Journal of Chemical Ecology, Vol. 8. No. 11, 1982

PROTEIN-PRECIPITATING CAPACITY OF TANNINS IN *Shorea* (DIPTEROCARPACEAE) SEEDLING LEAVES

PETER BECKER and JOAN STADLER MARTIN

Division of Biological Sciences
University of Michigan
Ann Arbor, Michigan 48109

(Received October 13, 1981; revised March 10, 1982)

Abstract—The protein-precipitating capacities of tanniferous extracts from immature and mature leaves of three *Shorea* spp. (Dipterocarpaceae) seedlings were measured by an adaptation of Goldstein and Swain's β-glucosidase precipitation assay. Protein precipitation by the extracts was not correlated with total phenolics (Folin-Denis assay) or proanthocyanidin content (BuOH—HCl assay) as measured in an earlier study. Extracts of *S. maxwelliana* mature leaves had much lower protein-precipitating capacity than those of *S. acuminata* and *S. leprosula*, but fewer insect species feed on and cause less damage to the foliage of *S. maxwelliana* compared with the other species' foliage. Immature leaf extracts of *S. leprosula* and *S. acuminata* had substantial protein-precipitating capacities which in the latter species exceeded that of its mature leaf extracts. Leaf extracts precipitated less protein when initial protein concentration was reduced, although not limiting, but no effect or the reverse effect occurred with quebracho tannin and tannic acid. Problems in the characterization of foliage astringency and the interpretation of its role as a potential antiherbivore defense are discussed.

Key Words—Tannins, *Shorea*, Dipterocarpaceae, insect herbivores, protein precipitation, β-glucosidase, phytochemical defenses.

INTRODUCTION

Tannins adversely affect the feeding, growth, or survival of certain insects (Bennett, 1965; Bernays, 1981; Bernays and Chamberlain, 1980; Bernays et al., 1980; Chan et al., 1978; Feeny, 1970; Fox and Macauley, 1977; Maxwell et al., 1967) and so may defend plants to some extent against herbivores (Feeny, 1976; Rhoades and Cates, 1976). An earlier study (Becker, 1981) found no correlation between insect attack and the concentrations of total phenolics

0098-0331/82/1100-1353$03.00/0

(Folin-Denis assay) or proanthocyanidins (BuOH-HCl assay) in seedling leaves of *Shorea maxwelliana*, *S. acuminata*, and *S. leprosula* from a Malaysian rainforest. The assays employed in that study have serious shortcomings as indices of tannin content (Swain, 1979) and, moreover, equal amounts of different tannins may have quite different protein-precipitating capacities (Haslam, 1974). Since the potential defensive function of plant tannins against herbivores may depend on their capacity to form complexes with proteins (Feeny, 1970), we compared this property in the three *Shorea* species by adapting Goldstein and Swain's (1965) β-glucosidase precipitation assay to the analysis of leaf extracts. In this assay tannins in a leaf extract cause the precipitation of β-glucosidase from solution by forming a complex with the enzyme. After centrifugation, the amount of protein precipitated, as enzyme, is measured by the difference between the enzyme activities of the supernatant and an appropriate control.

Although leaves of the three *Shorea* spp. studied have similar concentrations of both total phenolics and proanthocyanidins (Becker, 1981), the potencies of their tannins, as measured by the β-glucosidase precipitation assay, differed markedly. Protein-precipitating capacity of the leaf extracts depended on the initial protein concentration, so we further investigated this effect using commercial tannins.

METHODS AND MATERIALS

Preparation of Extracts. Mature and immature leaves were collected from 0.1–0.4-m-tall seedlings of the 1976 mast crop growing in the shaded, primary forest understory of Pasoh Forest Reserve (Negeri Sembilan, W. Malaysia) during October and November 1979. (See Becker, 1981, for details of the collection protocol; insufficient *S. maxwelliana* immature leaves remained after previous phytochemical assays for use in this study.) Dried leaves were ground in a Culatti® microbeater and mortar to pass a 0.25-mm (No. 60) sieve and stored for 17 months at room temperature. Samples were oven-dried to constant weight at 70–72°C prior to extraction. All solutions with tannins were processed in glassware. All solutions less than 5 ml in volume were dispensed by adjustable Pipetmans®. Leaf powder (80.0 mg dry wt) was extracted twice for 8 min with 3.4 ml of boiling 50% (v/v) aqueous methanol in a marble-capped centrifuge tube at 90–95°C. The combined extracts were centrifuged (12,000 *g*, 15 min, 5°C), and the resulting pellet was resuspended in 2 ml of 50% methanol and centrifuged as before. The volume of the combined supernatants was brought to 10.0 ml with 50% methanol. Because methanol inhibits β-glucosidase, solvent was removed from 5.0 or 10.0 ml of this extract at 27–38°C under reduced pressure in a rotary concentrator. The slightly moist residue was redissolved in 5.0 ml of acetate

buffer (0.1 M, pH 4.8) by rotary agitation for 15 min. Protein-precipitating capacities of *S. leprosula* extracts assayed after 15 and 60 min resolubilization did not differ ($F[1,8] = 0.882$, $P > 0.25$). The stock extract in acetate buffer was centrifuged (800 *g*, 10 min), and its clear supernatant was diluted with acetate buffer to obtain extracts of various concentrations.

Ideally, leaves should be extracted and assayed for protein-precipitating capacity immediately after collection. When this is impossible, freshly collected leaves should be lyophilized and stored in a freezer. A lack of suitable equipment prevented following either of these protocols in this study. Drying and prolonged storage of *Shorea* leaves very likely altered their tannins. Provided that approximately parallel changes occurred in the different *Shorea* species, our inter- and intraspecific comparisons of protein-precipitating capacity would not be affected. Although there is an apparent loss in tannin content caused by drying, the rank correlation between analyses of comparable fresh and dry leaf samples is very high (Waterman et al., 1980). The rank order of proanthocyanidin concentrations in six oak species was unaltered after 12 months of storage of lyophilized leaf material, despite an apparent decrease in absolute values (Martin and Martin, 1982). Unfortunately, there is no comparable information about the effects of drying and storage of leaves on the protein-precipitating capacities of their extracts.

β-Glucosidase Precipitation Assay. Goldstein and Swain (1965) established the appropriate chemical and spectrophotometric parameters of this assay, so our study focused on its adaptation for the analysis of leaf extracts. The substrate used, esculin, does not form a complex with tannins (Goldstein and Swain, 1965). Haslam (1974), using natural and synthetic polyphenols, showed that loss of activity in the supernatant is due to precipitation and not to inhibition of β-glucosidase by residual soluble polyphenols.

Leaf extract in acetate buffer (0.30 ml) was added to 0.30 ml of a solution of β-glucosidase (0.50 or 1.0 mg of almond emulsin per ml, Sigma G-8625, Lot 4OF-4017, activity: 6 units/mg) in phosphate buffer (0.1 M, pH 7.0), swirled on a vortex mixer, and left at room temperature for 15 min. Simultaneously, four standards were prepared by adding 0.30 ml of acetate buffer to 0.30 ml of the enzyme solution. After centrifugation of the extract–enzyme preparations (12,000 *g*, 15 min, 5° C; the standards were only refrigerated), the supernatant was drawn off with a Pasteur pipet to avoid disturbing the precipitated enzyme. The activities of the enzyme in the supernatants and the standards were determined in triplicate by combining 20- to 50-μl aliquots with 3.0 ml of acetate buffer containing esculin hydrate (Sigma, 0.50 mM) and aluminum chloride (3.75 mM) at 25° C and determining the change in absorbance at 385 nm over 3 min (self-zeroing Zeiss M4 QII spectrophotometer with 1-cm quartz cuvettes zeroed against water).

The change in absorbance was linear over this time interval, and the initial reaction rate thereby measured was linearly related (with zero *y*

intercept) to enzyme concentration up to 1.0 mg emulsin/ml phosphate buffer as tested by polynomial regression analysis. By weighing dispensed enzyme solutions, we found that most of the variance among triplicate determinations of enzyme activity could be accounted for by pipetting error. Protein precipitation did not increase when enzyme–extract (*Quercus rubra*) mixtures were held for 50 instead of 10 min prior to centrifugation. Intensified centrifugation (30,000 *g*, 30 min, 5° C) of the enzyme–extract (*Q. rubra*) mixture did not increase sedimentation of complexed β-glucosidase (F [1,32] = 1.781, $P > 0.10$).

Extracts must be combined with the enzyme immediately after their preparation. A delay of only 4 hr resulted in reduced protein precipitation (*Q. bicolor* extracts, F [1,24] = 200.379, $P < 0.001$). Bate-Smith (1973, 1975) likewise reported that tannin solutions loose their astringency on standing.

The pH of the acetate buffer was unaffected by the presence of *S. acuminata* leaf extract. Since Goldstein and Swain (1965) found that precipitation of β-glucosidase was constant between pH 2.0 and 7.5, the assay is probably insensitive to normal differences in acidity of leaf extracts.

The proportions of β-glucosidase and the other component proteins vary among different lots of emulsin, and this probably affects the determination of binding capacity (see Discussion). Therefore, any comparative study should use a single enzyme lot.

Nontannin Inhibitors. To assess whether residual methanol or nontannin phytochemicals in the leaf extracts contributed to enzyme inhibition, tannin-free extracts were prepared by treatment with hide powder and then combined with β-glucosidase. Gustavson (1956) found that hide powder fixes ca. 30% of its weight of mimosa tannins and tannic acid at pH 4.8, so the quantity of hide powder used here was presumably sufficient to remove all tannins from the extracts.

Hide powder (100 mg, Calbiochem, fine grind), soaked in deionized water for ca. 3 hr, was centrifuged (800 *g*, 5 min), and excess water was discarded. This wetted hide powder was stirred magnetically with 1.0 ml of extract in acetate buffer, derived from 8.0 mg (*S. maxwelliana*) or 4.0 mg (remaining samples) of leaf powder. After centrifugation (800 *g*, 10 min), 0.3 ml of the cloudy supernatant was combined with 0.3 ml of emulsin solution (1.0 mg/ml, $N = 2$) and assayed as described above. Acetate buffer that was similarly treated with hide powder contained no leached substances that affected enzyme activity (Kruskal-Wallis test, $H[7] = 10.95$, $P > 0.10$).

Extractability of Tannins. Bate-Smith (1977) showed that considerable amounts of *Acer* leaf proanthocyanidins may exist in nonextractable forms that nonetheless show tannin activity, so we measured the residual protein-precipitating capacities of the methanol-extracted marcs. Leaf powder (5.0, 10.0, and 15.0 mg dry wt) was extracted with methanol and centrifuged following a procedure similar to the preparation of extracts. The marcs (pellets) were

dried under a stream of nitrogen for 10 min and then stored in an evacuated desiccator for 19–23 hr to remove residual methanol. The desiccated marcs were stirred magnetically for 35 min with 0.30 ml β-glucosidase in phosphate buffer (0.50 mg emulsin/ml) and 0.30 ml of acetate buffer, then centrifuged; the activity of the supernatant was determined as usual.

Tannic Acid and Quebracho. We further investigated the effect of initial protein concentration on protein-precipitating capacity of tannins using unpurified commercial preparations of tannic acid (Sigma T-0125) and bisulfited quebracho (Pilar River Plate Corp., Newark, New Jersey). Various concentrations of these tannins were made up in acetate buffer and assayed immediately for β-glucosidase precipitation at initial emulsin concentrations of 0.25, 0.50, and 1.0 mg/ml according to the same procedure used for leaf extracts. Moisture content was determined for separate samples of tannins dried to constant weight at 60–68° C in a vacuum oven. We made a second set of assays at some of the initial protein concentrations after discovering that the relationship between enzyme precipitation and tannin concentration was sometimes nonlinear.

Statistical Analyses. Each of the different leaf powders was extracted and assayed twice with emulsin at 1.0 mg/ml and once at 0.50 mg/ml. Although the emulsin used was 100% protein according to the manufacturer, the actual proportion of β-glucosidase was unknown, so it was impossible to calculate the absolute amount of protein precipitated by the extracts. Because of day-to-day variations in the activity of enzyme standards (not attributable to weighing errors), the results were standardized by calculating the percentage of activity lost from the supernatant of enzyme–extract mixtures (mean of $N = 3$ determinations) relative to the activity of the standards (mean of $N = 4 \times 3$ determinations).

Because a threshold concentration of extract was sometimes required for protein precipitation, regression analysis was necessary to overcome the problem of nonzero y intercepts when comparing the protein-precipitating capacities of different extracts. However, standardizing the data as described above introduced a covariance in the dependent variable (% enzyme activity lost) which invalidated an assumption of regression analysis. An appropriate transformation of the independent variable (extract concentration) in linear or nearly linear regions of the curves permitted the calculation of regression coefficients with corrected standard errors as described in the statistical note appended to this report. Since the usual multiple-comparisons tests were inappropriate, regression coefficients (slopes) were compared by Behrens-Fisher tests at $\alpha = 0.0083$ (0.05 divided by 6, the number of comparisons) to compensate for the lack of independence among multiple comparisons. For clarity the results in Figure 1 and Table 1 are expressed in terms of the untransformed data with suitably corrected standard errors.

Since the marcs were all assayed on the same day, these problems were

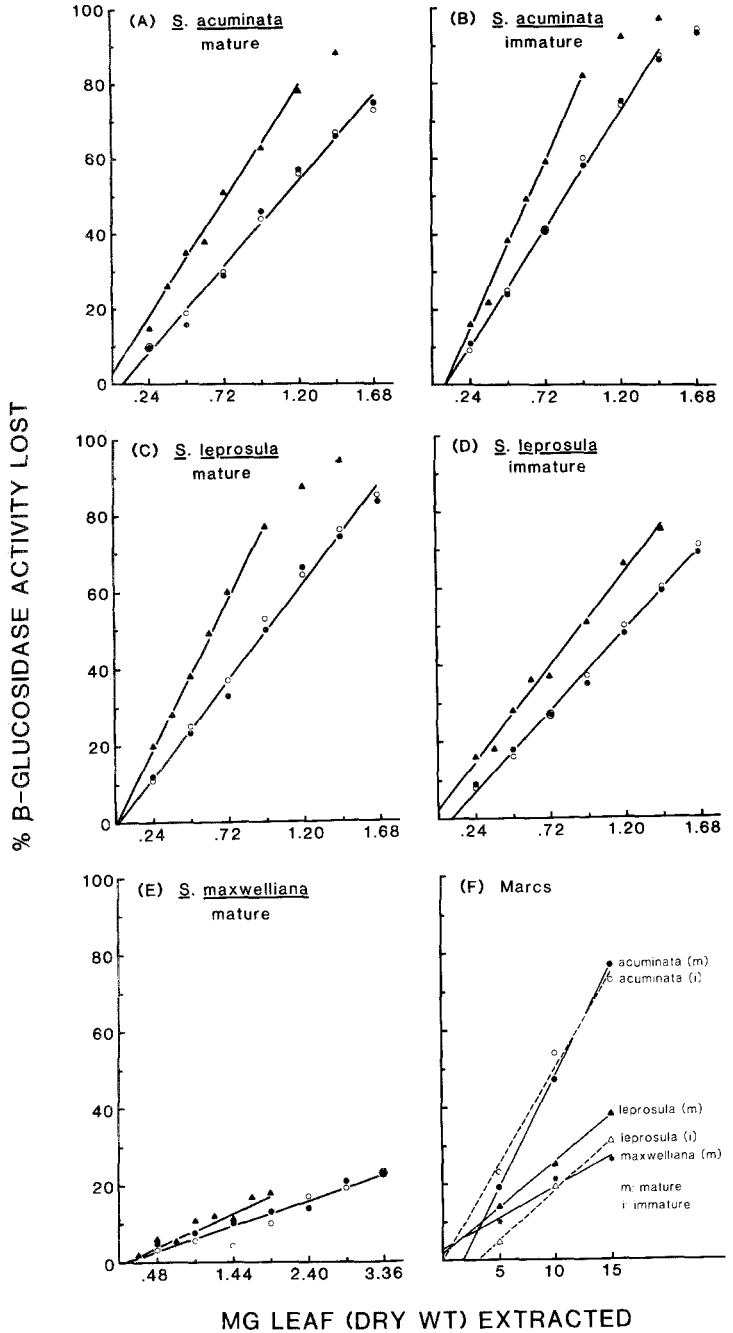

FIG. 1. Precipitation of β-glucosidase by tanniferous extracts (A–E) and marcs (F) of dipterocarp seedling leaves. The abscissa indicates the amount of leaf (per 0.3 ml acetate buffer) from which extractives or marcs were derived and combined with 0.3 ml phosphate buffer containing 0.15 mg (▲ of A–E and all runs in F) or 0.30 mg (○, ● of A–E) of emulsin. Lines represent linear, least-squares regressions.

TABLE 1. PROTEIN-PRECIPITATING CAPACITIES OF *SHOREA* SEEDLING LEAF EXTRACTS FOR TWO INITIAL PROTEIN CONCENTRATIONS

Initial emulsin conc. (mg/ml)	Leaf age	% β-glucosidase activity lost per mg leaf extracted (±SE)[a]		
		S. acuminata	*S. leprosula*	*S. maxwelliana*
1.0	Immature	65.5(±1.78)	43.4(±0.97) a	
	Mature	47.4(±2.04)A	52.3(±1.88)Aa	6.9(±0.54)
0.5	Immature	93.3(±3.43)	51.4(±1.99)	
	Mature	64.0(±3.07)	81.4(±2.07)	9.2(±1.09)

[a]Calculated as linear regression slopes from data in Figure 1A–E. Within lines or columns for a given initial emulsin concentration, values followed by the same letter did not differ ($P > 0.05$) by Behrens-Fisher tests actually made on transformed data (see Methods and Materials and Statistical Note). Y intercepts ± SE (df), lines 1–4 from left to right: −5.8±1.93(4), −3.4±1.26(5); −2.9±2.61(5), −0.7±2.37(5), −0.6±1.52(5); −7.8±2.16(4), 2.5±1.81(6); 2.7±2.33(5), −0.7±1.30(4), −0.6±1.52(6).

avoided by regressing the mean ($N = 3$) activity of the marc–enzyme mixtures on marc concentration. Regression coefficients (slopes) were compared by a simultaneous test procedure (Sokal and Rohlf, 1969). To facilitate comparison, the results in Figure 2 and Table 2 are expressed as for extracts.

Due to curvilinearity for some of the data, the protein-precipitating capacities of tannic acid and quebracho at different initial protein concentrations could not be compared by means of regression slopes. A given reduction of enzyme activity in a tannin–enzyme mixture, relative to its standard, corresponded to an unknown absolute amount of precipitated protein which varied directly with the standard's enzyme concentration. Therefore, to standardize the results relative to the highest protein concentration, the percentage of β-glucosidase activity lost was divided by 2 and 4 for the assays at 0.50 and 0.25 mg emulsin/ml, respectively, and expressed as arbitrary units of β-glucosidase precipitated. The resulting curves relating protein precipitation to tannin concentration were compared by profile analysis (Morrison, 1967) with appropriate adjustments for the covariance structure of the dependent variables.

RESULTS

Protein Precipitation by Extracts. Protein precipitation increased ($P < 0.001$) with increasing leaf extract concentration in all of the assays (Figure 1A-E). A threshold concentration of extract for β-glucosidase precipitation was apparently required for immature *S. acuminata* (0.50 and 1.0 mg

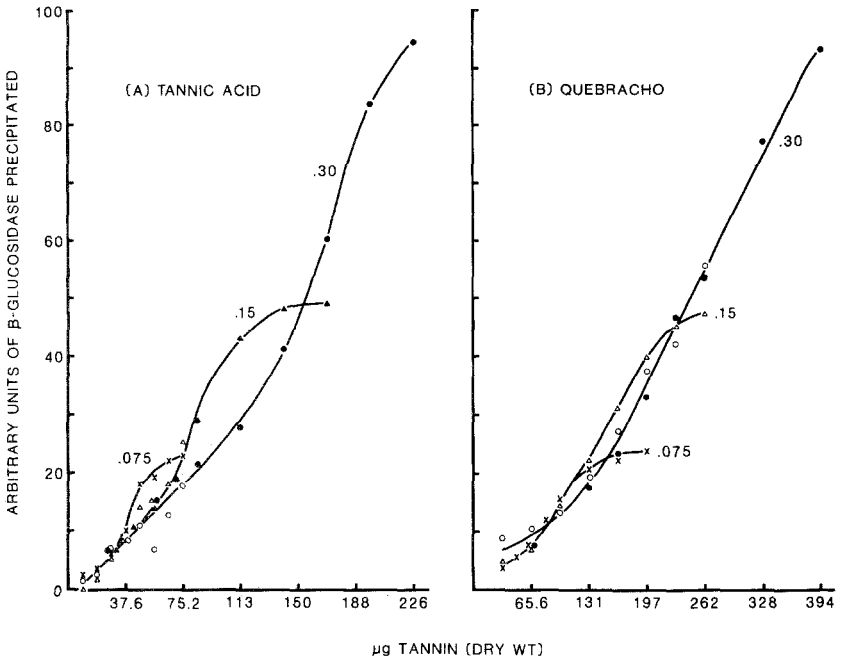

FIG. 2. Effect of initial protein concentration on precipitation of β-glucosidase by tannic acid (A) and bisulfited quebracho (B). The abscissa indicates the amount of tannin per 0.3 ml of acetate buffer combined with 0.3 ml of phosphate buffer containing 0.075 (×), 0.15 (△, ▲), or 0.30 (○, ●) mg of emulsin. Lines fitted by eye. Profile analysis compared protein-precipitation values corresponding to the following tannin concentrations (μg/0.3 ml, ×s and open symbols only)—tannic acid (0.25 vs. 1.0 mg emulsin/ml): 9.4–47.0; tannic acid (0.50 vs. 1.0 mg emulsin/ml): 9.4–65.8; quebracho (0.25 vs. 1.0 mg emulsin/ml): 32.8, 65.6, 98.4, and 131.2; quebracho (0.50 vs. 1.0 mg emulsin/ml): 32.8, 65.6, and 98.4–196.8.

TABLE 2. PROTEIN-PRECIPITATING CAPACITIES OF MARCS[a]

Leaf age	% β-glucosidase activity lost per mg marc[b]		
	S. acuminata	*S. leprosula*	*S. maxwelliana*
Immature	5.0 Aa	2.6 Ab	
Mature	5.8 a	2.4 Bb	1.6 B

[a] Initial emulsin concentration of 0.5 mg/ml.

[b] Calculated as linear regression slopes from data in Figure 1F. The slopes for immature *S. acuminata* and mature *S. maxwelliana* were not different ($P > 0.05$) from zero. Within lines or columns, values followed by the same letter did not differ ($P > 0.05$) by a simultaneous test procedure. Statistical tests were actually made on enzyme activity rather than the above derivative values (see Methods and Materials).

emulsin/ml) and immature *S. leprosula* (1.0 mg emulsin/ml only) since their regression lines had y intercepts (Table 1) different from zero (two-tailed t-tests, $P < 0.05$). Similar threshold effects have been noted in previous studies with leaf extracts (Bate-Smith, 1973; Martin and Martin, 1982) and tannins (Haslam, 1974; Schultz et al., 1981). The linearity of the non-asymptotic regions of the curves in Figure 1A-E permitted general comparisons of protein-precipitating capacity among extracts based on the regression slopes. However, the relative protein-precipitating capacities of the extracts at a particular initial protein concentration may be more similar or reversed from that suggested by such comparisons when protein-extract ratios are very high or low (Figure 1A-E).

The protein-precipitating capacity of *S. maxwelliana* extracts was much lower than that of the other two species (Table 1). Immature leaf extracts of both *S. acuminata* and *S. leprosula* had substantial protein-precipitating capacities which in the former species exceeded that of its mature leaf extracts. Differences among extracts were enhanced at the lower emulsin concentration; this affected the ranking of *S. leprosula* extracts only.

If tannins in the extracts bound and precipitated β-glucosidase in a stoichiometric manner, at least for the linear regions of the curves in Figure 1A–E, then halving the initial protein concentration should result in a doubling of the loss in β-glucosidase activity. This did not occur for any of the five extract types (Behrens-Fisher tests, $P < 0.05$), which had consistently lower than expected binding capacities at 0.50 mg emulsin/ml.

Nontannin Inhibitors. Significant residual inhibition of β-glucosidase activity by leaf extracts treated with hide powder to remove tannins occurred only in the cases of mature *S. leprosula* ($F[1,20] = 4.664$, $P < 0.05$) and *S. maxwelliana* ($F[1,20] = 18.041$, $P < 0.001$). Assuming that the inhibitory effect of the presumed nontannins (residual methanol or coextracted phytochemicals) was directly proportional to extract concentration, then they accounted for 25% of the observed (Table 1) reduction in enzyme activity by *S. maxwelliana* extracts but only 3.6% of that by mature *S. leprosula* extracts for assays at an initial emulsin concentration of 1.0 mg/ml. These results suggest that the protein-precipitating capacity of *S. maxwelliana* extracts relative to the other species was even lower than indicated by the data in Table 1.

Extractability of Tannins. Apparently there were no gross differences in tannin extractability to account for the basic differences in protein-precipitating capacity of leaf extracts. The cause of β-glucosidase inactivation by methanol-extracted marcs is uncertain since the enzyme may have been bound by tannins or other leaf constituents or otherwise inhibited, but the effect of the marcs was always small relative to that of the leaf extracts (Figure 1F, Tables 1 and 2). Absence of significant regressions for immature *S. acuminata* and mature *S. maxwelliana* was due to excessive residuals and

small sample sizes which probably also accounted for the statistical identity of the regression slopes for immature *S. acuminata* and *S. leprosula*.

Tannic Acid and Quebracho. Leaf extracts consistently precipitated less β-glucosidase at the lower initial protein concentration even though protein was still apparently in excess, as suggested by the positive linear relationship between enzyme precipitation and extract concentration. To determine whether this effect generally occurred with β-glucosidase, we studied its precipitation by commercial tannins. Solutions of these tannins treated with hide powder as described for leaf extracts had no residual inhibitory effect on β-glucosidase activity (tannic acid: $F[1,24] = 1.027$, $P > 0.25$; quebracho: $F[1,24] = 0.038$, $P > 0.75$).

For both tannic acid and bisulfited quebracho, the protein precipitation curves (Figure 2) were statistically indistinguishable (profile analysis, $P > 0.50$) over the compared regions, indicating no effect of initial protein concentration on protein-precipitating capacity of these tannins. For a given initial protein concentration, only protein precipitation values based on the same standards (to preserve balanced statistical design), occurring outside the asymptotic region, and corresponding to matching extract concentrations of the compared curve were tested. At tannic acid concentrations greater than those tested statistically and less than those at which protein precipitation had leveled off, however, there was a tendency for protein precipitation to increase as initial protein concentration was reduced. These results were contrary to those for leaf extracts.

Tannic acid precipitated about 1.7 times more β-glucosidase than an equal weight of quebracho. This is consistent with Haslam's (1974) findings for hydrolyzable and condensed tannins with this enzyme.

DISCUSSION

Tannin–Protein Chemistry. Our results underscore the need for suitable precautions when attempting to compare the protein-precipitating capacities of tannins. The robustness of differences in this property of tannins should be assessed by measurements made over a range of tannin and protein concentrations. Threshold and asymptotic effects of tannin concentration on protein precipitation limit the meaningfulness of measurements made at a single tannin concentration. While precipitation of β-glucosidase increased with increasing leaf extract or tannin concentration, the initial protein concentration affected the amount of protein precipitated in different ways, depending on the type and concentration of tannins.

Some insight into the complexities of tannin–protein interactions is provided by the finding of Van Buren and Robinson (1969) that gelatin and tannic acid form both soluble and insoluble complexes. They suggested that

the precipitability of tannin–protein complexes is affected by changes in their size and bonding configuration as the tannin–protein ratio varies. We suspect that the relative precipitability of a particular protein in a heterogeneous mixture may also be affected by interaction of tannins with the other proteins. This is suggested by our observation that *S. maxwelliana* leaf extract reduced β-glucosidase activity less than expected from the pellet's (tannin–protein precipitate) visible size.

Tannin Content and Astringency. For the three *Shorea* spp. studied here, Becker (1981) showed that seedling leaves of a particular age class have similar concentrations of both total phenolics and proanthocyanidins as measured by the Folin-Denis and BuOH-HCl assays, respectively. However, the protein-precipitating capacities of leaf constituents in these species, as measured here with β-glucosidase, differed markedly. The Folin-Denis assay includes all phenolic compounds, not just tannins, while the BuOH-HCl assay includes monomers of proanthocyanidins which do not precipitate proteins, so these results are not surprising (Rhoades and Cates, 1976; Swain, 1979). Other studies have also failed to find a correlation between the content and astringency of tannins in leaf extracts (Bate-Smith, 1977, 1981; Martin and Martin, 1982; Swain, 1979).

Given this discrepancy and the current beliefs about how tannins function defensively, we believe that the various assays for measuring tannin astringency (Bate-Smith, 1973; Martin and Martin, 1982) are more likely to be relevant in exploratory ecological studies than chemical, reactive group assays of tannin content (cf. Gartlan et al., 1980). There are at least three mechanisms by which tannins may defend plants from attack by herbivores: as feeding deterrents affecting palatability, as growth inhibitors affecting nutrient availability, and as direct toxic agents (Feeny, 1970; Bernays et al., 1980). All three mechanisms probably depend, at least partially, on the ability of tannins to form complexes with proteins.

Herbivory and Tannin Defense. Earlier attempts (Feeny, 1976; Rhoades and Cates, 1976; Cates, 1980) to generalize concerning the within-plant allocation of tannins between immature and mature leaves are questionable, as remarked previously by McKey (1979). Immature leaves of *S. acuminata* and *S. leprosula* contain substantial quantities of protein-precipitating phenolics; in the first species they appear to be more potent than those of mature leaves, contrary to the pattern predicted by the former investigators.

Extracts of *S. maxwelliana* leaves had much lower protein-precipitating capacity than those of *S. acuminata* and *S. leprosula* when measured by the β-glucosidase assay under favorable pH conditions, and differences in tannin extractability apparently did not account for this relationship. Superficially, these results suggest that the lower insect species richness and damage on *S. maxwelliana* foliage (Becker, 1981) are not due to its having more potent tannin defenses than the other *Shorea* species. However, the certainty of this

conclusion depends on whether the astringency of leaf extracts, as measured with β-glucosidase, adequately reflects the capacity of leaf tannins to bind with those other proteins (e.g., insect salivary proteins and digestive enzymes and leaf proteins) actually affecting the palatability, digestibility, and toxicity of *Shorea* foliage to insects. β-Glucosidase occurs in the guts of many insects (House, 1974), so its interaction with tannins has potential relevance to the digestibility of foliage. Although the degree of inhibition of different enzymes by a particular tannin or tanniferous extract can differ markedly (Griffiths, 1979; Mandels and Reese, 1963; Schneider and Hallier, 1970), it would still be possible to meaningfully rank foliage astringency if the component tannins vary similarly in their interaction with different proteins. There is a high correlation between the precipitability of β-glucosidase and bovine serum albumin (BSA) by tanniferous foliage extracts of six oak species (Martin and Martin, 1982), and extracts of the three species that have been tested rank identically with respect to their precipitation of BSA and ribulose 1,5-biphosphate carboxylase-oxygenase, the major soluble leaf protein (Martin and Martin, in press). A broader survey is required, but the results so far suggest that a consistent ranking of foliage astringency is possible.

Bernays (1981) has excellently reviewed available information concerning responses of insects to tannins and how some insects overcome the potential adverse effects of tannins and even benefit from their presence in food. She concluded that generalizations about the effectiveness of tannins as defensive compounds cannot yet be made, despite the earlier belief that this was possible (Feeny, 1976; Rhoades and Cates, 1976). We wish to emphasize that the utility of protein precipitation assays like the one described here lies in the identification of foliage in which tannins have a potential defensive role against herbivores. Confirmation or disproof of this role requires experimental study of the behavioral and physiological responses to tannins by potential and actual herbivores in the system. Nevertheless, correlative studies of insect feeding and foliage astringency importantly complement such experimental studies because of the difficulty of altering dietary tannin composition and concentration in a biologically relevant manner.

STATISTICAL NOTE

W.A. Ericson
Statistical Research Laboratory
University of Michigan
Ann Arbor, Michigan 48109

For the comparison of protein-binding capacities of leaf extracts by regression analysis, we assumed that the basic measurements of enzyme activity in the standards (S_{ij}) and supernatants of the enzyme–extract mixtures

(M_{ij}) had the forms:

$$S_{ij} = \mu + \gamma_i + \epsilon_{ij} \tag{1.1}$$

and

$$M_{ij} = \mu'_i + \gamma'_i + \epsilon'_{ij} \tag{1.2}$$

where μ and μ'_i are the means, γ_i and γ'_i are random preparation effects, and ϵ_{ij} and ϵ'_{ij} are random measurement (within preparation) effects. We further assumed that $E(\gamma_i) = E(\gamma'_i) = E(\epsilon_{ij}) = E(\epsilon'_{ij}) = 0$, $\mathrm{Var}(\gamma_i) = \mathrm{Var}(\gamma'_i) = \sigma_p^2$, and $\mathrm{Var}(\epsilon_{ij}) = \mathrm{Var}(\epsilon'_{ij}) = \sigma_w^2$.

The dependent variable in the regressions was

$$Y_i = (\bar{\bar{S}} - \bar{M}_i)/\bar{\bar{S}} \tag{2}$$

where $\bar{\bar{S}} = (\sum_{i=1}^{4} \sum_{j=1}^{3} S_{ij})/12$ and $\bar{M}_i = (\sum_{j=1}^{3} M_{ij})/3$.

From (1.1) and (1.2) it follows that $\mathrm{Var}(\bar{\bar{S}}) = (\sigma_p^2/4) + (\sigma_w^2/12)$ and $\mathrm{Var}(\bar{M}_i) = \sigma_p^2 + (\sigma_w^2/12)$. Expanding (2) in a first-order Taylor series around the means of $\bar{M}_i$ and $\bar{\bar{S}}$ and using the sample values to estimate these means gives

$$\mathrm{Var}(Y_i) \approx (\bar{\bar{M}}^2/\bar{\bar{S}}^4)\mathrm{Var}(\bar{\bar{S}}) + (1/\bar{\bar{S}}^2)\mathrm{Var}(\bar{M}_i) = (\bar{\bar{M}}^2/\bar{\bar{S}}^4)\,[(\sigma_p^2/4) + (\sigma_w^2/12)] + (1/\bar{\bar{S}}^2)\,[\sigma_p^2 + (\sigma_w^2/3)] \tag{3.1}$$

and

$$\mathrm{Cov}(Y_i, Y_j) \approx (\bar{\bar{M}}^2/\bar{\bar{S}}^4)\,[(\sigma_p^2/4) + (\sigma_w^2/12)] \tag{3.2}$$

where $\bar{\bar{M}} = (\sum_{i=1}^{n} \bar{M}_i)/n$.

Consequently, we assumed a regression model of the form

$$Y_i = \alpha + \beta(X_i - \bar{X}) + \epsilon_i$$

where X_i is extract concentration and the ϵ_is have a multivariate normal distribution with means all equal to zero. $\mathrm{Var}(\epsilon_i) = a + b = \mathrm{Var}(Y_i)$, and $\mathrm{Cov}(\epsilon_i, \epsilon_j) = a = \mathrm{Cov}(Y_i, Y_j)$, where a and b are evident from (3.1) and (3.2).

By means of an orthogonal transformation (e.g., Arnold, 1979), the above regression model can be reformulated almost like the standard, homoscedastic, uncorrelated error case. Inference above β is exactly as in the standard case, i.e.,

$$\frac{\hat{\beta} - \beta}{[s^2/\sum_{i=1}^{n}(X_i - \bar{X})^2]^{1/2}} \tag{4}$$

has a t distribution on $n - 2$ degrees of freedom, where $\hat{\beta}$ is the usual least squares estimator of β and s^2 is the usual regression error mean square. Comparisons between different slopes were based on the Behrens-Fisher distribution (Box and Tiao, 1973; Fisher and Yates, 1963) with significance levels adjusted according to the Bonferroni inequality approach (Miller, 1966).

Inference about α was based on the fact that, given α, a, and b, $\overline{Y}$ is normally distributed with mean α and variance $a + (b/n)$. Here the usual regression mean square error has an expected value equal to b. An unbiased estimate of the variance of $\hat{\alpha}$ is

$$\widehat{\text{Var}}(\hat{\alpha}) = (\overline{\overline{M}}^2/12\overline{\overline{S}}^4)\Sigma_{i=1}^{4}(\overline{S}_i - \overline{\overline{S}})^2 + (s^2/n) \quad (5)$$

where s^2 is as in (4). Inference about α then depended on the approximation that $(\overline{Y} - \alpha)/[\widehat{\text{Var}}(\hat{\alpha})]^{1/2}$ has a t distribution on 3 degrees of freedom (the smaller of the two variance component estimators' degrees of freedom).

Acknowledgments—S. Latif, T. Hussein, and M. Wong helped to collect leaves. Logistical and material support and living expenses were provided by the Forest Research Institute (Kepong) of Malaysia and the U.S. Peace Corps, respectively, during P. Becker's field study. Financial support during the laboratory study was provided to P. Becker by the University of Michigan's Division of Biological Sciences and to J. Martin by a grant from the National Science Foundation to M. Martin (PCM78-22733). We are grateful to M. Martin for advice and encouragement and to W. Ericson for statistical advice and modeling. D. Janzen, P. Marshall, M. Martin, D. Rabinowitz, B. Rathcke, and two anonymous reviewers commented helpfully on an earlier version of this paper. This research constitutes part of a doctoral dissertation to be submitted by P. Becker in partial fulfillment of the requirements for the Doctor of Philosophy in the Horace H. Rackham School of Graduate Studies at the University of Michigan.

REFERENCES

ARNOLD, S.F. 1979. A coordinate-free approach to finding optimal procedures for repeated measures designs. *Ann. Statist.* 7:812–822.

BATE-SMITH, E.C. 1973. Haemanalysis of tannins: The concept of relative astringency. *Phytochemistry* 12:907–912.

BATE-SMITH, E.C. 1975. Phytochemistry of proanthocyanidins. *Phytochemistry* 14:1107–1113.

BATE-SMITH, E.C. 1977. Astringent tannins of *Acer* species. *Phytochemistry* 16:1421–1426.

BATE-SMITH, E.C. 1981. Astringent tannins of the leaves of *Geranium* species. *Phytochemistry* 20:211–216.

BECKER, P. 1981. Potential physical and chemical defenses of *Shorea* seedling leaves against insects. *Malay. For.* 44:346–356.

BENNETT, S.E. 1965. Tannic acid as a repellent and toxicant to alfalfa weevil larvae. *J. Econ. Entomol.* 58:372–373.

BERNAYS, E.A. 1981. Plant tannins and insect herbivores: An appraisal. *Ecol. Entomol.* 6:353–360.

BERNAYS, E.A., and CHAMBERLAIN, D.J. 1980. A study of tolerance of ingested tannin in *Schistocerca gregaria*. *J. Insect Physiol.* 26:415–420.

BERNAYS, E.A., CHAMBERLAIN, D., and MCCARTHY, P. 1980. The differential effects of ingested tannic acid on different species of Acridoidea. *Entomol. Exp. Appl.* 28:158–166.

BOX, G.E.P., and TIAO, G.C. 1973. Bayesian Inference in Statistical Analysis. Addison Wesley, Reading, Massachusetts, pp. 106–107.

Cates, R.G. 1980. Feeding patterns of monophagous, oligophagous, and polyphagous insect herbivores: The effect of resource abundance and plant chemistry. *Oecologia (Berlin)* 46:22–31.

CHAN, B.G., WAISS, A.C., JR., BINDER, R.G., and ELLIGER, C.A. 1978. Inhibition of lepidopterous larval growth by cotton constituents. *Entomol. Exp. Appl.* 24:294–300.

FEENY, P. 1970. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51:565–581.

FEENY, P. 1976. Plant apparency and chemical defense. *Rec. Adv. Phytochem.* 10:1–40.

FISHER, R.A., and YATES, F. 1963. Statistical Tables for Biological, Agricultural, and Medical Research, 6th ed. Hafner, New York, Table VI_1, p. 61.

FOX, L.R., and MACAULEY, B.J. 1977. Insect grazing on *Eucalyptus* in response to variation in leaf tannins and nitrogen. *Oecologia (Berlin)* 29:145–162.

GARTLAN, J.S., MCKEY, D.B., WATERMAN, P.G., MBI, C.N., and STRUHSAKER, T.T. 1980. A comparative study of the phytochemistry of two African rain forests. *Biochem. Syst. Ecol.* 8:401–422.

GOLDSTEIN, J.L., and SWAIN, T. 1965. The inhibition of enzymes by tannins. *Phytochemistry* 4:185–192.

GRIFFITHS, D.W. 1979. The inhibition of digestive enzymes by extracts of field bean (*Vicia faba*). *J. Sci. Food Agric.* 30:458–462.

GUSTAVSON, K.H. 1956. The Chemistry of Tanning Processes. Academic Press, New York, pp. 169–170.

HASLAM, E. 1974. Polyphenol-protein interactions. *Biochem. J.* 139:285–288.

HOUSE, H.L. 1974. Digestion, pp. 63–117, *in* M. Rockstein (ed.). The Physiology of Insecta, Vol. 5. Academic Press, New York.

MANDELS, M., and REESE, E.T. 1963. Inhibition of cellulases and β-glucosidases, pp. 115–157, *in* E.T. Reese (ed.). Advances in Enzymic Hydrolysis of Cellulose and Related Materials. Pergamon, New York.

MARTIN, J.S., and MARTIN, M.M. 1982. Tannin assays in ecological studies: Lack of correlation between phenolics, proanthocyanidins and protein-precipitating constituents in mature foliage of six oak species. *Oecologia (Berlin)*. (in press).

MARTIN, J.S., and MARTIN, M.M. Tannin assays in ecological studies: The precipitation of ribulose-1,5-biphosphate carboxylase/oxygenase by tannic acid, quebracho, and oak foliage extracts. *J. Chem. Ecol.* (in press).

MAXWELL, F.G., JENKINS, J.N., and PARROTT, W.L. 1967. Influence of constituents of the cotton plant on feeding, oviposition, and development of the boll weevil. *J. Econ. Entomol.* 60:1294–1296.

MCKEY, D. 1979. The distribution of secondary compounds within plants, pp. 55–133, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

MILLER, R.G., JR. 1966. Simultaneous Statistical Inference, McGraw-Hill, New York, pp. 6–8.

MORRISON, D.F. 1967. Multivariate Statistical Methods. McGraw-Hill, New York.

RHOADES, D.F., and CATES, R.G. 1976. Toward a general theory of plant antiherbivore chemistry. *Rec. Adv. Phytochem.* 10:168–213.

SCHNEIDER, V., and HALLIER, U.W. 1970. Polyvinylpyrollidon als Schutzstoff bei der Untersuchung gerstoffgehemmter Enzymreaktionen. *Planta* 94:134–139.

SCHULTZ, J.C., BALDWIN, I.T., and NOTHNAGLE, P.J. 1981. Hemoglobin as a binding substrate in the quantitative analysis of plant tannins. *J. Agric. Food Chem.* 29:823–826.

SOKAL, R.R., and ROHLF, J.F. 1969. Biometry. Freeman, San Francisco, pp. 457–458.

SWAIN, T. 1979. Tannins and lignins, pp. 657–682, *in* G.A. Rosenthal and D.H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

VAN BUREN, J.P., and ROBINSON, W.B. 1969. Formation of complexes between protein and tannic acid. *J. Agric. Food Chem.* 17:772–777.

WATERMAN, P.G., MBI, C.N., MCKEY, D.B., and GARTLAN, J.S. 1980. African rainforest vegetation and rumen microbes: Phenolic compounds and nutrients as correlates of digestibility. *Oecologia (Berlin)* 47:22–33.