TANNIN ASSAYS IN ECOLOGICAL STUDIES
Precipitation of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase by Tannic Acid, Quebracho, and Oak Foliage Extracts

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Abstract—Tannic acid and quebracho precipitate many times their weight of the abundant leaf protein, ribulose-1,5-bisphosphate carboxylase/ oxygenase (RuBPC). The use of this protein in protein precipitation assays for tannin content is described. Extracts of mature foliage of pin, bur, and black oak precipitate 2.01, 0.69, and 0.09 mg RuBPC/mg (dry wt) of leaf powder extracted, respectively, at pH 6.1. From these measurements it can be calculated that all three of these oak species have sufficient tannins to precipitate all of the RuBPC present in their foliage. At mildly alkaline pHs, however (pH $\geqslant$ 7.5), RuBPC is not precipitated by tannins. Since RuBPC is the most abundant protein present in photosynthetic tissues, often constituting as much as 50% of the soluble proteins and 25% of the total proteins in leaf tissue, the interactions of this protein with tannins are highly relevant to an evaluation of the role of tannins as antiherbivore, digestibility-reducing substances. Our measurements provide no basis for arguing that differences in tannin levels in different species reflect differences in the digestibility of leaf proteins or that tannins have any effect whatsoever upon the digestibility of leaf protein under conditions which normally prevail in most insects' guts. These findings emphasize the need to test more of the assumptions underlying contemporary interpretations of the importance of tannins in plant herbivore interactions.

Key Words—Herbivory, chemical defense, allelochemics, tannins, digestibility reducing substances, RuBPC, *Quercus*.

INTRODUCTION

Tannins are water-soluble phenolic compounds which occur widely in vascular plants (Bate-Smith, 1957; Swain, 1979a). They are known to have

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adverse effects upon organisms as diverse as viruses, bacteria, fungi, insects, reptiles, birds, and mammals (Swain, 1979a). They have been accorded an important role in protecting plant tissues from herbivore attack (Feeny, 1976; Rhoades and Cates, 1976), although Bernays (1981) has recently stressed the variability of the effects of tannins on insect herbivores and has cautioned against premature generalizations concerning their evolutionary and ecological significance.

Defining the status of tannins as defensive chemicals in plants requires suitable procedures for measuring tannin content. The assay procedures which have been most frequently used in ecological studies have been the Folin-Denis assay for total phenols, the butanol–HCl assay for proanthocyanidins, and the vanillin–HCl assay for catechins. Unfortunately, these methods depend upon the presence of functional groups which are neither unique to tannins nor invariant features of tannin structure. Dissatisfaction with chemical, functional group assays has led to the development of other procedures based upon the ability of tannins to form insoluble complexes with proteins. Since it is the capacity of tannins to precipitate proteins which is postulated to be responsible for the adverse effects of these substances, these assays would seem to be particularly appropriate ones in studies of the significance of tannins in herbivory. Reliable assays for tannin content, based upon the precipitation of hemoglobin (Bate-Smith, 1973; Schultz et al., 1981), β-glucosidase (Becker and Martin, 1982) and bovine serum albumin (BSA) (Hagerman and Butler, 1978, 1980; Martin and Martin, 1982) have been described.

Since these assays use proteins which are not present in the diets of foliage-feeding insects, it is prudent to question whether the measure of protein-precipitating capacity they provide is of any relevance to the study of the role of tannins in plant–herbivore interactions. Proteins differ in the extent to which they are precipitated by tannins (Mandels and Reese, 1963; van Sumere et al., 1975; Griffiths, 1979; Hagerman and Butler, 1981), and a tannic extract which precipitates 1 mg of BSA or hemoglobin will not necessarily precipitate 1 mg of leaf protein or insect digestive enzyme.

In this study we have compared the precipitation of the abundant leaf protein, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC), with the precipitation of BSA by solutions of tannic acid and bisulfited quebracho and by extracts of the mature foliage of three oak species (pin oak, *Quercus palustris*; bur oak, *Q. macrocarpa*; black oak, *Q. velutina*). As expected, the two proteins differ in the extent to which they are precipitated by a given tannic solution. However, the rank order of protein-precipitating capacity of a series of tannic extracts established using the nonleaf protein, BSA, correctly predicts the rank order using the leaf protein, RuBPC. Since RuBPC often makes up as much as 25% of the total protein and 25–50% of the soluble protein in leaf tissue (Singer et al., 1952; Akazawa, 1970; Lyttleton, 1973;

Jensen and Bahr, 1977), it is a major dietary protein for any foliage-feeding insect. Its interactions with tannins are, therefore, particularly relevant to an evaluation of the potential role of tannins as digestibility-reducing substances. Our measurements of the amounts of RuBPC precipitated by oak leaf tannins over a range of pHs call into question some of the current interpretations of the role of tannins as antiherbivore defensive chemicals and emphasize the need for direct experimental investigations of interactions between tannins and relevant dietary proteins under conditions which prevail in an herbivore's gut.

METHODS AND MATERIALS

Processing of Plant Materials. Foliage was processed in a manner calculated to minimize chemical alterations in leaf constituents (Swain, 1979). Whole, mature undamaged leaves were frozen and lyophilized immediately after collection and then stored at $-15°C$ in a desiccator. Shortly before the leaves were to be extracted, the midribs were removed, and the remainder of the leaf was ground to a powder (60-mesh) in a Wiley mill. The leaf powder was temporarily stored in a desiccator in the dark at room temperature. At no time were the leaves or leaf powder exposed to any preservatives or to temperatures above 25°C. Although we have noted a gradual decrease in phenol and tannin content during the storage of leaf tissue processed in this way, these changes cause no alteration in the rank order of phenolic content in the foliage of six oak species, suggesting that parallel and roughly comparable changes occur in the different samples (Martin and Martin, 1982). Likewise Gartlan et al. (1980) showed that when foliage samples were sun- or oven-dried (60°C), there was an apparent decrease in phenolic content, but there was still a very strong correlation between the phenol values from fresh and dried samples. Lyophilization would be expected to result in much less chemical modification of leaf phenols than sun- or oven-drying, and we assume that what limited changes do occur are roughly comparable in all foliage samples.

Preparation of Extracts. Leaf powder (30–150 mg) was extracted twice for 8 min with 2 ml of boiling 50% (v/v) aqueous methanol in a centrifuge tube (capped with a marble) placed in a heat block at 95°C. After centrifugation (9600 rpm, 19000 g, 15 min, 5°C), the pellet was resuspended in a small volume of 50% methanol and centrifuged as before. The volume of the combined supernatants was adjusted to 5 or 10 ml, and dilutions appropriate to the assay were prepared from aliquots of the stock solution. Extracts were prepared immediately prior to use.

Protein-Precipitation Assay. The procedure is a variant of the method of Martin and Martin (1982). To a solution of 1.2 mg of protein (BSA, Sigma

A-4378, Lot 70F-9350; or spinach RuBPC, Sigma R-8000, Lot 98C-7140) in
1.8 ml buffer (0.1 M sodium succinate, pH 4.1, with BSA; 0.1 M sodium
2-(N-morpholino)-ethanesulfonate, MES, pH 6.1, with RuBPC) containing
0.17 M sodium chloride was added 0.3 ml of a 50% aqueous methanolic
solution of tannic acid (Sigma T-0125, Lot 40F-0253, 6.4% moisture),
bisulfited quebracho (Pilar River Plate Corp., 18% moisture), or an aliquot of
foliage extract. After vortexing, the mixture was centrifuged (9600 rpm, 19000
g, 15 min, 5° C). Separate experiments demonstrated that the precipitation of
the tannin–protein complex occurs rapidly and that waiting as long as 30 min
before centrifugation does not increase the amount of protein precipitated.
Separate experiments also demonstrated that the presence of methanol in the
tannin solution, which produced a final mixture containing 7% methanol,
does not affect the amount of protein precipitated.

After centrifugation, the pellet was rinsed very gently with 0.4 ml of
buffer and centrifuged as before. The combined supernatants were applied to
a 1.7 × 5.0-cm column of Sephadex G-25 (Pharmacia Fine Chemicals, PD-10
Columns), which had been equilibrated with buffer containing 0.17 M sodium
chloride and 6% methanol. Proteins were eluted completely in 3.5 ml of the
same solvent mixture. This step removes all materials from the supernatant
which absorb at 595 nm. The amount of protein in the eluent was determined
by mixing a 50-μl aliquot with 2.5 ml of Coomasse brilliant blue G-250 dye
reagent (Bio Rad Protein Dye Reagent), and determining A_{595} after 6 min
(Bradford, 1976) using a blank consisting of 50 μl of buffer containing 6%
methanol plus 2.5 ml of the dye reagent. The absorbance at 595 nm was
transformed into mg of BSA or RuBPC by the use of a calibration curve
constructed on the same day as the assay. From a determination of the
amount of protein in the original solution, the amount precipitated by the
addition of the tannin or foliage extract could be calculated. Separate
experiments verified that the presence of 6% methanol, which is the final
concentration of methanol in these experiments, does not interfere with the
determination of protein using the Bradford procedure.

The protein-precipitating capacity was measured as the slope of the linear
regression of amount (mg) of protein precipitated on amount (mg dry wt) of
tannin or leaf powder extracted. By obtaining measurements at several
concentrations of tannin or foliage extract, it is possible to ensure that the
determinations are being performed under conditions which generate a linear
relationship between the amount of protein precipitated and the amount of
tannin or extract being used and to determine how close the y intercept is to
zero. Some protein-precipitation assays are characterized by significant
negative y intercepts, indicating a threshold concentration of extract below
which no protein is precipitated (Bate-Smith, 1973; Schultz et al., 1981;
Becker and Martin, 1982; Martin and Martin, 1982). In this study, the
relationship between the amount of BSA or RuBPC precipitated and the

amount of tannin added to the test solution of protein was linear over a wide range of concentrations of tannic acid, quebracho, and foliage extracts which brought about the precipitation of 1.5–89% of the protein present. Correlation coefficients greater than 0.96 were obtained in every assay. In most cases the y intercepts were not significantly different from zero (Table 1). Regression coefficients (slopes) and y intercepts were calculated assuming that the independent variable (dry weight of tannin in solution or leaf powder extracted) was measured without error, employing data in which there was more than one value of the dependent variable per value of the independent variable (Sokal and Rohlf, 1969). In calculating standard errors of regression coefficients, mean squares were not pooled.

Buffers used in the pH studies were 0.1 M sodium citrate (pH 3.1), 0.1 M sodium succinate (pH 4.1, 5.6), 0.1 M sodium acetate (pH 5.0, 5.1), 0.1 M sodium 2-(N-morpholino)-ethanesulfonate (MES) (pH 6.1), 0.1 M sodium piperazine-N,N'-bis-2-ethanesulfonate (PIPES) (pH 6.6, 7.1), 0.1 M sodium N-2-hydroxyethylpiperazine-N-ethanesulfonate (HEPES) (pH 7.6), and 0.1 M sodium N-2-hydroxyethylpiperazine-N'-3-propanesulfonate (HEPPS) (pH 8.0). All buffers contained 0.17 M sodium chloride. The ionic strengths of the succinate (pH 5.6) buffer and both PIPES buffers were between 0.35 and 0.40. All of the other buffers had ionic strengths between 0.22 and 0.24. Ionic strengths in this general range have been reported for the gut fluids of representative Lepidoptera (Giordana and Sacchi, 1978) and Ortoptera (Dow et al., 1981).

RESULTS AND DISCUSSION

As expected, BSA and RuBPC differ in the extent to which they are precipitated by Tannins (Table 1). At favorable pHs for precipitation (4.1 for BSA, 6.1 for RuBPC), a given amount of tannin or foliage extract precipitates much more RuBPC than BSA. The rank order of protein-precipitating capacity of the various tannin solutions or foliage extracts is the same whether measured with BSA or RuBPC. Using either BSA or RuBPC, tannic acid (a hydrolyzable tannin) precipitates more protein than quebracho (a condensed tannin), and the relative protein-precipitating capacities of the three oak foliage extracts are in the sequence, pin oak > bur oak > black oak. However, the actual numerical values for the relative protein-precipitating capacities of the different tannins or foliage extracts depend upon which protein is used in the measurement (Table 2). For example, pin oak foliage has a protein-precipitating capacity 22.6 or 8.3 times greater than black oak foliage, depending upon whether it is measured using RuBPC or BSA. Furthermore, the disparity between relative protein-precipitating capacities of different tannins or different foliage samples measured using the two different proteins is not a constant factor. Thus, while the activity of pin oak

TABLE 1. AMOUNTS OF BSA AND RuBPC PRECIPITATED BY SOLUTIONS OF
TANNIC ACID, BISULFITED QUEBRACHO, AND EXTRACTS OF MATURE
FOLIAGE OF THREE OAK SPECIES[a]

Precipitating solution or extract	BSA pptd (mg/mg)[b] (pH 4.1)	RuBPC pptd (mg/mg)[c] (pH 6.1)
Tannic acid	4.92 ± 0.47 (4,6,20)	20.84 ± 0.99 (4,5,13)
Quebracho	1.92 ± 0.04 (2,6,12)	4.00 ± 0.21 (2,6,11)
Q. palustris (pin)	0.357 ± 0.007 (2,6,12)	2.01 ± 0.11 (2,5,10)
Q. macrocarpa (bur)	0.257 ± 0.008 (2,6,12)	0.691 ± 0.064 (2,5,10)
Q. velutina (black)	0.043 ± 0.005 (2,6,12)	0.089 ± 0.006 (2,6,11)

[a] Entries are regression coefficients (slopes ± SE) of mg protein precipitated vs. mg (dry weight) of tannin or of leaf powder extracted. The number of separate solutions or extracts used, the number of different concentrations examined, and the total number of measurements performed are indicated, in that order, in the parentheses.
[b] y intercepts: tannic acid, −0.08; quebracho, −0.04; pin oak, −0.008; bur oak, −0.014; black oak, −0.015. None of the y intercepts are significantly different from zero ($P > 0.01$).
[c] y intercepts: tannic acid, −0.58; quebracho, −0.09; pin oak, −0.44; bur oak, −0.015; black oak, −0.063. Only the y intercepts for tannic acid and pin oak are significantly different from zero ($P < 0.01$).

relative to black oak foliage is 2.7 times higher when determined with RuBPC than when determined using BSA (22.6 vs. 8.3), the activity of bur oak relative to black oak foliage is only 1.3 times higher (7.8 vs. 6.0). Considering the extraordinary complexity of tannin–protein interactions (Goldstein and Swain, 1965; Calderon et al., 1968; Van Buren and Robinson, 1969; Haslam, 1974; van Sumere et al., 1975; Becker and Martin, 1982), it comes as no surprise that relative protein-precipitating capacity is not a simple, invariant property of a tannin.

These results help to define the value of protein-precipitation assays in ecologically oriented studies designed to probe the role of tannins in plant–herbivore interactions. Measurements of the protein-precipitating capacity of a set of foliage extracts using BSA, almond emulsin, or hemoglobin as the test

TABLE 2. RELATIVE PROTEIN-BINDING CAPACITIES OF
DIFFERENT TANNINS AND FOLIAGE EXTRACTS USING
BSA AND RuBPC AS TEST PROTEINS

Test protein	Tannins Tannic acid/quebracho	Oak foliage Pin/bur/black
BSA	2.6/1.0	8.3/6.0/1.0
RuBPC	5.2/1.0	22.6/7.8/1.0

proteins provide consistent rank orders of tannin contents which can be very useful in correlative ecological studies of plant–herbivore interactions. However, none of the above-named proteins occur in leaves. Consequently, these measurements cannot be used to estimate the actual fraction of dietary protein which might be precipitated in an herbivore's gut by the tannins in their food plants. The use of the abundant leaf protein, RuBPC, as the test protein is a step in the direction of making measurements of protein-precipitating capacity more useful in assessing the likelinood that tannins function as digestibility-reducing substances.

It is revealing to examine the implications of the RuBPC-precipitating potentials of the three oak extracts. Lawson et al. (1982) have reported that the mature foliage of pin, bur, and black oak contain 2.27, 2.92, and 2.11% nitrogen, respectively. Using the factor 6.25 to convert "% nitrogen" into "crude protein," and assuming that 25% of the total leaf protein is RuBPC, it follows that mature pin, bur, and black oak foliage contain 0.035, 0.046, and 0.033 mg RuBPC/mg (dry wt), respectively. Thus, all three oak species have sufficient tannins to precipitate all of the RuBPC present in their foliage (Table 1). Even black oak, the species with the lowest potential for precipitating proteins, has sufficient tannins to precipitate more than 2.5 times the amount of RuBPC present. Thus the more than 20-fold greater protein-precipitating potential of pin oak relative to black oak foliage does not reflect a corresponding difference in the extent to which the RuBPC in the two species might be precipitated by the tannins present. This finding clearly provides no basis for arguing that the more tannin-rich pin oak foliage is less digestible than black oak foliage. Of course it is still possible that high levels of tannins are required to precipitate RuBPC in the presence of other tannin-binding leaf constituents, to precipitate other leaf proteins which are important dietary components, or to precipitate insect digestive enzymes. At the very least, however, we urge ecologists to be wary of the assumption that differences in tannin levels necessarily reflect differences in nutritive value or differences in extent of protection against herbivores.

The amount of BSA or RuBPC precipitated from an aqueous solution by the addition of a solution of tannic acid or quebracho depends upon pH (Figure 1). Maximum precipitation of BSA by tannic acid occurs at a pH around 4. Hagerman and Butler (1978) have also reported an optimal pH between 4 and 4.5 for the formation of an insoluble complex between BSA and sorghum tannins. The dependence of RuBPC precipitation by tannins on pH (Figure 1) raises significant questions about the potential efficacy of these substances as antiherbivore defensive chemicals. Extensive precipitation occurs between pH 5.6 and 7, but there is very little precipitation at pHs above 7.5. At pH 8 no RuBPC was precipitated from a solution by the addition of an extract of pin oak leaf powder which would have precipitated all of the RuBPC at pH 6.1. Midgut pHs in excess of 9 are not uncommon in foliage-
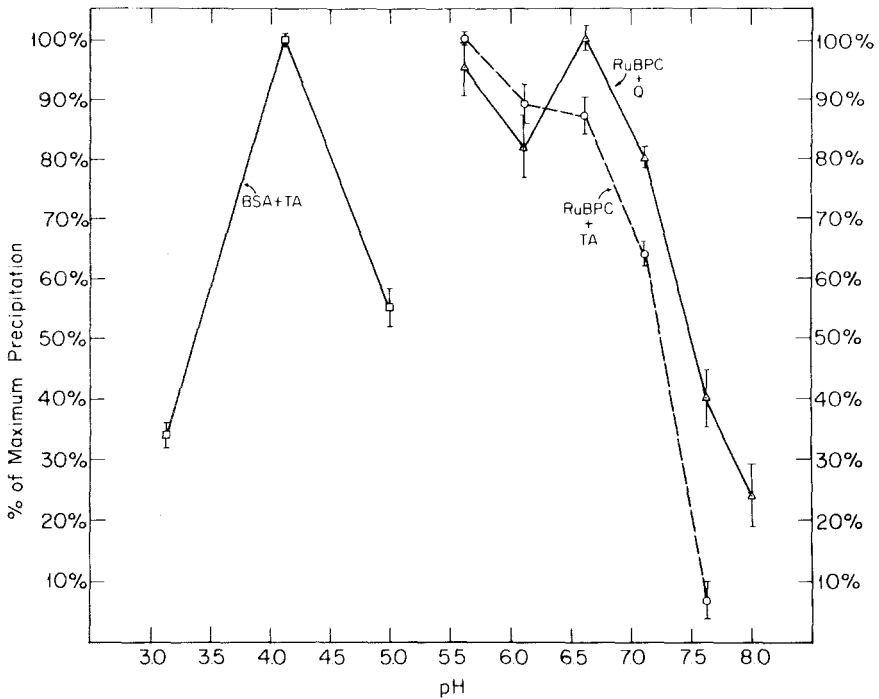
FIG. 1. Effect of pH on the precipitation of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBPC) and bovine serum albumin (BSA) by tannic acid and quebracho. Incubation mixtures contained 1.2 mg of protein and either 180 μg of tannic acid (with BSA), 90 μg tannic acid (with RuBPC), or 300 μg quebracho (with RuBPC). Tannic acid precipitated 0.806 mg of BSA at pH 4.1 and 0.999 mg of RuBPC at pH 5.6, while quebracho precipitated 1.04 mg of RuBPC at pH 6.6. All points are the mean of five measurements; bars give the standard error of the mean. Abbreviations: BSA, bovine serum albumin; RuBPC, ribulose-1,5-bisphosphate carboxylase/oxygenase; TA, tannic acid; Q, quebracho.

feeding Lepidoptera (Berenbaum 1980), and Feeny (1970) and Berenbaum (1980) have suggested that the high gut alkalinity in herbivores could be an adaptive mechanism to prevent or reverse the binding of proteins by tannins. While it is certainly true that the maintenance of modestly alkaline conditions in the gut would be a useful mechanism to prevent the precipitation of dietary proteins by tannins, our results show that extremely high pHs are not necessary. Indeed, many insects, including some non-tannin-adapted species, have sufficiently alkaline guts to prevent the precipitation of RuBPC by tannins. Of course, it is still possible that highly alkaline conditions are required to prevent the precipitation of some other important dietary proteins by tannins or that the RuBPC in oak foliage binds more tenaciously to tannins

at high pHs than does spinach RuBPC. However, until those possibilities have been tested experimentally, it is prudent to take cognizance of possible alternative explanations for the adaptive significance of an alkaline gut which have no connection with a need to overcome the presumed digestibility-reducing properties of tannins.

Correlative studies have generated many important and original hypotheses concerning strategies of chemical defense and the role of secondary metabolites in influencing interspecific interactions. In this paper we have emphasized how few of the assumptions underlying these hypotheses have been tested. Experiments to test them are quite feasible, and it is our opinion that the next major advances in clarifying the role of tannins in plant-herbivore interactions will come from chemically oriented studies of interactions of proteins and tannins actually present in an insect's food plant under conditions which might reasonably be expected to prevail in the gut.

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