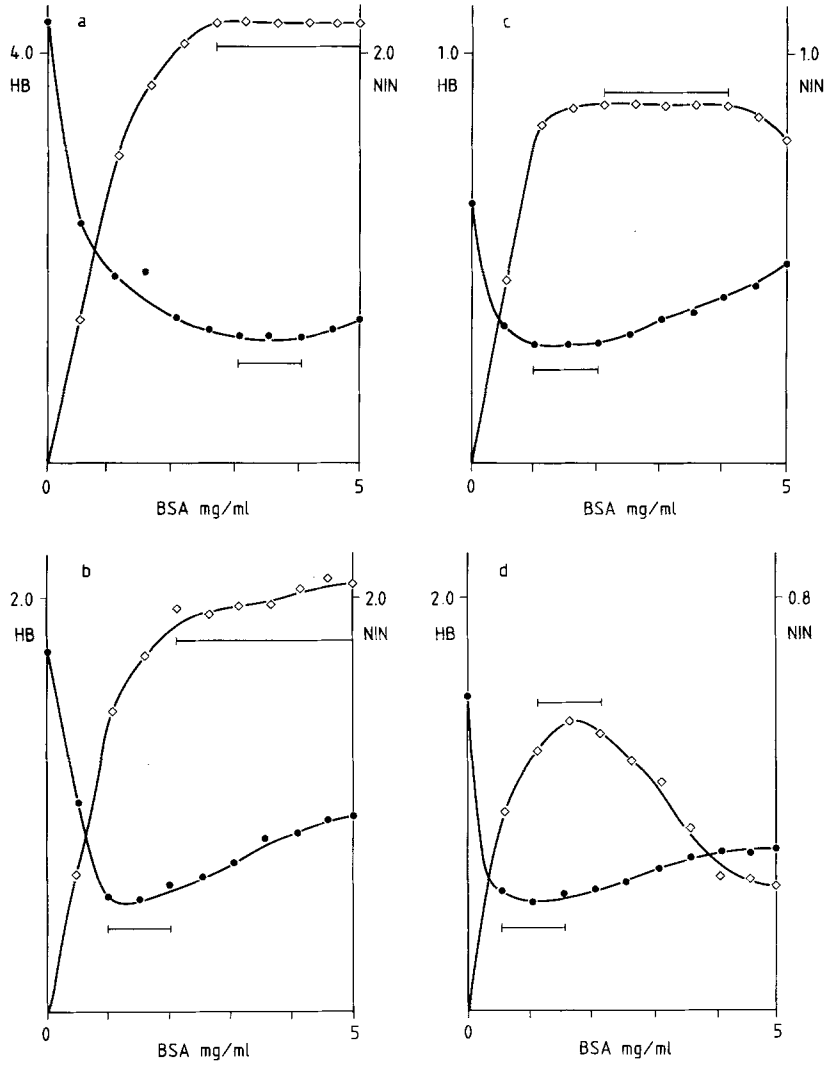

Fig. 1a–d. Plots of results for protein precipitation experiments using (**a**) quebracho tannin, (**b**) tannic acid, (**c**) *Pinus radiata* tannin and, (**d**) *Quercus* sp. tannin. The x axis indicates the strength of the BSA solution (mg/ml) titrated against the standard strength tannin. The quantity of protein in the precipitate is indicated by the NIN absorbance scale (points depicted as ◇) and the levels of phenolics remaining in the supernatant by the HB absorbance scale (points depicted as ●). Horizontal bars indicate the range of data points used for calculation of values presented in Tables 1 and 2

that many non-tannin plant constituents absorb at 578 nm and so interfere with this assay.

Both Martin and Martin (1983) and Hagerman and Butler (1978, 1980) report that BSA is a suitable protein for quantifying the activity of tannins. Hagerman and Butler (1978) initially measured the loss of tannin by precipitation from solution, but whilst of potential value in indicating the amount of tannin present this measure alone does not quantify the ability of that tannin to precipitate protein. They then developed a radiochemical method for detecting the precipitation of ^{14}C labelled BSA by tannins (Hagerman and Butler 1980). By a combination of these two techniques they were able to measure what they termed the specific activity of a tannin in terms of protein precipitation on a "per total phenolics" basis (Hagerman and Butler 1980). Unfortunately their radiochemical techniques require sophisticated instrumentation and as a consequence have not been adopted in ecological surveys.

In the work described here the method of Hagerman and Butler (1980) is employed to the stage where a tannin-protein precipitate is generated for analysis. Thereafter the protein in the precipitate is analysed by its alkaline hydrolysis followed by a ninhydrin assay for the amino acids generated in the hydrolysate. It is of note that both Martin et al. (1985) and Butler (Asquith and Butler 1985) have now changed their techniques for measurement of protein in the presence of tannins to what they consider to be more satisfactory methods. Although neither use a ninhydrin technique, Marks et al. (1985) have reported the suitability of this method for measuring proteins in the presence of tannins. The use of an alkaline hydrolysis in 4 M NaOH followed the recommended procedure for hydrolysing proteins (Mahler and Cordes 1971). Alkali, not acid, was chosen for the hydrolysis because only alkali dissolved the tannin protein precipitate to give a clear solution at the start of the hydrolysis stage.

Of the seventeen tannin extracts studied, the data for four are plotted in Fig. 1a to d. Each shows the level of protein precipitated by the tannin when a tannin solution of constant strength is titrated with the protein solution,

the concentration of the latter varying from zero to 5 mg/ml in 0.5 mg increments. Also shown is the concomitant change in the level of phenolics which remain in the supernatant solution. The anticipated outcome to the experiments is seen in the results obtained for quebracho tannin (Fig. 1a). Here there is a rise in the precipitation of protein in the experiment up to a plateau beyond which there is no more precipitation as the concentration of protein in the reaction increases. This is exactly the situation reported by Hagerman and Butler (1980). The fall in supernatant phenolics, which they did not record, does not exactly reciprocate the pattern seen in the curve for protein precipitation. There is no flat minimum, but rather a curve which begins to rise again at the higher concentrations of protein. This tendency indicates that at the higher protein concentrations, protein which is in the precipitate is associated with relatively less and less tannin whilst phenolics which were demonstrably tannins by virtue of their inclusion in a precipitate are tending to occur in solution with protein. Such a scenario is in keeping with the results of Van Buren and Robinson (1969) who reported redissolution of tannin-protein complexes in the presence of an excess of either component. As a final observation, it should be noted that the level of maximum protein precipitation occurs at a lower concentration of protein than does maximum phenolic precipitation.

The plots for tannic acid (TA, Fig. 1b) are similar in that there is a rapid rise in protein precipitation followed by a "levelling off", but not to a flat plateau. The curve for supernatant phenolics shows a clear minimum attained after an initial and steep fall. Thereafter there is a rise, again indicating that with increasing protein concentration more protein is associated with less tannin in the precipitate. For this and several other extracts it proved impossible to manipulate experimental conditions so as to obtain a flat plateau maximum for protein precipitation. One possible explanation for this could be the entrapment of protein solution within the precipitate, which was often quite bulky. Unlike quebracho tannin, the minimum for supernatant phenolics with TA occurs prior to the levelling off of protein precipitation.

Figures 1c and 1d show results for extracts Pr and Qs and illustrate the range of results obtained in the survey. For Pr a plateau for protein precipitation is seen but there is a clear fall from this at high protein concentrations. Figure 1d shows a further and extreme case of this for Qs, where there is a clearly peaked response in the curve for protein precipitation. This illustrates the same type of curve reported by Van Buren and Robinson (1969 – their Fig. 2). In both Fig. 1c and d the minimum in supernatant phenolics occurs before the maximum level of protein precipitation is reached. The decision as to where these maxima and minima occur is somewhat subjective. However, considering data for all the extracts it is interesting that there is a clear distinction to be seen with quebracho the only tannin where maximal protein precipitation is attained prior to maximum phenolic precipitation (Fig. 2). This suggests that the observation that there is a declining proportion of tannin relative to protein in precipitates may be generally true, the consequence of this being an ultimate decline in levels of precipitation as the condition is reached where precipitates become less stable due to the smaller percentage of tannin incorporated within them (Van Buren and Robinson 1969). From a methodological viewpoint, the combination

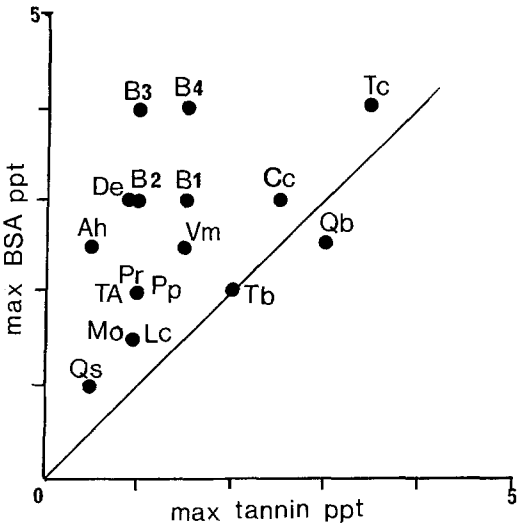

Fig. 2. A plot indicating the lower concentration limit of BSA at which maximum precipitation of the BSA and the tannin extract occurs in the protein-precipitation experiments (see Fig. 1a–d – ranges of maximum precipitation depicted by horizontal bars and values given in Table 1). Both axes are calibrated in ml of BSA solution (concentration 5 mg/ml). Codes for extracts are given in Table 1

of threshold precipitation effects (van Buren and Robertson 1969; Hagerman and Butler 1978; Schultz et al. 1981) and precipitates redissolving in excess protein make this protein precipitation assay difficult to manage when working with a range of tannins. For example, it proved impossible to conduct these experiments with our seventeen different extracts using a concentration of tannin set by a given measure of total phenolics and to obtain precipitation maxima within the range of protein concentrations used. The results of this can clearly be seen in Table 1 where the concentrations of phenolics employed with each extract tested are recorded.

The objective of these experiments was to calculate the specific activity (*sensu* Hagerman and Butler 1980) of the tannin extracts, with tannin concentrations being assessed on the basis of total phenolics in the extracts. In attempting this two estimates of specific activity (SA) were calculated. The first followed Hagerman and Butler (1980) and calculated SA1 as the maximum amount of protein precipitated in the assay divided by the total phenolic content (TPS) of the extract in the system (i.e. supernatant and precipitate), TPS (Table 2) being obtained using the method of Hagerman and Butler (1978) for total phenols. The value used for the protein precipitated was obtained from the ninhydrin assay for hydrolysed protein averaged over the values surrounding the highest level attained (as indicated in Fig. 1) and converted into mg BSA by reference to a calibration curve. Table 1 provides the data used to calculate specific activities for all the extracts, as derived from plots of experimental data such as that displayed in Fig. 1a–d.

As has already been observed the phenolics present in a precipitate with protein may not necessarily constitute all the phenolics capable of acting as tannins in the extract. Nevertheless, given that some non-tannin phenolics are certainly present in the extracts, a second specific activity measure (SA2) was calculated. SA2 used a value calculated for

Table 1. Maximum levels of protein precipitation ("protein") and lowest levels of phenolics remaining in solution after precipitation ("phenolics") based on the protein precipitation assays. The mean values (x) and standard deviation (sd) for the "protein" are based on absorbance values for the ninhydrin assay and are calculated over the range (mg/ml of BSA) of maximum precipitation (as indicated by the horizontal bar in Fig. 1a–d). Data for phenolics in solution was obtained in the same manner using absorbance values from the Hagerman and Butler (1980) assay. The absorbance value for total phenolics for each extract in the experiment are given as TPS in Table 2.

Tannin source	Code	"Protein"				"Phenolics"			
		range	x	sd	n	range	x	sd	n
Aesculus hippocastanum	Ah	2.5–5.0	0.17	0.000	5	0.5–2.5	0.17	0.008	5
Callistemon citrinus	Cc	3.0–5.0	1.20	0.067	5	2.5–4.0	0.89	0.000	4
Diospyros ebenum	De	3.0–5.0	1.13	0.019	5	1.0–2.5	0.23	0.028	4
Loropetalum chinense	Lc	1.5–2.5	1.16	0.018	3	1.0–2.0	0.24	0.031	3
Myrtus obcordata	Mo	1.5–4.0	1.03	0.069	6	1.0–2.0	0.60	0.004	3
Pomaderris phylloides	Pp	2.0–5.0	0.80	0.036	7	1.0–2.0	0.26	0.009	3
Pteridium aquilinum	B1	3.0–5.0	1.43	0.040	5	1.5–5.0	0.34	0.008	8
Pteridium aquilinum	B2	3.0–4.0	0.28	0.046	3	1.0–5.0	0.77	0.018	9
Pteridium aquilinum	B3	4.0–5.0	0.83	0.013	3	1.0–5.0	0.25	0.016	9
Pteridium aquilinum	B4	4.0–5.0	1.17	0.014	3	1.5–3.0	0.29	0.004	4
Quercus sp.	Qs	1.0–2.0	0.53	0.025	3	0.5–1.5	0.22	0.010	3
Taxus baccata	Tb	2.0–5.0	0.80	0.045	7	2.0–5.0	0.70	0.180	7
Vaccinium myrtilis	Vm	2.5–5.0	1.13	0.015	6	1.5–5.0	1.00	0.010	8
Tannic acid	TA	2.0–5.0	2.00	0.060	7	1.0–2.0	0.56	0.030	3
Quebracho tannin	Qb	2.5–5.0	2.15	0.010	6	3.0–4.0	1.22	0.010	3
Terminalia chebula	Tc	4.0–5.0	1.98	0.060	3	3.0–5.0	1.89	0.040	5
Pinus radiata	Pr	2.0–4.0	0.87	0.000	5	1.0–2.0	0.29	0.004	3

Table 2. Specific activity calculations for phenolic extracts. TPS = total phenolics in the system, as estimated by the Hagerman and Butler (1980) method; %T = the maximum percentage of TPS participating in the precipitation reaction (calculation from TPS and the minimum absorbance value for phenolics remaining in solution – see Table 1); SA1 = maximum protein precipitated/TPS, the value for protein precipitated (PROT) being obtained from the absorbance values for the ninhydrin assay (Table 1) and reference to a calibration curve for this assay against BSA; SA2 = (SA1/%T) × 100

Extract code	PROT	TPS	%T	SA1	SA2
Ah	0.40	0.25	31	1.61	5.20
Cc	2.85	1.79	50	1.59	3.18
De	2.69	0.79	71	3.36	4.73
Lc	2.75	0.80	70	3.42	4.89
Mo	2.44	1.10	45	2.22	4.92
Pp	1.90	0.54	52	3.49	6.72
B1	3.39	0.64	47	5.27	11.20
B2	0.66	1.30	40	0.51	1.27
B3	1.97	0.37	32	5.30	16.60
B4	2.77	0.70	59	3.94	6.68
Qs	1.26	1.51	63	0.83	1.32
Tb	1.90	0.96	27	1.96	7.25
Vm	2.68	1.29	22	2.66	12.10
TA	4.74	1.74	68	2.71	3.99
Qb	5.10	4.30	72	1.18	1.64
Tc	4.69	3.72	49	1.25	2.56
Pr	2.06	0.63	54	3.26	6.04

the minimum amount of soluble phenolics found in the supernatant (Table 1); this being used to express the maximum proportion of the total phenolics present in the extract (%T) which entered into precipitates, thus achieving a best estimate of functional tannins. The estimate SA2 expresses the activity of the extract in terms of these phenolics alone and is calculated as (SA1/%T) × 100. The results for all these variables (SA1, SA2, %T, TPS) are presented in Table 2.

The value obtained for the SA1 of TA is very close to that reported by Hagerman and Butler (1980). The values for SA1 of other tannins ranged from about twice to less than 20% of the value of TA. There is thus a clear spread of values for the variable SA1 and inspection of Table 2 shows a similar range for SA2. Values of SA2 are all higher than those for SA1 as values of %T are less than 100% (range 22% to 72%). There is no correlation between SA1 and %T evident in the data, thus indicating that a tannin or extract may be active as a precipitant whilst not necessarily being subject to precipitation in proportion to that activity. Indeed, such a relationship would not have been expected. Comparing the data in Table 2 to that for chemical analyses (Mole and Waterman 1987a) shows that not a single chemical variable correlates well with the measures of SA. Of particular note is the absence of a relationship with either estimates of polymer length or of the percentage of condensed tannin in the extracts. As it was found that procyanidin-rich condensed tannins were the predominant feature in the phenolic components of most of these extracts the absence of any correlation was disappointing.

However, considering data from individual extracts does provide some useful results. The De extract was suspected of containing hydrolysable tannins and so the low %CT1 value (= 23% – see Table 2 in Mole and Waterman 1987a) should not have indicated low astringency and indeed both SA1 (3.36) and %T (71) indicate a substantial amount of tannin material present in the extract. In contrast, Tc, with a (suspected) low concentration of hydrolysable tannin and no condensed tannin, has lower specific activities than TA and De. The data is also clearly indicative

of the presence of other precipitable phenolics in some extracts though these do not necessarily add to SA values. For example, consider the data for B1 (%CT1 = 6%, %T = 47%, SA1 = 5.27) and B2 (%CT1 = 6%, %T = 40%, SA1 = 0.51), suggesting that other phenolics contribute to precipitation in B1 but not B2. Conversely, extracts with %CT1 and %CT2 values of around 100% (Ah, Pp, Tb, Pr) can have comparatively low proportions of precipitable phenolics.

The results outlined above confound the belief that satisfactory interspecific comparisons of plant tannins can be made by the use of chemical analysis if the ultimate objective is to make inferences concerning tannin-protein interactions. The range in SA values found for extracts of equal phenolic concentrations, and lack of correlation between these activities and chemical variables illustrate the enormous diversity of this property in different phenolic extracts. Thus, these results reinforce points previously made by Martin and Martin (1983) in recommending the use of protein precipitation assays to measure tannins. This is not to say that intraspecific measurements cannot be based on chemical measurement techniques, but here errors will still occur if the components making up the plant phenolics change between samples (as between B1 and B2).

It could be argued that if chemically pure and defined sources of tannin had been used in this work, then some correspondence between (say) condensed tannin polymer length and specific activity might have been obtained. This does indeed seem plausible (Haslam 1974) but misses the point that herbivores do not consume pure chemicals but crude extracts. An important outcome of this study has been to determine that these ecologically significant relationships can not be distinguished against the background of the other phenolics present in the plant. Having established this, then there appears to be no justification from an ecological point of view in examining the specific activities of the component phenolics of an extract individually unless the commitment is made to do this exhaustively for the whole extract and then to further consider the effects of synergism between components.

On the positive side the results indicate a successful method for measuring the activities of tannin-containing extracts and several important sources of tannin-tannin variation have come to light. The existence of peaked responses seen in the curves for protein precipitation suggests that for some tannins the conditions where precipitates form are quite narrow. Given that the assay conditions are designed to be very favourable to precipitate formation then perhaps values for SA1 and SA2 are not the only biologically important variables, and parameters such as %T and threshold precipitation levels (very approximately indicated by TPS) should be included when assessing the potential effect of a tannin in a biological environment. The significance of these criteria would eventually require in vivo testing, but it should be noted that their utility is crucially dependent on the notion that precipitation phenomena are central to understanding the role of tannins as anti-feedants, an assumption brought into question by both Martin et al. (1985) and Mole and Waterman (1985).

The amino acid precipitating properties of the extracts

It is generally thought that tannins produce precipitation reactions with proteins, and not with their constituent free amino acids. Handley (1954) did, however, show that certain amino acids precipitated some tannins from solution, his observations being based upon the behaviour of fresh unbuffered plant extracts to which amino acid solutions were added dropwise. Generally these reactions involved basic amino acids and tannins from species whose litter tended to form mor humus. In the course of this investigation an attempt has been made to confirm these findings and to examine the influence of pH on any reaction. Table 3 presents the results for the precipitation of arginine and histidine, the two most reactive amino acids, with the extracted phenolics at a range of pH values.

Table 3. Formation of tannin – amino acid precipitates involving arginine (A) and histidine (H) over a range of pH values. + = precipitate observed

Extract code	pH								
	2	3	4	5	6	7	8	9	10
	A H	A H	A H	A H	A H	A H	A H	A H	A H
TA	+	+	+	+	+	+			
Qs	+	+	+	+					
De	+	+	+	+	+	+			
Pp	+	+	+						
B4	++	++	++	++	++	++			
Mo	+	+	+	+	+	+			
Lc	+	+	+	+	+	+	+		
Qb[a]									

[a] None of the remaining extract showed precipitation with these or any other amino acids

Handley's observations are confirmed by these results in that the amino acids which do give precipitates are of the expected (basic) type. No conclusion could be drawn as to the type of tannin most likely to produce precipitation, whilst the absence of precipitation cannot be taken as evidence that no interaction has occurred between the tannin and amino acid. The absence of precipitation at high pH indicates that ionisation of the non-alpha amino group may be important to the precipitation reaction. All precipitates formed were fully pH reversible.

The conclusion drawn from this experiment is that no simple relationship exists between the chemical structure of tannins and their interaction with single amino acids. Tannin-protein interactions can thus be expected to prove at least as complex, with the amino acid complement of the protein and its tertiary structure (Hagerman and Klucher 1986) likely to be as significant as the tannin. It is also of note from an ecological point of view that the interaction of single amino acids with tannins may act to inhibit the amino acid nutrition of herbivores by impairing specific amino acid absorption in the gut, thus extending the allelochemical action of tannins beyond that envisaged by Feeny (1976).

Effect of tannins on cellulase activity

In contrast to the precipitation techniques which make the most accurate detection and determination of tannins in terms of their operational definition, this section is concerned with measurement of the impact of tannins on digestive systems. Whilst the notion that tannin-protein precipitates are indigestible is long standing (Feeny 1969; Rhoades 1977) it is curious that measurements of the im-

Table 4. Cellulase digestion of substrate under various conditions. Mean values (x) are for average % weight loss of substrate; se = standard error. Conditions used were; "bound" = tannin impregnated substrate after leaching; "free" = tannin in solution, released from impregnated paper by leaching; "unmasked" = paper impregnated with tannin extract only; "masked" = paper impregnated with BSA and tannin extract

Extract code	"Bound"		"Free"		"Unmasked"		"Masked"	
	x	se	x	se	x	se	x	se
Ah	28.6	0.21	16.2	0.82	23.5	3.25	27.5	0.62
Cc	21.1	0.11	20.5	0.23	18.3	3.72	19.9	4.32
De	6.7	1.20	12.2	0.81	14.3	0.49	0.9	0.00
Lc	15.9	0.98	16.6	1.95	18.0	0.50	13.7	5.01
Mo	14.2	1.78	22.9	0.81	17.5	0.14	14.6	1.69
Pp	15.5	0.35	20.2	0.81	17.6	1.41	21.1	2.76
B1	20.5	4.30	23.6	0.97	18.9	3.53	19.2	3.51
B2	23.0	0.17	18.9	2.12	23.4	1.37	19.9	0.56
B3	21.9	1.14	18.0	0.98	18.4	0.96	1.1	0.25
B4	1.7	0.57	15.4	1.39	15.3	2.79	13.5	0.05
Qs	23.1	0.11	18.6	0.97	15.9	3.15	13.8	2.13
Tb	20.1	1.20	22.1	0.74	19.4	5.22	17.1	0.43
Vm	22.4	0.96	16.6	0.20	19.7	1.41	13.4	0.00
TA	11.9	0.11	15.2	3.38	21.7	0.79	20.3	0.94
Qb	19.5	0.74	17.8	0.84	21.6	2.87	11.5	1.57
Tc	19.3	2.35	18.9	0.40	19.9	0.13	20.3	1.88
Pr	18.1	0.62	20.1	0.22	20.9	0.44	14.1	2.19
Catechin	20.1	2.23	21.5	0.56	19.5	2.04	15.4	1.94
Gallic acid	17.3	2.23	19.6	0.46	20.9	0.13	18.2	0.44

pact of tannins on proteolytic or other digestive systems have been largely ignored in favour of protein precipitation techniques in ecological studies, even though they are widely employed in the agricultural sciences (see for example, Faithful 1985). By returning to a digestive system, the divide between the in vitro and in vivo situations is narrowed and inferences about the latter from the former may be more reasonably made.

Digestive systems do however represent fundamentally different reactions to those of the precipitation type. In a precipitation reaction the tannin is generally given a single substrate protein to interact with whereas in a digestive system at least two components are present besides tannin (e.g. enzyme and substrate). The tannin may interact with either or both of these, depending on the specificity of the interactions involved (Hagerman and Butler 1981; Mole and Waterman 1987b). The obvious system to employ is one where the effect of tannins on proteolysis is observed. However, the complexity of this reaction, which may involve soluble and insoluble tannin-protein complexes, changes in substrate structure and possibly interactions involving the enzyme renders proteolysis a difficult technique to employ for a meaningful assay of tannins, particularly as we do not know to what extent proteolytic enhancement by tannins (Mole and Waterman 1985) may occur in vivo. Here we have chosen to examine a cellulolytic system where to some extent the effects of substrate-bound and soluble fractions of tannin can be separated.

The set of experiments reported here investigates the ability of the phenolic extracts characterised previously to inhibit the action of cellulase on substrates prepared by impregnating cellulose (chromatography paper) with solutions of these extracts. The solutions used were all made up to contain a total phenolic concentration equivalent to that of 0.1% tannic acid. A further variation required making a second set of substrates using paper previously impregnated with BSA with the object of examining the influence, if any, of protein on the binding of tannins onto the cellulase's substrate. Extract free samples of paper (+ or − BSA) were employed as controls. The digestion system employed was the cellulase technique of Choo et al. (1981) although in the experiments described below the pepsin pretreatment has been omitted.

In all four series of experiments were carried out with this system. In the first, tannin impregnated substrates were pretreated with 19 ml of the cellulase buffer (pH 4.6). At the end of the pretreatment this buffer was removed and added to a fresh sample of untreated paper in a replicate set of vials. To each set of vials cellulase solutions of required concentrations were then added to constitute a standard cellulase digestion system. The second set of vials will contain in the buffer the fraction of the tannin leached out of that substrate whilst the original "leached" substrates retained the tannin which was adsorbed onto the solid substrate. This system thus allowed for a distinction to be made between what may be characterised as "direct" versus "substrate level" inhibition of cellulase.

In a further set of experiments cellulase digests of substrates prepared with and without BSA were performed to see if masking of cellulose from cellulase through interaction between tannin and protein could be observed. All the digests were performed in duplicate and additionally a set of tannin free controls were made for comparison. All the digests were performed simultaneously with the same batch of cellulase and are therefore directly comparable.

The results from the first experiments on the full range of extracts (Table 4) showed that most substrates were degraded at a rate close to that of the control (20%). The weight loss from the leached extract of Ah is anomalously high, which is almost certainly due to the elution of non tannin material incorporated into the substrate from this

phenol-deficient extract. Weight losses clearly less than control were caused by three extracts (B4, De and TA) for systems where cellulase is inhibited both in solution and by cellulose-bound material. Indeed the precipitation of some cellulase could be seen as the enzyme was added to the buffer containing tannin in solution. It is notable that these three extracts do not have unusually high specific activities and that the cellulase inhibition by extracts that do is not distinguishable from the average. Inspection of the data thus reveals that protein precipitating ability is, on this evidence, not a good predictor of an extract's ability to inhibit cellulase activity. Differences in the relative effects of substrate-bound and soluble tannins were noted in a number of cases, but with no clear pattern between the two forms of enzyme inhibition.

Comparing the effects of the presence/absence of protein in this cellulase inhibition system indicates that the presence of protein does increase the inhibitory effects of tannin (Table 4). Otherwise the results are similar to the previous set with De and B4 still the most inhibitory extracts.

Considering all four sets of data (Table 4) shows, not surprisingly, that the cellulase digestion of BSA-masked material produced the lowest weight losses. Also the correlation between these results and those for the digest inhibited by cellulose-bound tannin is very high ($+0.92$, $P< 0.001$, $n=19$). All four sets of results suggest that tannin structure must contribute significantly to the variation in the results, yet there is clearly additional variability introduced by the way tannin is combined in the cellulose substrate. None of the results correlate well with the chemical characteristics reported previously for these extractives (Mole and Waterman 1987a).

Once again the hope that results from a simple biochemical assay might correlate well with the chemical analyses of the tannins present is confounded. The two most inhibitory extracts span the range for angiosperm tannins, i.e. B4 contains procyanidin condensed tannin whilst De contains hydrolysable tannin and prodelphinidin condensed tannin. One positive gain from this work, however, has been to demonstrate cellulose masking *sensu* Handley (1954) involving a range of tannin types as a potentially significant factor mediating the rate and degree of cellulose digestion.

Discussion

To the best of our knowledge the information brought together in this and the preceeding paper (Mole and Waterman 1987a) represents the most comprehensive analysis of chemical and biochemical properties of tannins extracted from a taxonomically diverse range of plants (at the family level) so far attempted. The results of this analysis indicate the severe problems relating to the interpretation of these data with regard to the impact of tannins as allelochemics.

The only general conclusion that can be drawn from these analyses is that all of these tannin-containing extracts did to some extent precipitate protein, so it would appear that a positive result for a chemical assay procedure can be used with some confidence to predict an ability to precipitate protein. This fact should be trivial but given the general lack of correlation between chemical and operational measures on a numerical basis then perhaps it represents the limits of our present predictive ability when considering interspecific comparisons rather than intraspecific comparisons. This unfortunate state of affairs is made more likely when it is considered that all of these in vitro experiments are one stage removed from the in vivo effects that tannins may or may not have. For example, the demonstrable effects of gut surfactants on tannin-protein interactions (Martin et al. 1985; Mole and Waterman 1985) and the variety of in vivo effects tannins are reported to exert (Mole and Waterman 1987c) serve to widen the divide between measures such as protein precipitation and the real physiological effects of tannins on digestion which ecologists wish to infer.

To illustrate the dangers of interpreting proximate data we consider a case from the recent literature, the report by Cooper and Owen-Smith (1985) that condensed tannins deter feeding in browsing ruminants in a South African savanna. We do not dispute their contention, and indeed would strongly support the hypothesis that tannins can have a considerable negative impact of food selection (Mole and Waterman 1987c). However, we do dispute the use of their data to advance a number of other hypotheses which we regard as untenable. Cooper and Owen-Smith (1985) studied crude plant extracts as we did here. They measured condensed tannins by the proanthocyanin method and total phenolics by a reaction with a ferric reagent (Jerumanis 1972) which from our own results and their observations, gives a comparable measure to the Folin-Denis method. By reference to the work of Deiber (1975), who found that sorghum polyphenols inhibited enzymic activity and that the Jerumanis method was the best to predict enzyme inhibition, they concluded that total phenol measurements gave an estimate of polyphenolic enzyme inhibition in their samples. To assume such a correlation on an intraspecific level is defensible, if factors such as plant age, growing conditions and plant part are taken into consideration. To extend it to extracts from a taxonomic diversity comparable to that used in this study is unacceptable both in terms of the information that the assays yield on the type and structure of condensed tannins and on the enormous diversity in their ability to interact with proteins and other substrates. In short we would assert that their claim to have measured enzyme inhibiting phenolics as opposed to total phenolics is unfounded. We would further argue that from their measurements of total phenols and condensed tannins they can make no reliable prediction of protein precipitating ability of the crude extracts of the leaves that their study animals eat.

We would also reject their argument that, because the correlation between condensed tannin and total phenolics contents in their data is low, then "the enzyme inhibiting polyphenols [present in their samples] include mainly hydrolysable tannins which have been found to have a stronger protein-precipitating action than condensed tannins". As they have no direct measure of the quantity of hydrolysable tannins or even qualitative evidence for them from chromatographic analysis we also have to dismiss their claims regarding the presence and role of hydrolysable tannins. Our own results (%CT1, %CT2) clearly reveal that where there are measurements of total phenols and condensed tannins the latter cannot be reliably expressed as a percentage of the former. The presence of hydrolysable tannins (or any other specific type of phenolic) can thus in no way be inferred from their estimates of total phenolics and condensed tannins.

The purpose of this critique is to illustrate the numerous problems arising in any attempt to use this proximate chemical information in the analysis of feeding ecology. This is not to say that such measures are never of value; for example their employment in investigations of resource allocation within species (Waterman et al. 1984) and within ecosystems (Gartlan et al. 1980; Coley 1983) can be highly informative.

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